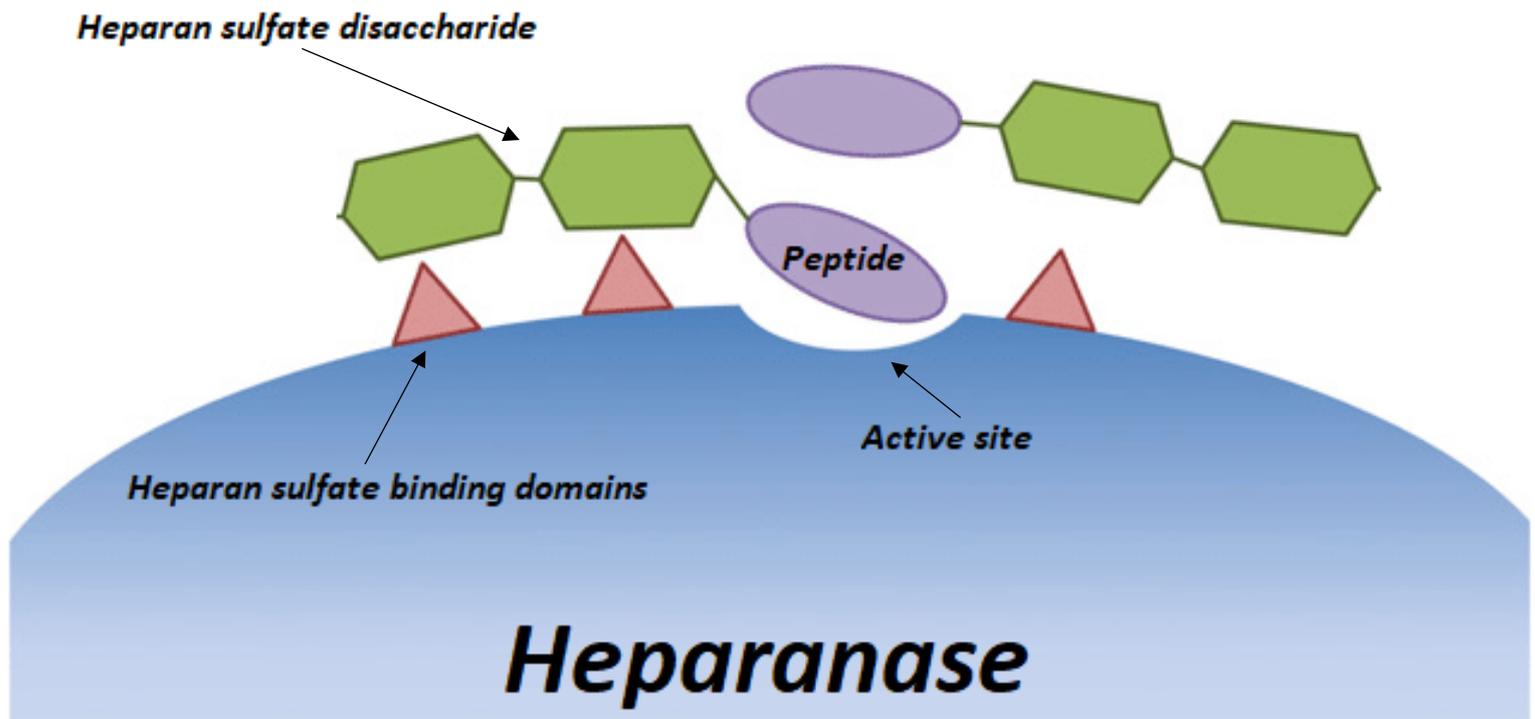


Covalent modification of peptide libraries to target the active site of heparanase in mRNA display



Master's Internship Thesis

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Date: 21-02-2022

Table of contents

List of abbreviations	3
Abstract	4
Laymen's summary.....	4
Introduction.....	5
Targeting heparanase's active site	9
Results and discussion.....	12
▪ Optimisation of the CuAAC reaction with synthetic peptide	12
▪ Optimisation of the CuAAC reaction with <i>in vitro</i> translated peptide	16
▪ Immobilization of heparanase.....	19
▪ Activity assessment of biotinylated heparanase.....	20
▪ Selection RaPID	21
▪ Design of the cDNA-TRAP libraries.....	23
▪ Conclusion and Future directions.....	25
Experimental methods	26
▪ 1. F-moc solid phase peptide synthesis.....	26
▪ 2. CuAAC click reaction with synthetic alkyne peptide	26
▪ 3. Aminoacylations of tRNA ^{fMet} (based on 10 µL volume).....	27
▪ 4. CuAAC click reaction with <i>in vitro</i> translated peptide.....	27
▪ 5. Heparanase biotinylation	29
▪ 6. Assessment of biotinylated protein binding capacity on beads	29
▪ 7. Colorimetric activity assays	30
▪ 8. Library preparation.....	31
• 8.1. Random NNK15 library, RaPID display:.....	31
• 8.2. Fixed Methionine NNK15 library, cDNA-TRAP display:	31
▪ 9. Selection protocol RaPID system.....	32
• 9.1. RNA purification in between selection rounds	33
• 9.2. DNA purification in between selection rounds.....	33
Acknowledgements	34
References.....	35
Supplementary Information	38

List of abbreviations

- **CuAAC:** Copper(I)-catalysed alkyne-azide cycloaddition
- **ECM:** Extracellular matrix
- **FAM:** Fluorescein Amidite
- **Hpg:** Homopropargylglycine
- **HPSE:** Heparanase
- **HPLC:** High-performance liquid chromatography
- **HS-DS:** Heparan-sulfate disaccharide
- **LC-MS:** Liquid-chromatography Mass-spectrometry
- **PAGE** Polyacrylamide gel-electrophoresis
- **PCR:** Polymerase Chain Reaction
- **qPCR:** Quantitative Polymerase Chain Reaction
- **RaPID:** Random nonstandard Peptide Integrated Discovery

Abstract

Heparanase (HPSE) cleaves the chains of heparan sulfate proteoglycans at cleavage sites between glucuronic acid and sulfated glucosamine residues (endo- β -glucuronidase). Heparan sulfate proteoglycans are crucial in maintaining structural integrity of basement membranes, cell surfaces and extracellular matrixes (ECM). Heparan sulfate chains bind to a vast array of signaling molecules, such as growth factors, chemokines and cytokines. By cleaving heparan sulfate chains and proteoglycans, these signaling molecules will be released and ECM and membranes will be remodelled. In adults, the basal level of HPSE activity is rather low. HPSE expression and activity are increased in many diseases, and more importantly in most types of tumors. In tumors, elevated HPSE activity plays a role in angiogenesis, metastasis, cell proliferation and cell invasion. This makes HPSE an interesting target for anti-cancer therapies. Current HPSE inhibitors in clinical trials are all sulfated polysaccharides, which entail anticoagulant side-effects. Peptides are a potential class of HPSE inhibitors without anticoagulant effect, but they remain understudied. In this thesis, a method for chemical modification of peptide libraries to selectively target the enzyme's active site in high-throughput screenings was developed and optimised. The modification is a heparan-sulfate disaccharide with an azide-bearing aromatic aglycone, being attached to the peptide libraries by a well-established copper(I)-catalysed alkyne-azide cycloaddition (CuAAC). The glycan-modified libraries were used in RaPID mRNA display, and will be used in the future in cDNA-TRAP display, to screen for potential HPSE inhibitors. RaPID mRNA display against HPSE did not lead to hits because of recurring truncation issues with the libraries, but the cDNA-TRAP display system could overcome this. The optimised click conditions can also be exploited in future selections with similar goals to modify libraries with different substrates for more specific targeting of the target.

Laymen's summary

Heparanase (HPSE) is an enzyme that cleaves polysaccharide chains (heparan sulfates) that bind to signaling molecules, such as growth factors. By cleaving these heparan sulfate chains, HPSE facilitates the release of these growth factors and biologically active saccharides. In adults, the basal level of HPSE expression is low, but in most types of tumors, the enzyme is overexpressed. Because of the overexpression, the enzyme plays a role in formation of new blood vessels, tumor cell spreading, cell proliferation and cell invasion. This makes HPSE an interesting target for anti-cancer therapies. Currently, all drugs in clinical trials targeting HPSE are polysaccharides with sulfate groups, which entail anticoagulant side-effects (blood thinning). It is important to find molecules that inhibit HPSE activity, that do not have these anticoagulant side-effects. Peptides are short chains of aminoacids that are suitable therapeutics without such side-effects. To identify peptide inhibitors, a selection process called mRNA display was carried out. In this selection it was important to target the enzyme's active site, since the peptides need to bind in the active site and block the activity. To achieve this, this project was used to develop and optimise an approach to attach the peptides to a disaccharide, resembling the heparan sulfate substrate, which should specifically target the peptides to the active site. A first mRNA display selection did not result in the selection of peptides that could inhibit HPSE. Nevertheless, the developed and optimised approach can be used in different mRNA display selections, with the same aim to find peptides that inhibit the enzyme. This project lays the basis for the future selection and development of therapeutic peptides targeting HPSE that could contribute to a novel anti-cancer therapy.

Introduction

Heparanase (HPSE) is an endo- β -glucuronidase of the glycoside hydrolase family GH79^{1,2}. The enzyme cleaves heparan sulfate (HS) chains of heparan sulfate proteoglycans at cleavage sites between glucuronic acid (GlcA) and sulfated glucosamines, especially N-sulfo-glucosamine GlcN(NS) (*figure 1*). Human heparanase has two isoforms; heparanase 1 (HPSE1) and heparanase 2 (HPSE2), from which only HPSE1 exhibits enzymatic activity. HPSE2 plays an important role in HPSE1 inhibition and tumor suppression^{3,4}. HPSE1 is expressed as in-active preproheparanase (65 kDa) consisting of a 8 kDa fragment, a 50 kDa fragment, a 6 kDa linker peptide and a signal peptide (*figure 2*). Preproheparanase is converted to proheparanase by folding and cleaving of the signal peptide by peptidases. Proheparanase is processed to the active enzyme in lysosomes and secreted to extracellular matrixes (ECM) where it encounters HS chains. Activation occurs upon excision of the linker by cathepsins, after which the 8 kDa and 50 kDa fragments remain associated as a heterodimer^{5,6}. Crystal structures of the enzyme revealed that the linker peptide is located partially within an extended binding cleft (TIM barrel), where it blocks HS chains from entering the active site (*figure 2*)^{2,5,7,8}.

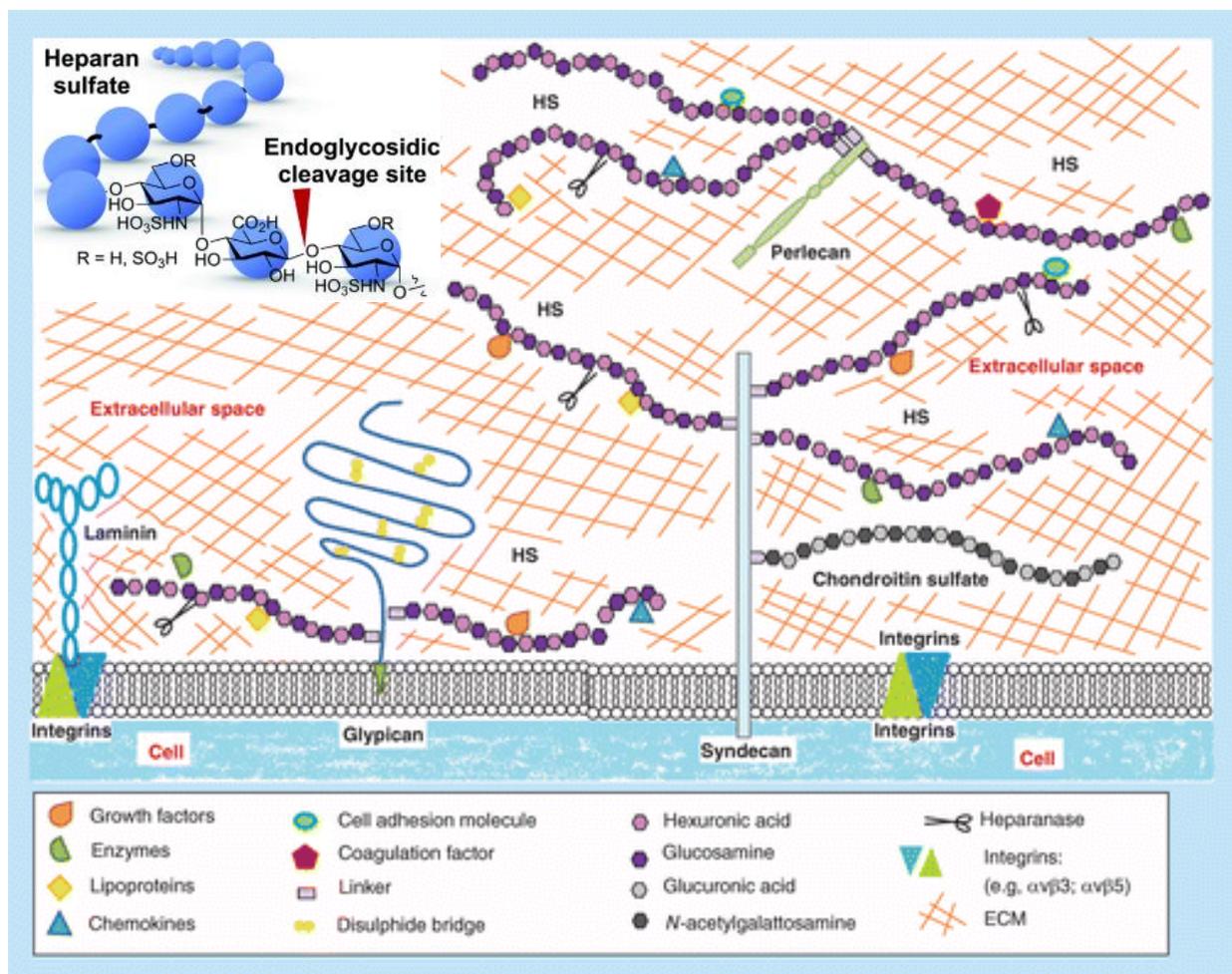


Figure 1: Membrane- and ECM-linked heparan sulfate chains cleaved by HPSE. Top left box: HPSE's cleavage site. Blue circle: monosaccharide. Red triangle: cleavage site between GlcA and GlcN(NS)^{1,2}. From: Rivara et al. (2016).

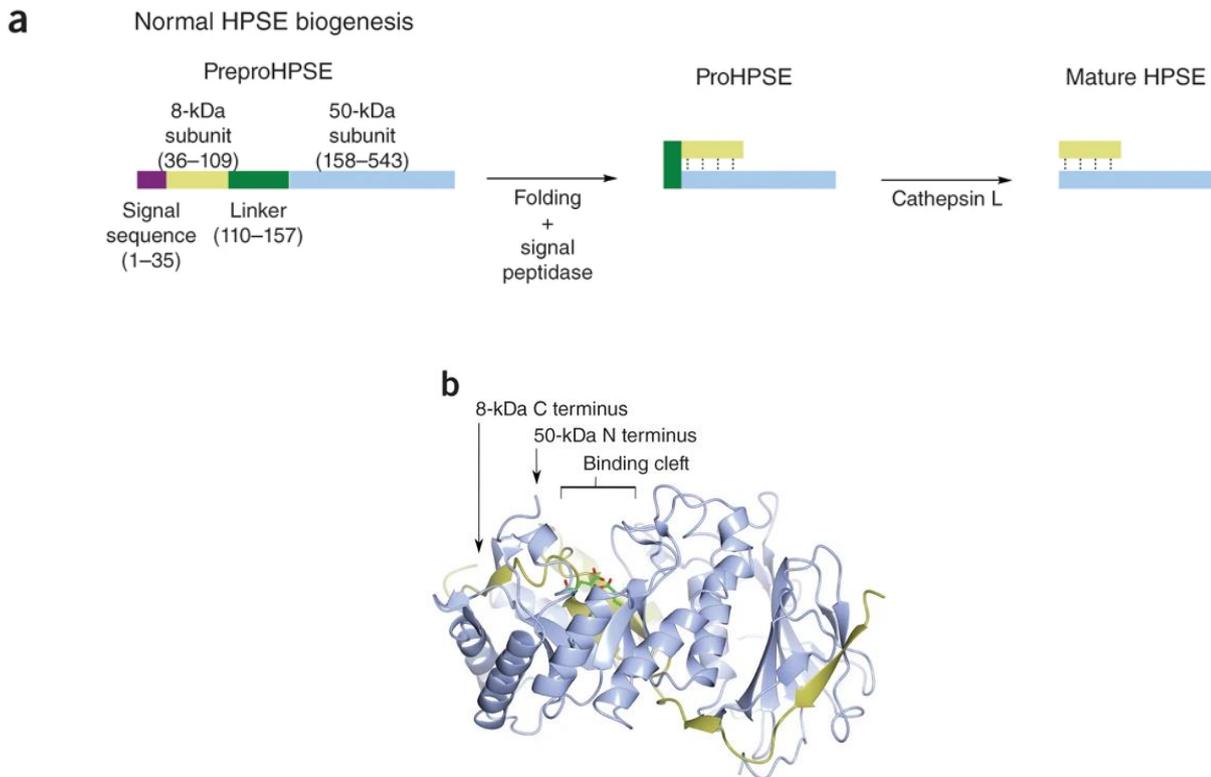


Figure 2: Sequence and structure of human HPSE. A) Schematic view of preproheparanase and proheparanase expression and progression to active HPSE. B) Structure of dimerized active HPSE, with the catalytic site (green) in the binding cleft. Yellow: 8 kDa subunit, blue: 50 kDa subunit ⁶. From: Wu et al. (2016).

Heparan sulfate proteoglycans are abundantly present on the basement membrane, cell surface and ECM ^{1,2} (figure 1), where they are crucial in maintaining the structural integrity. Besides that, HS chains bind to a broad range of signaling molecules. These molecules include growth factors, chemokines and cytokines, thus HS chains function as a biological reservoir that allows the release of these molecules when activation of their signaling pathways are required. Also, processing of HS chains releases carbohydrates capable of promoting cellular proliferation ⁹. In short, under physiological conditions, the enzymatic activity of HPSE is responsible for a crucial part of cellular behavior: releasing signaling molecules bound to HS chains; remodeling of the ECM and liberating biologically active carbohydrates. A problem arises when HPSE expression and activity are increased, which is observed in diseases such as chronic inflammation, diabetic nephropathy, thrombosis and atherosclerosis, and in most types of tumors. In tumors, elevated activity causes over-release of HS chain bound growth factors, cytokines and chemokines, resulting in increased angiogenesis, metastasis and cell invasion ¹⁰.

These adverse functions make HPSE an intriguing target for anti-cancer therapies that inhibit HPSE's biological activity ^{2,9,10}. In the last decade(s), different classes of (potential) inhibitors were reported, including nucleic acids, proteins, monoclonal antibodies, vaccines, sulfated saccharides and small-molecules ^{2,9,11,12}. Despite all these classes of inhibitors that have been researched, a rather low number of potential inhibitors have entered clinical trials. Nowadays, inhibitors in ongoing clinical trials are all from the class of sulfated polysaccharides (figure 3) ².

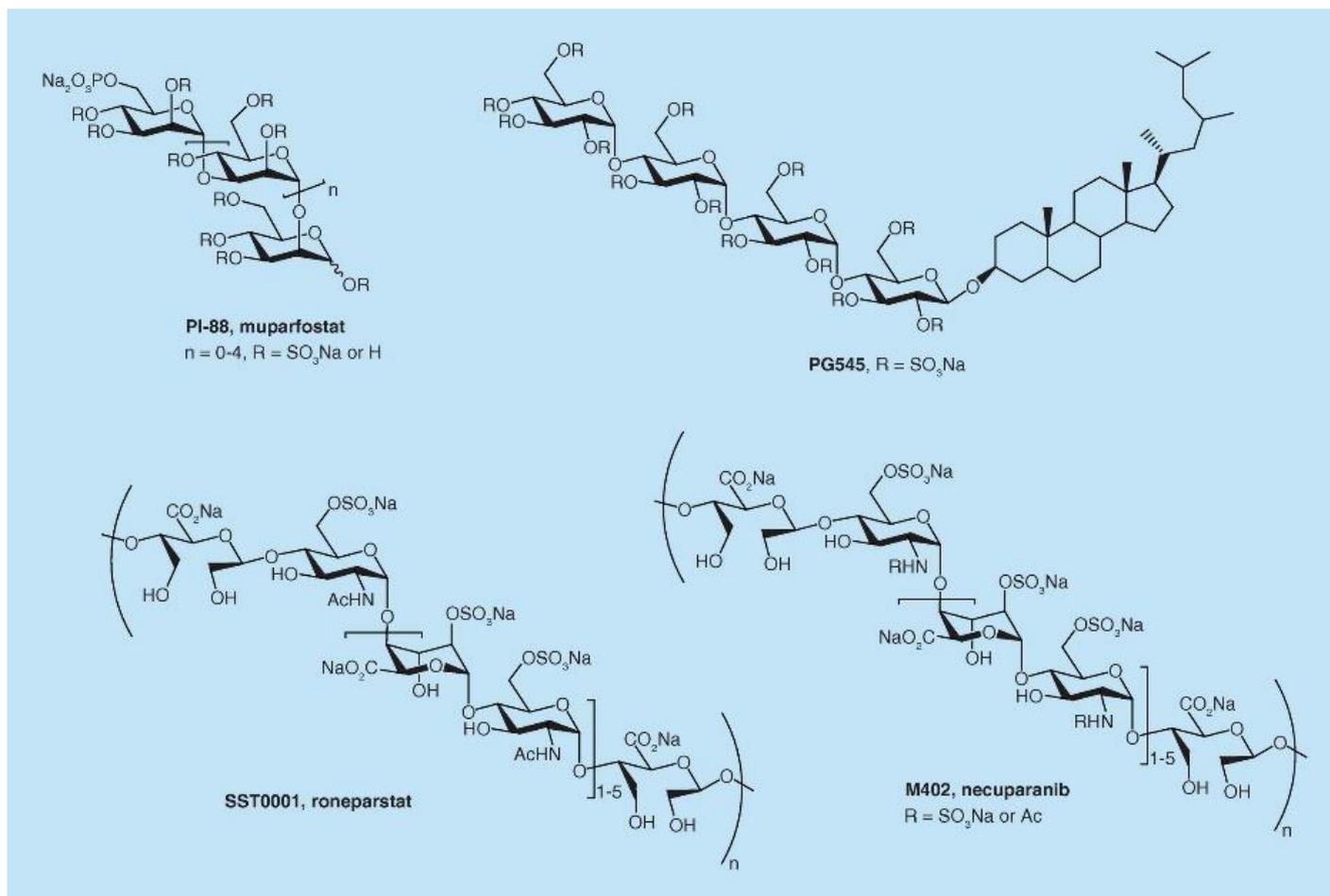


Figure 3: Structural formulas of HPSE inhibitors in clinical trials. All inhibitors are sulfated polysaccharides (SO_3Na), since the enzyme has shown to prefer cleavage at the nonreducing side of highly sulfated HS chains². From: Rivara et al. (2016).

Natural polysaccharides are found to have a broad range of (potential) clinical applications, such as anti-inflammatory or anti-cancer therapies^{13,14}. Unfortunately, they exert anticoagulant effects as well^{15,16}. Despite numerous chemical modifications of the polysaccharides that have been reported to separate the anticoagulant activity from inhibitory activities, it remains a major disadvantage of the polysaccharide class as therapy option.

Synthetic peptides have been shown to be very potent inhibitors for many drug targets, however, up to now, a limited number of studies have been reported for the discovery of such HPSE inhibitors^{2,9,17-19}. They have the additional advantage of having no anticoagulant effect, compared to polysaccharide HPSE inhibitors. Peptides stand out to be potential drug target inhibitors, as they entail multiple advantages over other small molecules inhibitors, such as higher binding affinity by low flexibility and large surface area, high specificity, low toxicity and immunogenicity²⁰. Besides that, peptide synthesis can be easily automated and a large array of modifications can be incorporated in the amino acid sequence. Synthetic peptides do also have some disadvantages, such as low oral availability, rapid clearance and poor stability, although these drawbacks are still more beneficial than having anticoagulant side-effects²¹.

Compared to linear peptides, macrocyclic peptides are known to show higher biological activity and stability, as they are resistant to exopeptidases, since they have no free C- and N-terminus. Potential (macrocyclic) peptide inhibitors can be found by using mRNA display systems, targeting the drug target of interest.

Multiple different mRNA display systems have been validated, of which RaPID display, TRAP display and cDNA-TRAP display possess the most advantages for this project ²²⁻²⁴. The key aspect of these displays involves a covalent linkage between the peptides and their encoding RNA, to generate a template that allows specific mRNAs to be enriched from a high-diverse library, by pulldown of a peptide library against target and mRNA/cDNA read-out by qPCR ²⁵. In all approaches, puromycin provides the covalent linkages by its tyrosine- and adenosine-like moieties ²⁶. The adenosine is attached to the 3' end of the mRNA sequence, and the tyrosine enters the ribosomal A-site where it can be attached to the peptides' C-terminus. TRAP and cDNA-TRAP display involve a different puromycin ligation strategy compared to RaPID, enabling continuous execution of transcription, ligation and translation, instead of consecutive time-consuming steps ^{23,24}. Despite the much faster workflow of (cDNA-)TRAP, RaPID display will be the first choice mRNA display system in this project, as the system components are already in hand.

RaPID (Random nonstandard Peptide Integrated Discovery) is a high-throughput screening method (libraries of up to 10^{13} peptides) that enables the rapid discovery of peptide inhibitors against a drug target, and that allows incorporation of non-natural aminoacids in the peptide libraries (*figure 4*) ²⁷. The system relies on cell-free peptide translation, in which flexizymes, or aminoacylating RNAs, are used to reprogram non-natural amino acids into the genetic code ²⁸.

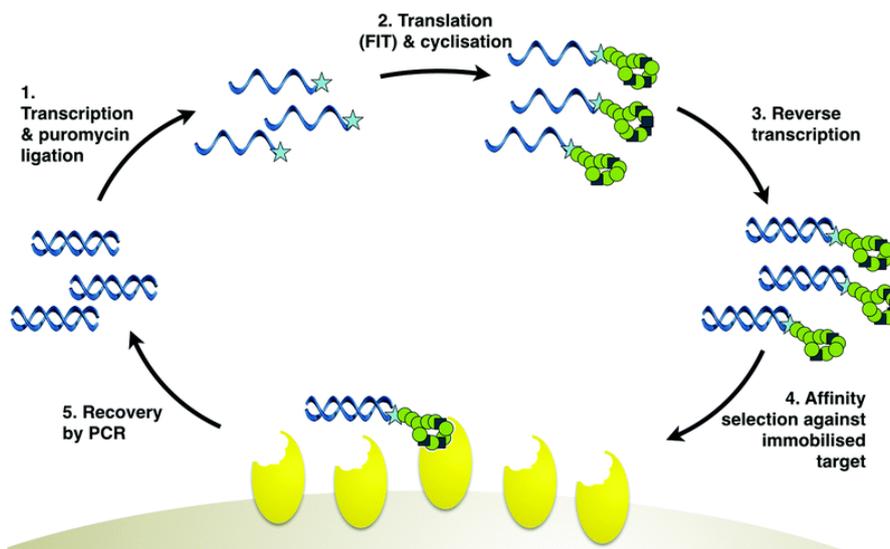


Figure 4: Schematic overview of the RaPID system. A DNA library with randomised codons is transcribed to mRNA and conjugated to puromycin. The pool of random puromycin-linked mRNAs is translated to (macrocyclic) peptides. The peptides are tested on affinity to an immobilized target of interest (selection). The puromycin covalently attaches each translated peptide to its encoding mRNA. Reverse transcription of the mRNA of the peptides that bind to the target leads to cDNA, which can be analysed and recovered by (q)PCR. This recovery gives a library of cDNA of peptides that bind to the target of interest. By consecutive rounds of selection, the strongest binders will eventually be recovered ²⁹. From: Passioura et al. (2017).

Targeting heparanase's active site

Four selections against HPSE have been executed before the start of this project (*unpublished data*). Table 1 shows an overview of the peptide library reprogramming and most important conclusions of these selections.

Table 1: Overview of the previously executed selection against HPSE. Repr = reprogramming. Met = methionine.

Selection	Repr. initiating Met	Repr. elongating Met	Conclusion
1	CIAC-D-Tyr / CIAC-L-Tyr	-	No enrichment; no binders were found at all
2	CIAC-D-Tyr / CIAC-L-Trp	-	Enrichment; but none of the peptides showed activity in inhibition assays
3	CIAC-D-Tyr / CIAC-L-Tyr	Phe-SO ₃ (mimics negative charges of heparan sulfate)	Enrichment; but none of the peptides showed activity in inhibition assays
4	CIAC-D-Tyr / CIAC-L-Tyr	L-DOPA (chelates the catalytic carboxylate side chains)	Enrichment; but sequencing showed that something went wrong during the selection's translation. No full length peptides and no DOPA were observed

The information in table 1, specifically from the 2nd, 3rd and 4th selection, suggests that HPSE has binding sites, additional to the active site, that are more dominant in binding these peptide hits. It is not exactly clear where these peptides bind to HPSE. What is known, is that HPSE does have multiple glycosaminoglycan (GAG) binding sites, that do not all play a direct role in enzymatic activity³⁰. These binding sites could be a plausible hypothesis for why the peptides encoding for the recovered sequences did bind to HPSE, but did not inhibit the enzyme's biological activity in inhibition assays.

The library used in a subsequent selection has to be modified in such a way that the translated peptides target the active site of HPSE. Krauss *et al.* (2014) reported successful results from using an *in vitro* selection of peptide libraries with unnatural amino acids, modified with glycans by a so-called copper(I)-catalysed alkyne-azide cycloaddition (CuAAC) to design glycopeptide vaccines against HIV³¹. Their aim was to use the peptide libraries as a carrier for the glycoclusters, that in turn bind to the target of interest and mimic the HIV antibody 2G12 epitope. In this project, a sulfated disaccharide will be covalently attached to the peptide library by CuAAC click chemistry³²⁻³⁴. The use of a substrate to help targeting and binding the peptide library more specifically to the enzyme's active site to find peptide inhibitors is what makes the method described in this thesis novel. In our ideal case, the substrate is just needed in mRNA display for targeting the peptide libraries to the active site, after which the substrate is cleaved off. When these hits have been found, they can be synthesised and tested on their ability to inhibit the enzyme without clicking on the substrate.

CuAAC is a cycloaddition between an azide and a Cu(I)-activated alkyne to yield a 1,2,3-triazole. In this project, the sulfated disaccharide substrate (**1**, *S.I. figure S 1*), with an azide-bearing aromatic aglycone, can react with the alkyne group of homopropargylglycine (Hpg, **2**) that is reprogrammed as non-natural amino acid on a methionine codon to yield the clicked product (**3**) (*figure 5*)³⁵.

Hpg is a methionine analogue, which enables the methionyl-tRNA synthetase to load this unnatural amino acid on the elongator tRNA encoding for methionine, recognizing CAU codons ($tRNA_{CAU}^{Met}$)³⁶. By doing this, the unnatural amino acid can be incorporated into a peptide sequence. Translation can also be initiated with Hpg by omitting methionine and adding formyl donor³⁷. By formylating the Hpg initiator-tRNA $tRNA_{CAU}^{fMet}$ (Hpg-*ini*- $tRNA_{CAU}^{fMet}$), initiator factors recognize this formylated tRNA complex and translation is initiated with the unnatural amino acid Hpg.

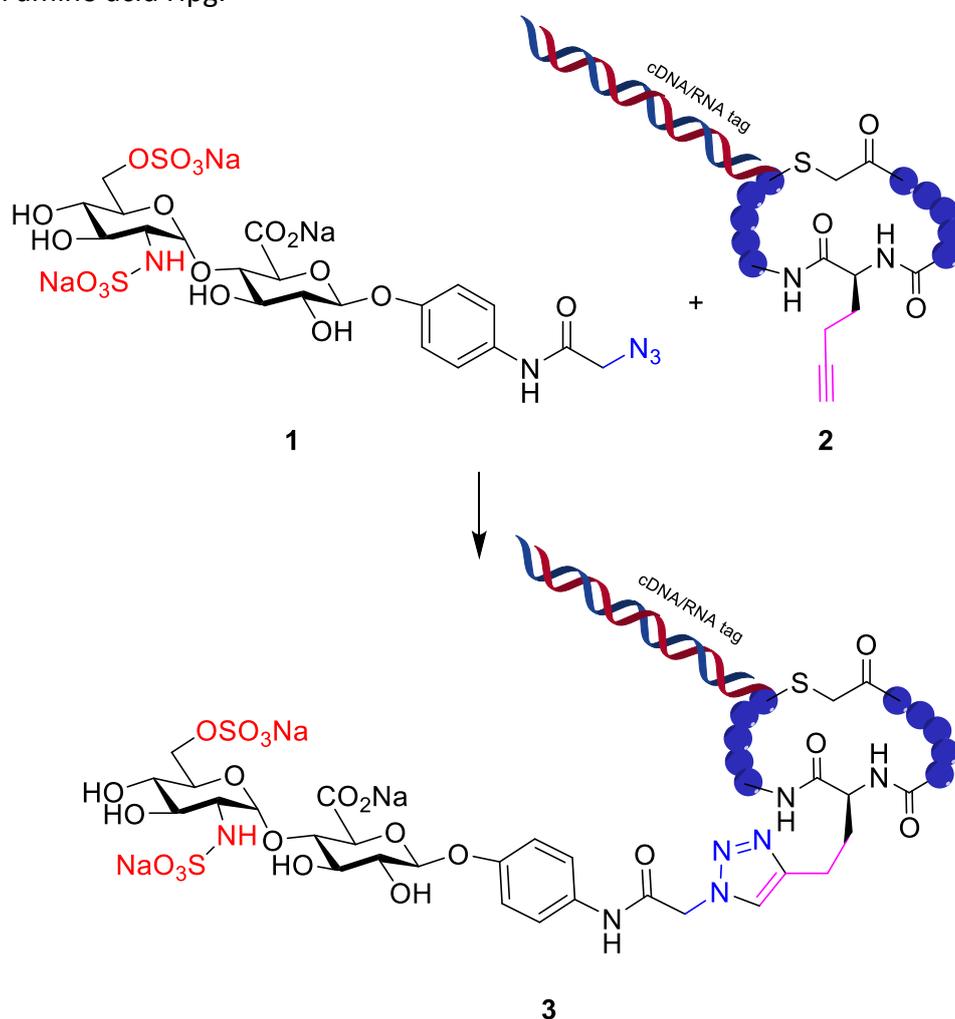


Figure 5: Overview of CuAAC reaction of HS-DS with macrocyclic peptide with homopropargylglycine, linked to mRNA/cDNA. **1** = HS-DS. **2** = homopropargylglycine in a macrocyclic peptide, covalently linked to mRNA/cDNA. **3** = product after CuAAC reaction.

The aim of this project is to develop and optimise an approach to selectively target HPSE's active site by using CuAAC click reaction in mRNA display to discover novel peptide inhibitors of HPSE. One of the two screened peptides library includes the unnatural amino acid chloroacetyl tyrosine (ClAc-Tyr) at the beginning of a sequence of 15 random amino acids, incorporated by reprogramming an initiating methionine codon. A cysteine at the end of the sequence enables the formation of a macrocyclic peptide through a spontaneous S_N2 reaction between the chloroacetyl group and thiol group, yielding a thioether bond³⁸. Cysteines that end up at any other position in the sequence could also form macrocycles, which increases the number of different ring sizes in the peptide library. Besides the ClAc-Tyr, the unnatural amino acid homopropargylglycine (Hpg) will be incorporated by reprogramming elongating methionine codons randomly incorporated at any position in the peptide sequence. To increase the chance of finding a biologically stable inhibitor, only the D-enantiomer of ClAc-Tyr is used³⁹. The second library will be initiated with Hpg by adding formyl donor, besides omitting methionine as in the first library³⁷. This library consisted of linear peptides, with one or multiple Hpgs ending up in the translated sequence. Hpg will be used in CuAAC to 'click' the aforementioned disaccharide to the library to target the HPSE active site^{31,32,34}. This library modification, together with the use of both linear peptides and cyclic peptides with ring sizes ranging from 3 – 17 by initiating with ClAc-D-Tyr, will increase the chance of finding a suitable inhibitor. By using this strategy, we hope to obtain peptides targeting the HPSE active site, thereby inhibiting HPSE's biological activity.

Results and discussion

As mentioned in the introduction, the CuAAC reaction to chemically modify peptide libraries in *in vitro* selections has been successfully used and described before³¹. However, Krauss *et al.* (2014) used a different mRNA display setup, with multiple library purification steps (capturing on oligo cellulose, filtration, ethanol precipitation and Ni-NTA agarose purification) and they required multiple modifications of the peptide library. Thus, their described click chemistry conditions do not transfer directly to the conditions in this project, but are a good starting point. Especially for the strategy to target HPSE's active site, it is crucial to collect evidence that the click reaction works as efficient as possible, to ensure that the peptide libraries are directed to the active site as much as possible. Therefore, before starting any preliminary assays and the actual mRNA display, optimisation of the CuAAC protocol is needed.

Optimisation of the CuAAC reaction with synthetic peptide

A peptide with sequence YILFRST~~X~~WSHPQFEKQ (~~X~~ = *Fmoc-Propargylglycine*, MW = 2160 g/mol), was synthesised on solid phase as described in experimental methods section 1. This peptide was used as alkyne-bearing template in optimisation click reactions. Azidohomoalanine was used as standard azide-bearing substrate. All click reactions were incubated at 42°C (*experimental methods section 2*). Table 2 gives an overview of the different conditions that were tested and their results.

Table 2: Results from the click test reactions that led to the optimal reaction conditions. The '[Alkyne]/[azide]' column indicates the final concentration of alkyne peptide and azide substrate in the reaction. 'CuAAC components' shows the component that deviates from the standard conditions given in table 2, Std = standard (500 μ M CuSO₄, 2.5 mM THPTA, 5 mM aminoguanidine, 5 mM sodium ascorbate and 50 mM phosphate buffer pH 6.5). 'Conversion of SM' is an estimation of the conversion of starting peptide to clicked product, calculated by comparing the peak area of most abundant extracted ion of starting peptide at different timepoints.

[Alkyne] / [azide] (μM)	CuAAC components	Reaction time	Conversion of SM
100 / 1000	Std	1 hr	97%
10 / 100	Biotin-PEG4-azide	2 hr	N.A.
25 / 100	Std	2 & 5 hr	85% & 94%
25 / 100	HS-DS	2 & 3 hr	98%
25 / 100	Std	0.5 & 1 hr	75% & 91%
25 / 100	50% Na Asc	1 hr	87%
25 / 100	500 μ M CuI / 2.5 mM THPTA	0.5 & 1 hr	30% & 47%
25 / 100	pH 8.3 (selection)	1 & 2 hr	N.A.
25 / 100	pH 8.3 (selection)	10 min	99%

The optimisation included multiple click reactions with varying conditions, checking the conversion by LC-MS. The major problem in analysing these LC-MS spectra, is the fact that the peaks of starting peptide and clicked product were overlapping in case of the azidohomoalanine substrate. Biotin-PEG4-azide was used as an alternate test azide with the idea to yield a product with a different retention time than the starting peptide itself, however no product peak was observed at all (*S.I. figure S 4 and S 5*). This may be because of poor ionisation. It would be ideal to see the starting material peak disappear, and to see a product peak with different retention-time appearing over time. This overlap makes it impossible to analyse the conversion by UV-traces. For this reason, the conversion of peptide to clicked product was estimated by using the peak area of the most abundant extracted ion of starting material at different timepoints, but this has a larger associated error because of potential differences in ionisation. In mRNA display, the concentration of translated peptide is 1 – 2 μM . The test click reactions should be as close to that low micromolar concentration as possible. 100 μM was too far away from the 1 – 2 μM and 10 μM synthetic peptide was barely visible on LC-MS, thus 25 μM was the best compromise as test concentration. Only one test was done with HS-DS due to scarcity of this component, but the 98% conversion indicates a good click efficiency. 0.5 and 1 hour reaction time seemed to give a comparable yield to 2 or 5 hours, which should enable a faster workflow. A reaction starting with 50% sodium ascorbate was tested to see whether this component depletes over time, and the remaining 50% was added after 30 minutes. This did not significantly improve the conversion, which indicates that the sodium ascorbate does not get depleted. CuI was used instead of CuSO_4 as source of Cu^+ , but the conversion of starting material decreased significantly to 43% and no extracted ions of clicked product were found. In the RaPID protocol, the reverse transcription, directly before click reaction, is executed at pH 8.3, thus the effect of this pH was tested on the efficiency of the click reaction. As shown in table 2, it was not possible to determine the conversion, since the starting material already disappeared at $t = 0$ hr (*S.I. figure S 16 and S 17*). This suggests that the reaction rate is relatively high at pH 8.3. The reaction was set-up again, with taking LC-MS samples at $t = 0$ hr, prior to adding CuSO_4 , THPTA and aminoguanidine, and at 10 minutes after adding these components. The estimated conversion is 97-99% after only 10 minutes. These optimisation tests led to the most efficient conditions, as stated in table 3. Figure 6 and 7 show the LC-MS spectra and extracted ions of the reaction with these optimal conditions. All LC-MS spectra, extracted ions and peak areas can be found in Supplementary Information Figure S 2 – 17.

Table 3: Optimal conditions for the click reaction, as found in the optimisation tests.

Component	Concentration
Alkyne test peptide	25 μM
Azide	100 μM
CuSO_4	500 μM
THPTA	2.5 mM
Aminoguanidine	5 mM
Sodium Ascorbate	5 mM
Tris HCl buffer (pH 8.3)	50 mM
Incubate at least 10 minutes at 42°C	

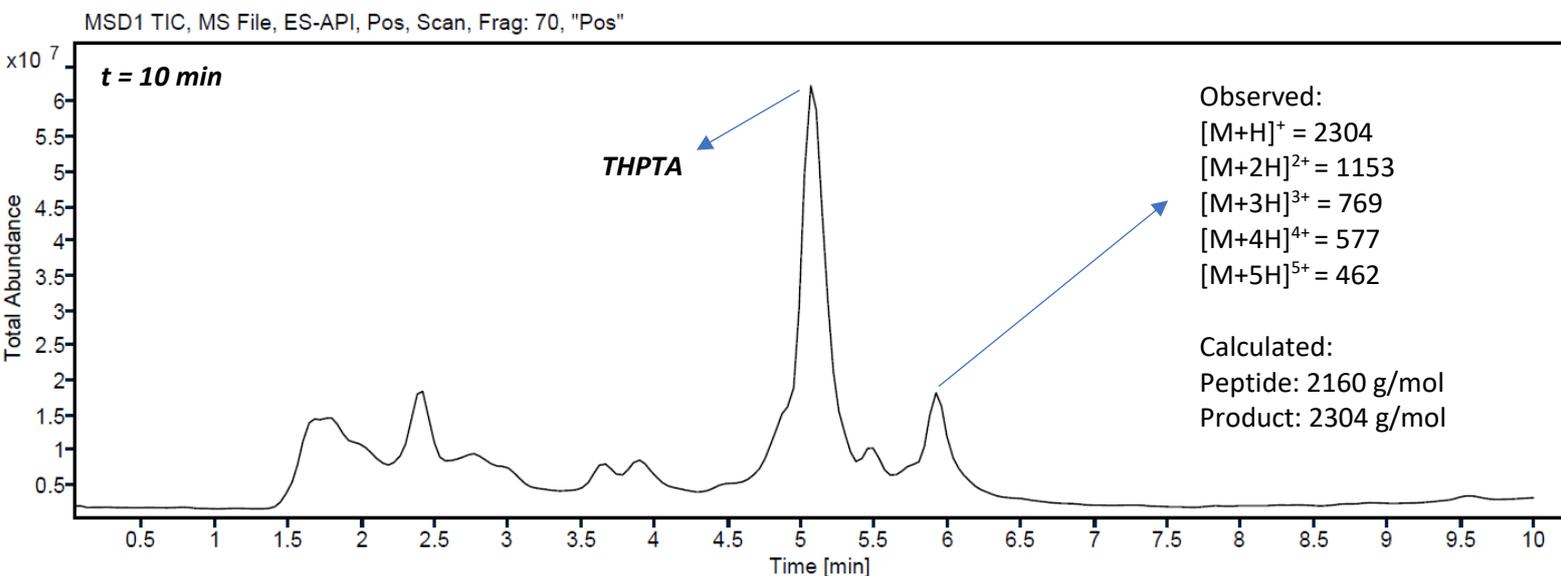
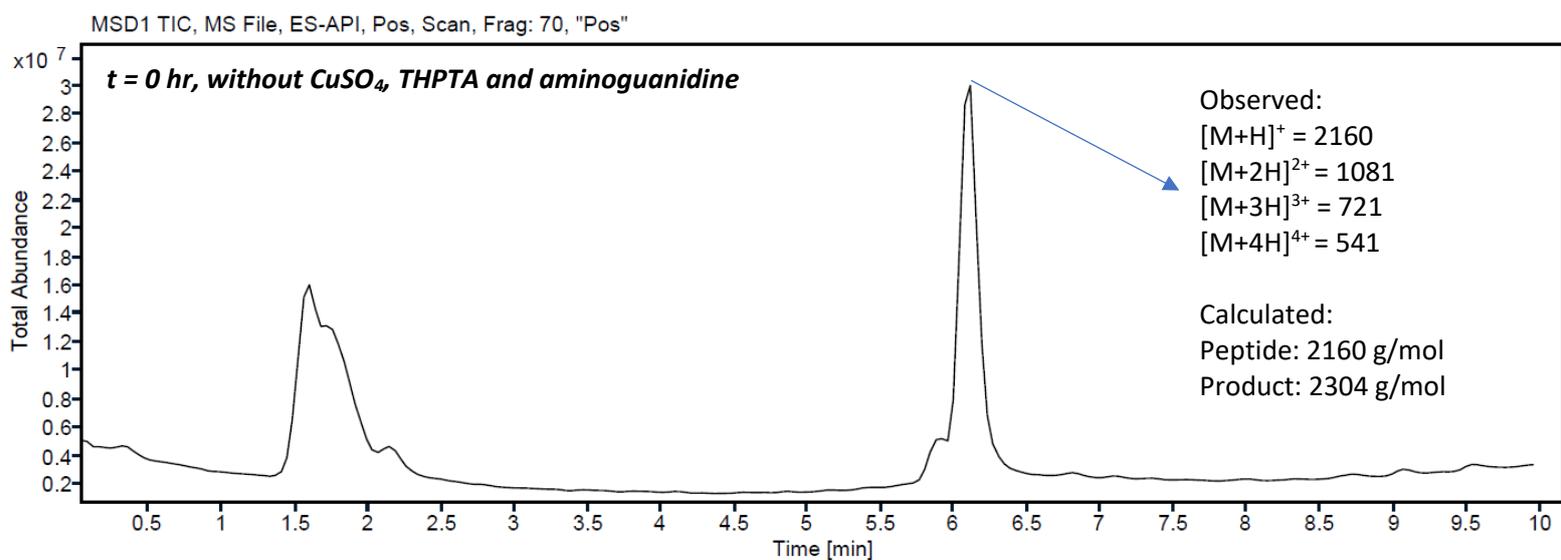


Figure 6: Total ion chromatograms showing the click reaction at optimised conditions, measured at timepoints 0 and 10 minutes in Tris HCl buffer pH 8.3.

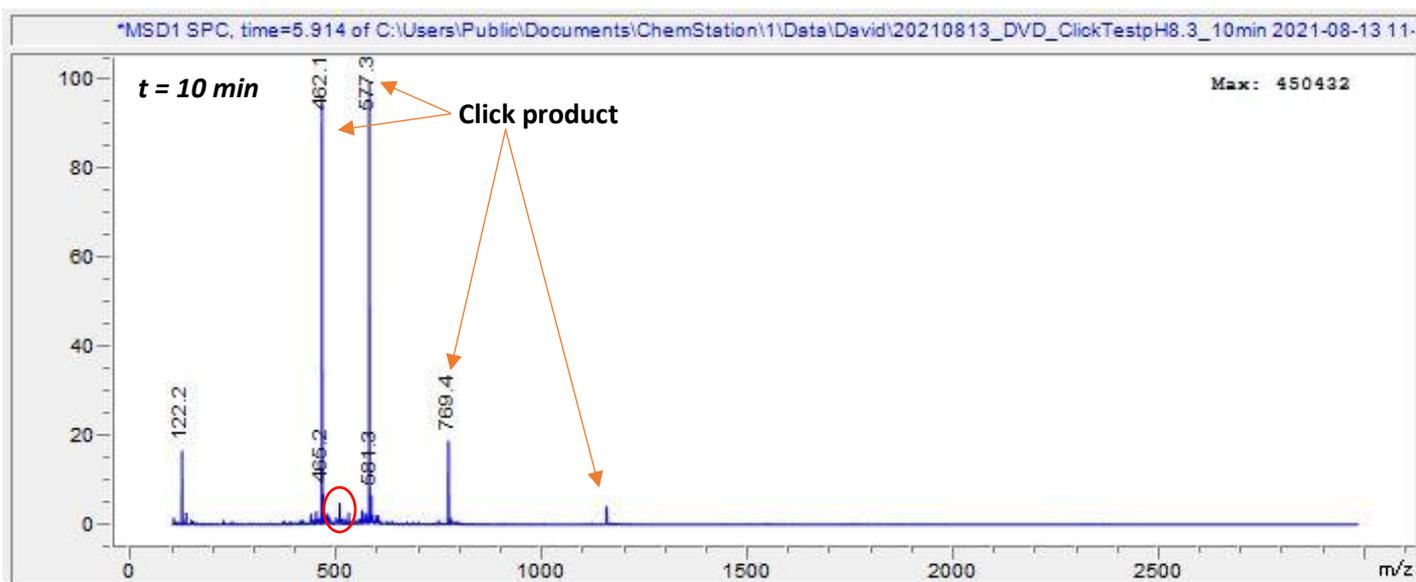
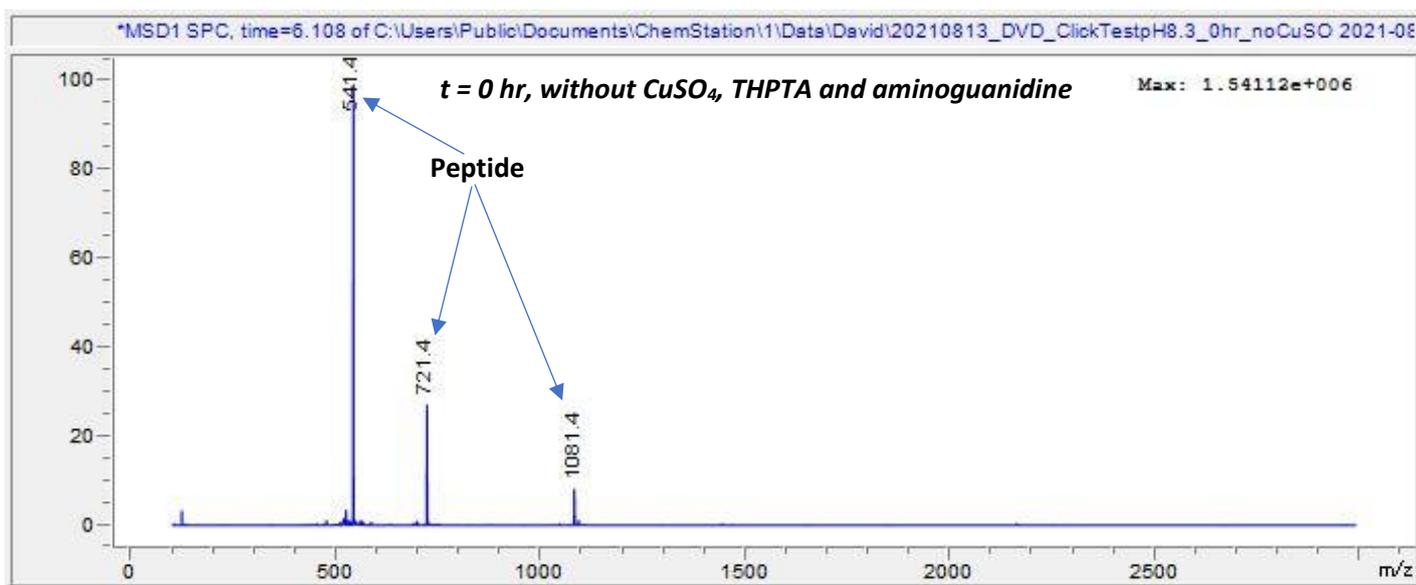


Figure 7: Extracted mass spectra at retention times 6.1 and 5.9 min (before and after click reaction), shown in figure 6 at timepoints 0 and 10 minutes.

The peak area of the most abundant extracted ion at t = 0 (541) was used to roughly estimate the conversion of starting material:

t = 0 hr, peak area: 121 million

t = 10 minutes, peak area: 2.2 million (peak in red circle) → 99% of starting material converted

Optimisation of the CuAAC reaction with *in vitro* translated peptide

After finding the optimal conditions for the click reaction on synthetic peptide, these conditions must still be tested and validated on *in vitro* translated peptide, resembling the scale and conditions of the RaPID system. For this, an *in vitro* translation using an RNA template encoding an arbitrary sequence ('Streptag') was executed, initiating with ClAc-D-Tyr and reprogramming elongating methionines with homopropargylglycine (*experimental methods section 3 and 4*). After translation, the resulting peptide was used in click reactions, to further optimize the reaction conditions. At first, the translated clicked peptides were purified by C18 extraction, after which the products were analysed on LC-MS. The selection protocol gives translated peptides at approximately 2 μ M scale, which turned out to be too low to detect on LC-MS (*data not shown*). To attempt to bypass this low scale problem, biotin-PEG4-azide was clicked on the Streptag peptide, after which the product was pulled-down with magnetic M-280 Streptavidin beads. For this experiment, the template RNA was first ligated to puromycin, and reverse transcribed to cDNA after translation. The pulled-down product was amplified and analysed by qPCR, but this also did not give a clear result (*data not shown*).

Finally, UREA-PAGE gels were used to visualize the click reaction with *in vitro* translated peptide while attached to mRNA. Template, ligated mRNA, translated peptide and clicked product were visualised on the gels by staining the RNA, present in each sample due to the puromycin ligation, with SYBRTM GREEN II RNA gel stain. These attempts gave gels with completely stained lanes, which was hypothesized to be caused by interference of the gel with click components. However, an experiment testing the influence of each separate click component on the gel staining, did still show black lanes for each component (*S.I. figure S 19*). The control of translation mix without any click component was stained completely black as well. It is unlikely that the black smearing is caused by all click components. Besides that, after phenol/chloroform extraction and EtOH precipitation were implemented in the protocol after translation and EtOH precipitation after click reaction to remove the proteins and click components, the lanes were still completely stained. This suggests the persistent presence of undesired RNAs added during translation (e.g. tRNAs and/or rRNA) that can not be removed by these clean up methods. These undesired RNAs most likely cause the smeared staining of the gels (*S.I. figure S 18-20*).

This problem was solved by two measures. Firstly, the 8% urea-PAGE gel was changed to 8% urea-PAGE gel with 0.05% SDS, with a stacking and separating layer (*experimental methods section 4*)²⁴. The use of a stacking layer sharpens all bands and make small changes clearer, and SDS improves electrophoretic behaviour of protein and peptide components. Secondly, the RNA will be annealed to a FAM-labelled fluorescent oligo (FAM - Fluorescein Amidite), which enables visualisation by emission at 472 nm instead of staining RNA with SYBRTM GREEN II. This ensures a more specific visualisation of just the desired product RNAs. Figure 8 shows the gel using these conditions that enabled visualisation of ligated and translated product for the first time. This technique also enables the quantification of puromycin-ligation and translation reaction yield by densitometry. These gels show that with the mRNA display ligation and translation conditions, 75% of template mRNA is ligated to puromycin, and 25% of this product is translated to peptide-puromycin-mRNA.

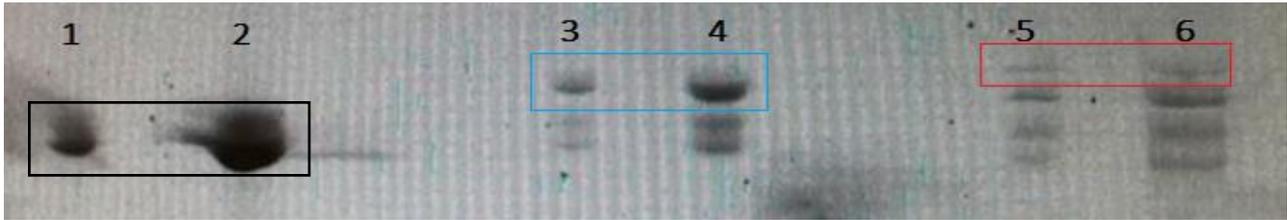


Figure 8: 8% urea-0.05% SDS-PAGE gel analysis of ligation and translation products annealed to FAM fluorescent oligo. The bands were visualised by excitation at 472 nm. Template mRNA is indicated in black box. Puromycin-ligated mRNA is indicated in blue box. Peptide-puromycin-mRNA translation product is indicated in red box. S.I. Figure S 21 for raw image.
Lanes: 1) 2 μ M template mRNA; 2) 5 μ M template mRNA; 3) 2 μ M puromycin-ligated mRNA; 4) 5 μ M puromycin-ligated mRNA; 5) 2 μ M peptide-puromycin-mRNA; 6) 5 μ M peptide-puromycin-mRNA.

A ligation product contains an extra covalently linked puromycin compared to template, which is visible as a band shifted upward (figure 8). A translation product contains an extra covalently linked peptide compared to template-puromycin, which is also visible as an upward shifted band. The same counts for the clicked product, it should show an additional band shifted upward. Thus, the ability to display ligation and translation by urea-SDS-PAGE gels, entails the ability to display the click reaction with *in vitro* translated peptide. Figure 9 shows the first attempt to visualize this click reaction.

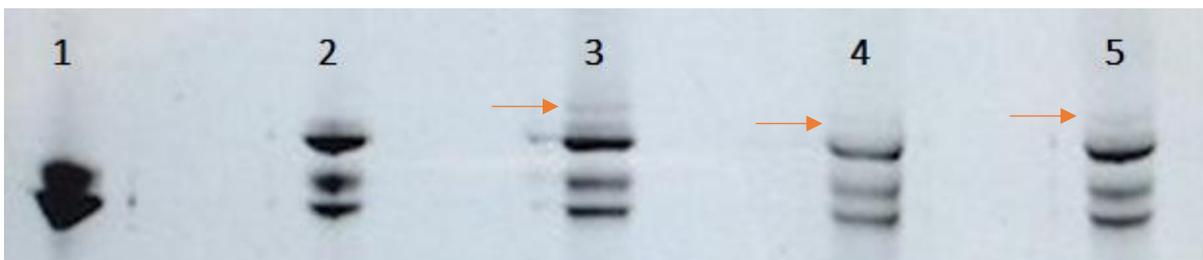


Figure 9: visualisation of click reaction by 8% urea-0.05% SDS-PAGE gel, visualised by excitation of FAM oligo at 472 nm. Translation product indicated with red arrows. S.I. Figure S 22 for raw image.
Lanes: 1) 2 μ M Streptag template mRNA; 2) 2 μ M puromycin-ligated mRNA; 3) 2 μ M translation product;
4) 2 μ M translation product clicked to 100 μ M 3 kDa azidopeptide;
5) 2 μ M translation product clicked to 100 μ M iFluorTM 647 azide.

As shown in figure 9, the translation product is visible as a faint band in lanes 3, 4 and 5. However, there is no sign of an additional band caused by clicked peptide. This suggests that the shift caused by clicking an 3 kDa azidopeptide or azido fluorophore may be too small to detect on gel. If there is a clicked product, it most likely overlaps with the band from the translated peptide. This problem was finally circumvented by using iFluorTM 647 azide as click substrate, but now visualising the clicked product at 742 nm (iFluorTM 647 azide), besides still visualising the template, ligation and translation with FAM oligo at 472 nm. Figure 10 and 11 show the results from these click reactions.

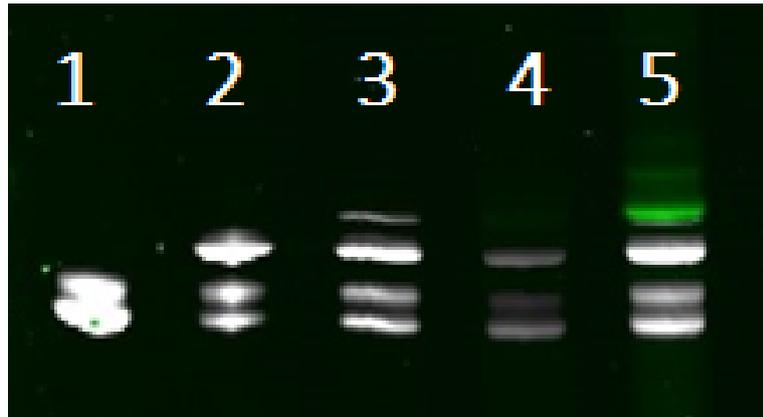


Figure 10: Conformation of click reaction in mRNA display by visualisation on 8% urea-0.05% SDS-PAGE gel at 472 nm (grey, FAM oligo) and 742 nm (green, iFluor™ 647 azide).

Lanes: 1) 2 μ M Streptag template mRNA; 2) 2 μ M puromycin-ligated mRNA; 3) 2 μ M translation product; 4) 2 μ M translation product clicked with 100 μ M iFluor™ 647 azide Overnight at 42°C; 5) 2 μ M translation product clicked with 1 mM iFluor™ 647 azide 45 minutes at 42°C.

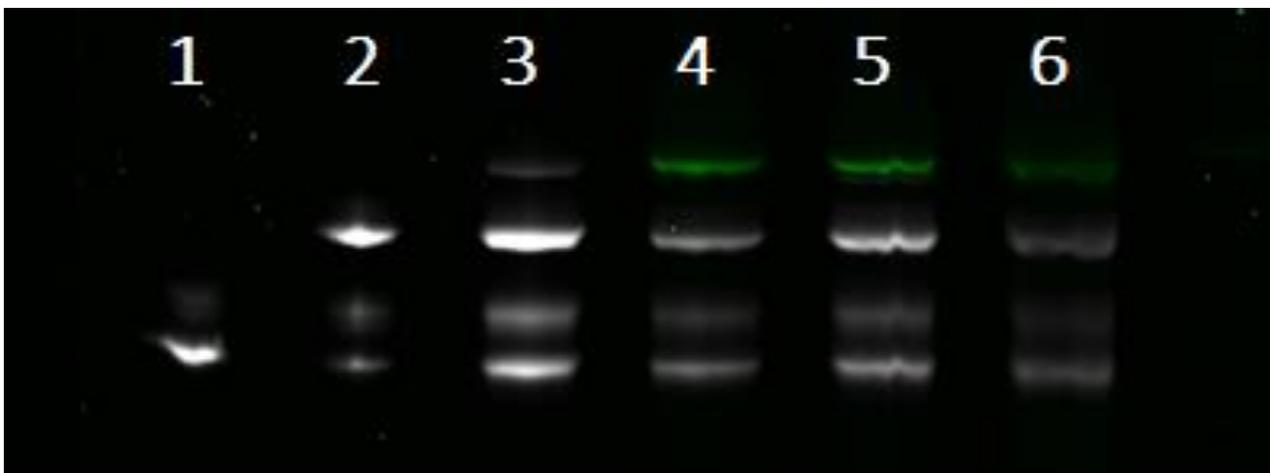


Figure 11: Optimisation of click reaction conditions in mRNA display by visualisation of click reaction on 8% urea-0.05% SDS-PAGE gel at 472 nm (grey, FAM oligo) and 742 nm (green, iFluor™ 647 azide).

Lanes: 1) 2 μ M Streptag template mRNA; 2) 2 μ M puromycin-ligated mRNA; 3) 2 μ M translation product; 4) 2 μ M translation product clicked with 1 mM iFluor™ 647 azide 2 hours at 42°C; 5) 2 μ M translation product clicked with 0.5 mM iFluor™ 647 azide 2 hours at 42°C; 6) 2 μ M translation product clicked with 1 mM iFluor™ 647 azide overnight at 42°C.

These results confirm the most important aspect of this project: the click reaction does work with *in vitro* translated peptide. However, a problem remains that this method is not quantitative, since the clicked product is visualised in a different channel than the template mRNA.

These results, optimisation of the click reaction with synthetic peptide and *in vitro* translated peptide, led to the following three conclusions: 1) analysing the click reaction with synthetic peptide by LC-MS shows an estimated conversion of 97-99% using the optimised conditions; 2) analysing the click reaction with synthetic peptide and HS-DS by LC-MS shows a rough estimated conversion of 98%; 3) urea-SDS-PAGE gel analysis of the click reaction with *in vitro* translated peptide confirmed that the click reaction gives detectable product on 2 μ M peptide scale. In these experiments, at least 0.5 mM substrate and a 2 hour 42°C incubation of the click reaction, seem to give the best conversion. Overnight incubation of the reaction significantly reduces the yield, possibly because of degradation of the mRNA-displayed peptide (*figure 10*).

These results were considered as sufficient evidence that the modification of the peptide libraries in the mRNA display selection will work. However, because of the risk that the reaction will be less efficient at the lower concentration of mRNA display in the selection, more forcing conditions in terms of HS-DS final concentration (1 mM) and reaction time (2 hours at 42°C) were used, to further push the reaction to the desired product. Besides that, the click reaction will be set up under N₂ flow and incubated at 42°C in sealed tubes filled with N₂, to prevent unwanted oxidation of the click components as much as possible. With the click reaction optimised and validated, the immobilization of the target protein can proceed.

Immobilization of heparanase

Part of the RaPID system involves immobilization of the target of interest on magnetic beads (*figure 4*). To enable this, the protein must be biotinylated (*experimental methods section 5*). After biotinylation, an assessment of streptavidin bead binding was done to find the percentage of biotinylated protein (*S.I. figure S 23*) (*experimental methods section 6*). This is needed to calculate the amount of biotinylated protein and magnetic beads to be used in the selection's pulldown. The results of the bead assessment assay are shown in *figure 12*, with 69% of the enzyme being biotinylated in the reaction with 8 equivalents of biotin-NHS.

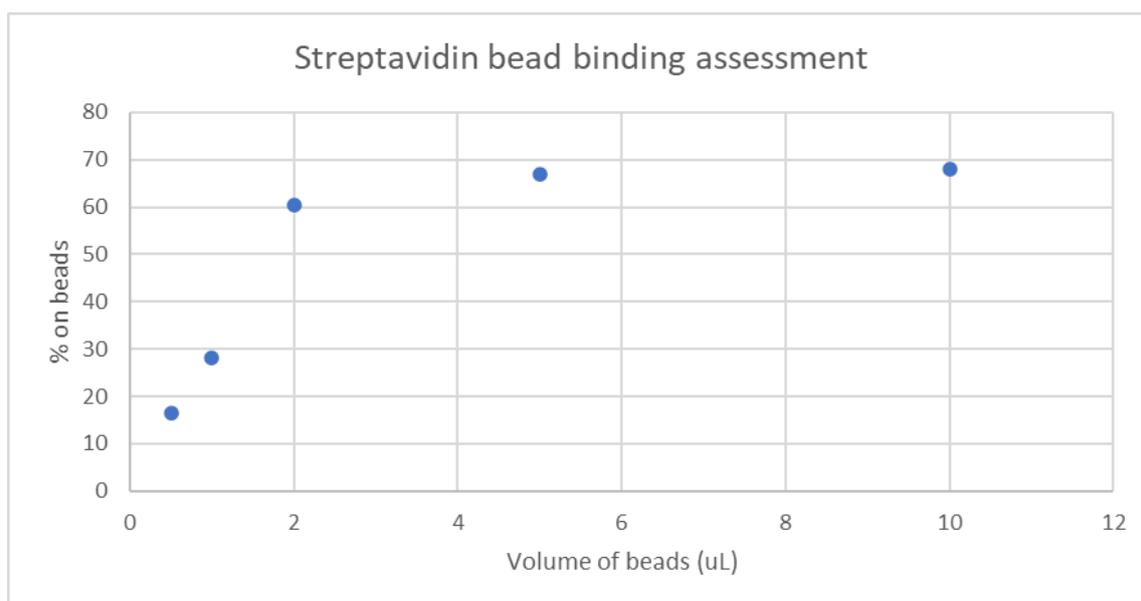


Figure 122: Streptavidin bead binding assessment to determine the percentage of biotinylated HPSE. X-axis shows the volume of beads, Y-axis indicates the corresponding percentage of proteins on beads, as quantified from densitometry on SDS-PAGE (Figure S 23).

Activity assessment of biotinylated heparanase

After biotinylation of HPSE, the activity must be verified before the biotinylated protein can be used in the selection. To be able to selectively target the active site, the enzyme must be in its active conformation. To prove that the biotinylation does not interfere with this active conformation, and for instance blocks off the active site, an activity assay was executed (*experimental methods section 7*). For this, a pentasaccharide substrate (fondaparinux) was incubated with the biotinylated protein at 37°C for 45 minutes. Cleavage of the substrate yields a disaccharide with a reducing end, which develops a blue color after 1 hour incubation with WST-1 at 60°C. Absorbance at 485 nm is used to detect the amount of product formation, which is a representation of the enzyme's activity⁴⁰. The absorbance value of unbiotinylated HPSE after 45 min was set at 100% activity (Abs of 4.3 at 485 nm), the other samples' relative activities to this 100% value were calculated using their own absorbance values. The results of the in solution activity assay are shown in figure 13. A standard curve of 0.2 μ M – 0.25 mM D-glucose was made to show the absorbance plateau, which is at 12 (S.I. Figure S 24).

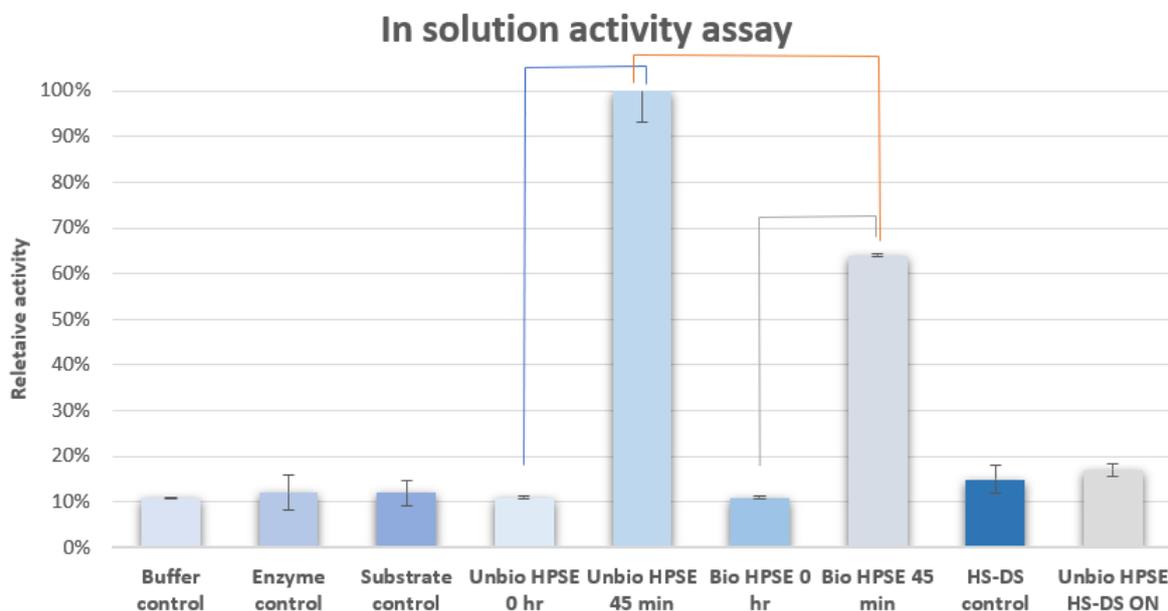


Figure 133: Colorimetric activity assay of biotinylated HPSE. The relative activity is the percentage of absorbance (Abs) at 485 nm compared to absorbance of Unbio HPSE 45 min, which was set to 100% activity. All measurements were executed in duplicate, the average absorbance is used to calculate the relative activity. The standard substrate is fondaparinux, HS-DS was only used in the last two measurements (HS-DS control and unbio HPSE HS-DS ON). Unbio = unbiotinylated, bio = biotinylated, HPSE 45 = Un-avitagged HPSE, HS-DS = heparan-sulfate disaccharide, ON = overnight. Standard deviations are shown. Important comparisons are indicated with blue, orange and grey lines.

From the assessment of protein binding to magnetic beads, 31% of HPSE was known to not be biotinylated. As shown in figure 13, a relative activity of 64% was found from reaction by the biotinylated HPSE after 45 min (Abs of 2.6 at 485 nm). It was concluded that that 64% conversion can not be caused by just 31% of the HPSE that is not biotinylated. From this, the conclusion can be drawn that (part of) the biotinylated HPSE must still be active.

Selection RaPID

With all click chemistry conditions optimised and the target protein prepared, the selection was initiated. From round 2, the amount of false-positive peptides unspecifically binding or sticking to the beads was minimised by incubating the peptide libraries with beads without proteins (negatives) prior to each incubation with immobilized proteins (positives). The percentage of recovered cDNA after each round's pulldown is shown in figure 14, as determined by qPCR.

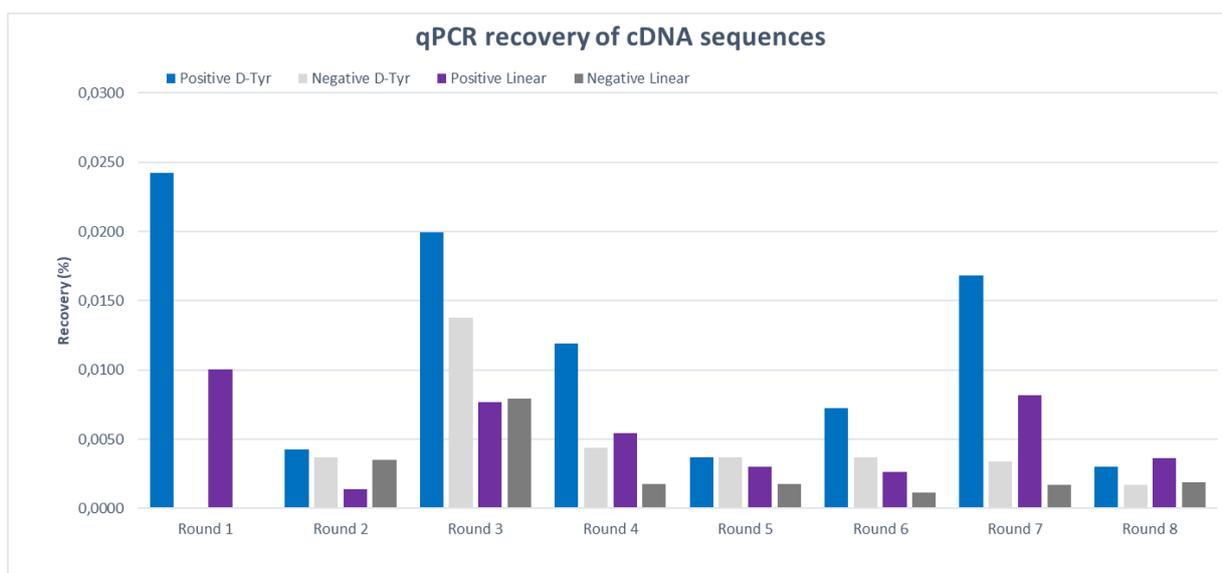


Figure 144: Selection results of a cyclic D-Tyr initiated library and linear Hpg initiated library against HPSE, showing the recovery of cDNA sequences across 8 rounds using RaPID mRNA display system. From round 5 to 7, a promising upward trend of the positives was ongoing, while the negatives stayed on the same level. The recovery of positives after round 8 decreased strongly, breaking this upward trend. This indicates that the libraries were not enriched with strong HPSE binders. X-axis indicates round numbers, Y-axis indicates the % of recovery calculated by dividing the amount of output cDNA (after pulldown by immobilized target) by input cDNA (after click reaction). Negatives show the % of cDNA recovered from beads without target protein. Positives show the % of cDNA recovered in the actual selection against immobilized target.

Typically, 0.1% recovery is considered as successful enrichment in RaPID display. As shown in figure 14, the recovery does not go above 0.025% and the ratio of positive to negative recovery does not increase consistently, indicating no selection and amplification of strong HPSE binders. The main problem in this selection was a recurring truncation of DNA after PCR and qPCR. Figure 15 shows the PCR product on 3% agarose gel after round 1 and round 4 of the selection. Around 50% of the DNA after round 4 is truncated product, compared to the 100% pure DNA libraries after round 1.

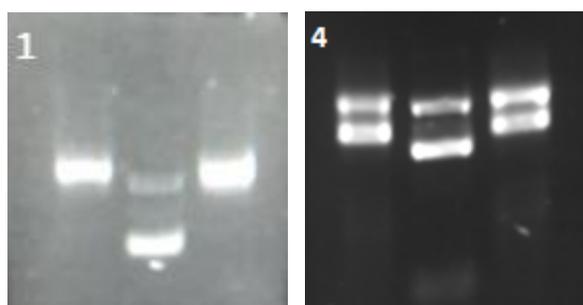


Figure 155: 3% agarose gel showing the PCR products after round 1 and round 4. Left: D-Tyr library PCR product, Middle: Marker, Right: Linear library PCR product. For both libraries after round 4, the top band represents the library DNA, the bottom band represents the truncated DNA. This truncated product was not present after round 1. Marker sizes: 132 base pairs, 98 base pairs and 67 base pairs.

The truncations are most likely caused by incorrectly annealing primers and primer-dimers. Our hypothesis is that these products are much shorter than the peptide's DNA sequences, causing them to be amplified faster, since the PCR is biased towards these shorter sequences. Thereby, the amplification goes exponentially, thus the desired product is quickly outcompeted. However, in theory, all sequences should be amplified equally with a long and efficient enough extension step. After round 3 and 7, the *in vitro* transcribed RNA product was purified from the truncated contamination by denaturing urea-PAGE gel (*experimental methods section 9.1*) and after round 4, the DNA product was purified from truncated DNA by an agarose gel DNA extraction kit from Macherey-Nagel (*experimental methods section 9.2*). Unfortunately, these steps were not sufficient to recover the selection. The truncation started from round 3 and came back stronger every round, even after the purification measures. All 3% agarose gels are shown in S.I. figure S 25, which shows the impact and persistency of this problem. There is a small chance that the truncation masks the qPCR results, and thereby the recovery calculation. However, if hits were enriching well, they would bind to the target and compete against the truncations which would not bind. Thus, it is very unlikely that binders were still selected after these 8 rounds. The increase in recovery from round 5 – 7 looks promising, but round 8, executed with round's 7 purified RNA, gave a strong decrease in recovery.

Design of the cDNA-TRAP libraries

The RaPID selection results leave three possibilities. Firstly, this RaPID selection can be repeated, by going back to early rounds. The first rounds are critical in determining the selection's chance of success. The selection should be re-started from round 1 or 2 and the desired product should be purified from truncated product after every round. Switching between (q)PCR primers could be implemented to reduce the chance of primer-dimers and truncation formation. Secondly, similar libraries could be prepared for another RaPID selection using a different spacer in the primers, and thus a completely new primer set. In theory, only the binding peptides from the library should then be amplified and selected. A likely cause for the primer issues could be the supply issues of filter tips we encountered. Consistently using filter tips in another RaPID attempt, should increase the chance of success as well.

Finally, the selection against HPSE can be tried with the TRAP or cDNA-TRAP system, as mentioned in the introduction. Normally, in a successful selection, >0.1% enrichment of the cDNA sequences is seen after 5 or 6 consecutive rounds. The TRAP and cDNA-TRAP display systems allow 6 rounds of selection in ± 14 hours, whereas the executed 8 rounds of RaPID took multiple weeks. The difference between TRAP and cDNA-TRAP display is that in TRAP the peptide is covalently linked to its encoding mRNA, whereas in cDNA-TRAP the peptide is covalently linked to the cDNA (*figure 16 B*)^{23,24,41}. Taking into account the CuAAC modification modifications done with the libraries, cDNA-TRAP is the preferred display system, since cDNA is more stable than RNA in these reaction conditions. Thus, by being much faster than RaPID and more stable than TRAP, cDNA-TRAP is the preferred option as next mRNA display system. For the future cDNA-TRAP selection, novel NNK15 libraries were designed and assembled by PCR (*experimental methods section 8*).

In the RaPID system, a standard random cyclic D-Tyr initiated NNK15 library and a linear Hpg initiated NNK15 library were used. In the cyclic library, it was assumed, by probability, that at least one methionine would be randomly incorporated into each peptide, which would become a Hpg through reprogramming. The linear library, with at least one Hpg ending up in the sequence, was used since a linear sequence may be able to fit better in the binding cleft. The cDNA-TRAP libraries are random NNK15 libraries, with methionines at fixed positions 3, 6, 9 or 12 in the sequence. As a result, it can be said with 100% certainty that every peptide contains at least one Hpg by reprogramming, thus a HS-DS can be clicked on every peptide. For the cDNA-TRAP libraries, initiation with both D- and L-enantiomers of ClAc-Tyr will be used instead of only the D-enantiomer and a linear library. This will cause both libraries to be cyclic through S_N2 reaction with an established cysteine at the end of every peptide sequence. As mentioned in the introduction, ClAc cyclisation can form cyclic peptides with cysteines randomly ending up at position three in the peptide. A 17 amino acid peptide with such a small cycle of three amino acids, will essentially be a linear peptide. The use of two cyclic libraries is solely a hypothesis, it might work, but there is no substantiated evidence that it will lead to better inhibitors than linear peptides. Nevertheless, both libraries consisting of macrocyclic peptides, instead of using one linear library, does entail an advantage. If eventually a hit is found, it will be a macrocyclic and not linear peptide. Macrocyclic peptides have the advantage to be more resistant to proteolysis and are known to have a higher potency in being drugs than linear peptides⁴².

Conclusion and Future directions

In the aforementioned selection of Krauss *et al.*, an uptrend in the positives eventually led to a major spike to significantly higher recovery percentages after 10 rounds of selection, indicating the enrichment of libraries with glycopeptides binding to their target. Sequencing of their libraries and testing the hits indeed demonstrated the enrichment of multivalent glycopeptides binding to their target of interest with low nM K_D 's³¹. In this project's RaPID selection against HPSE, the results from round 5, 6 and 7 showed a promising upward trend as well, however this trend was broken by a strong decrease in recovery in the positives of round 8. This indicates that no strong HPSE binders were selected from the modified libraries in the RaPID mRNA display selection. The main issue in this selection was the recurring truncation of the libraries. Despite not having demonstrated the *in vitro* selection of potential HPSE inhibitors from glycan-modified peptide libraries in RaPID mRNA display, the aim to develop and optimise an approach to use the combined CuAAC click reaction and mRNA display systems to selectively target the active site of HPSE has been achieved.

Besides the developments and knowledge gained regarding selection against HPSE, more insights have been gained in this project. The ability to display low micromolar modifications of peptide libraries is a novel purpose for the urea-SDS-PAGE gels. This can, besides being used in solely selections, be used for monitoring other peptide modification reactions. Also, the optimised conditions for the click reaction can be used in future selections (i.e. RaPID, cDNA-TRAP) with similar purposes. Click chemistry in mRNA display has been described by Krauss *et al.* and is, on itself, not novel. However, the use of a combination between commercial translation kits, an easier mRNA display protocol without multiple, extensive purification steps and the use of the CuAAC modification to target the peptide library to an enzyme's active site are novel. This use of the previously described click reactions in mRNA display allows modification of peptide libraries with different substrates, for more specific targeting of peptide libraries to the active site of drug targets.

At the time of writing this thesis, the puromycin-oligo used in the cDNA-TRAP system is still being assembled (*figure 16 B*). The selection results and potential sequencing and testing results will not be available yet. The DNA libraries for cDNA-TRAP were already prepared, thus the oligo is the last preparation needed before a consecutive selection can be executed. If this selection demonstrates no enrichment as well, there are other interesting aspects of HPSE that could be used to develop novel peptide inhibitors. Firstly, as mentioned in the introduction, there is the 6 kDa linker peptide in the active site cleft (TIM barrel) of in-active preheparanase, where it blocks HS chains from entering the active site². Peptides resembling this linker and binding noncovalently at the same site, may inhibit HPSE (activation). Secondly, as also mentioned in the introduction, the HPSE2 isoform lacks enzymatic activity, but plays a role in the inhibition of HPSE1 activity by inhibiting (pro)preheparanase internalisation and activation or by directly binding to active HPSE1^{3,4}. Peptide sequences that mimic this HPSE2 binding sequence might be potential HPSE inhibitors. A successful future mRNA display selection or development of therapeutic peptides targeting HPSE by another approach could contribute to a novel anti-cancer therapy.

Experimental methods

1. F-moc solid phase peptide synthesis

Alkyne test peptide, with amino acid sequence YILFRSTXWSHPQFEKQ ($X = Fmoc\text{-propargylglycine}$), was synthesised by automated Fmoc solid phase peptide synthesis using a Syro I (Biotage, Sweden) and Liberty Blue Microwave (CEM, USA) synthesizers. The peptide was synthesised on 114 mg (25 μmol) rink amide resin, Tentagel S RAM (0.23 mmol/g) (RAPP Polymere, Germany). Four equivalents of amino acid solutions (0.33 M) with 0.33 M oxyma in DMF were activated and coupled to the growing peptide sequence in two coupling steps of 30 minutes each by using 8 equivalents of activating reagent DIPEA (N,N-Diisopropylethylamine) (1.33 M) and 4 equivalents of the coupling reagents HATU ((1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium, 3-oxidhexafluorophosphate) (0.33 M) and PyBOP (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate) (0.33 M). The Fmoc-protected amino acids were deprotected by 25% Piperidine in DMF in two 10 minute reaction steps. After final deprotection, the peptide was completely deprotected and cleaved from Resin with cleavage solution (90% TFA, 5% H₂O, 2.5% TIPS, 2.5% DODT) for 2 – 3 hours, while shaken. Peptide was precipitated with cold diethyl ether and purified by preparatory-HPLC using a linear gradient 20-80% elution buffer (MeCN with 0.1% TFA) over 38 minutes on a Gemini 10u C18 Axia Packed, 250 x 21.20 mm 10 micron (Phenomenex) column. The peptide fraction was assessed for purity by LC-MS using a linear gradient of 5-90% elution buffer (MeCN with 0.1% FA) in 20 min on a InfinityLab Poroshell 120 EC-C18, 6.4 x 100 mm, 2.7 Micron column (Agilent). The peptide-containing fraction was lyophilised, after which the peptide was used in optimisation of the copper-catalysed click reaction.

2. CuAAC click reaction with synthetic alkyne peptide

In a microcentrifuge tube, CuSO₄, THPTA and Aminoguanidine were pre-mixed. For the test click reaction with the optimal conditions, the following was added:
25 μM alkyne test peptide
100 μM azide-bearing substrate
500 μM CuSO₄, 2.5 mM THPTA, 5 mM aminoguanidine
5 mM sodium ascorbate
50 mM Tris HCl buffer pH 8.3
Incubated at 42°C for 10 minutes.

Optimal conditions, these conditions were varied in each different optimisation click reaction. Variations on these optimised conditions for each different test are shown in table 2.

All reactions were on 30 or 45 μL scale, from which 15 μL was injected on LC-MS at every time-point. The conversion of starting peptide to clicked product was roughly estimated by comparing the peak area of the most abundant extracted ion at different timepoints. Extracted ion chromatograms were generated for starting material and product mass peaks of a calculated mass from 2+ - 5+ ionisation states.

3. Aminoacylations of tRNA^{fMet} (based on 10 μ L volume)

For 2 pellets of 125 pmol aminoacylated tRNA, the following protocol was followed: 25 μ M tRNAⁱⁿⁱ, 25 μ M eFX (for CME activation), 50 mM HEPES.KOH buffer pH 7.5 were mixed by pipetting, the volume was adjusted to 6 μ L with MilliQ water. The mixture was heated at 95°C for 2 minutes, followed by incubation at room temperature for 5 minutes. 0.6 M MgCl₂ was added, the mixture was first incubated at room temperature for 5 minutes and then cooled on ice. 5 mM of CME-activated amino acid in DMSO was added to give a final volume of 10 μ L. The reaction was left on ice for 2 hours. The 10 μ L was split to two times 5 μ L, equal to two times 125 pmol tRNA. The tRNAs were precipitated by addition of 20 μ L 0.3 M acetic acid, 50 μ L 100% EtOH. The mixture was vortexed and centrifuged at 15000 rcf for 15 minutes, the supernatant was discarded. The pellets were washed two times by addition of 30 μ L 0.1 M acetic acid, 70% EtOH in water, vortexed and centrifuged at 15000 rcf for 10 minutes, after which the supernatants were removed. Finally, the pellets were washed with 20 μ L 70% EtOH, followed by 3 minutes centrifugation at 15000 rcf, without vortexing. The supernatants were discarded and the pellets were dried for 3-5 minutes and stored at -80°C until further use in translation reactions.

4. CuAAC click reaction with *in vitro* translated peptide

For *in vitro* translations, the commercially available PURExpress Δ RF123 kit (NEB, UK) was used. The kit has two solutions that were used for translation: Solution A and Solution Δ B -RF. Solution A consists of small molecules, e.g. tRNAs, amino acids, energy supply and formyl donor. A homemade solution A was prepared with the similar components of the PURExpress solution A, being tRNA⁺ and either Fd⁻ (for Met initiation suppression) or Fd⁺ (for Met initiation)²⁸. Solution Δ B -RF consists of the ribosomes and proteins, and is missing release factors 1, 2 and 3 (Δ , -RF).

For *in vitro* translation click tests, a Streptag template was used, with sequence:

DNA:

TAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATatgtggagccatccgcagtttgagaagac
cgaatacgaatacctggattacgattttctgccggaatggaaccgctgggcagcggcagcgcTAGGACGGGGGCG
GAAA

Amino acid:

MWSHPQFEKTEYEYLDYDFLEPEMEPL(GS)₃, (**M** will be reprogrammed to Hpg)

Before translation, the template mRNA was ligated to puromycin in a 40 μ L at 1X reagents. The reaction was incubated 30 minutes at room temperature. The product was precipitated by addition of 1x vol. MilliQ, 2x vol. 0.6 M NaCl, 10 mM EDTA, 2 μ L 10 mg/mL glycogen and 8x vol. 100% EtOH, followed by vortexing and centrifugation at 15000 rcf for 15 minutes. The pellet was washed with 70% EtOH, centrifuged at 15000 rcf for 3 minutes. Supernatants were discarded and the pellet was air dried and resuspended in 4 μ L MilliQ to yield a 10 μ M puromycin-ligated mRNA solution.

5 μ L scale *in vitro* translation was started on ice using:
30% PURExpress Solution Δ B -RF
14.3% Homemade Solution A Fd-
25 μ M ClAc-D-Tyr acylated tRNAⁱⁿⁱ
0.5 mM 20 amino acid mix (-Methionine, +Homopropargylglycine)
2 μ M puromycin-linked RNA Streptag template
Volume was adjusted to 5 μ L with MilliQ water

The translation was incubated at 37°C for 30 minutes, at RT for 12 minutes and finally 1 μ L 100 mM EDTA was added prior to a final 30 min incubation at 37°C. After translation, the reaction mixture was diluted to 30 μ L with MilliQ water. The aqueous layer was extracted by consecutively 30 μ L P/C/I solution and 30 μ L C/I solution. The product was precipitated by addition of 1 eq. 0.6 M NaCl, 10 μ g glycogen and 2 eq. 100% EtOH. The solution was vortexed, centrifuged at 15000 rcf for 15 min, the supernatant was discarded and the pellet was washed with 50 μ L 70% EtOH. The sample was centrifuged at 15000 rcf for 3 min, the supernatant was removed and the pellet was air dried for 3-5 min.

The pellet was resuspended in the CuAAC reaction:

1 mM azide-bearing substrate
500 μ M CuSO₄, 2.5 mM THPTA, 5 mM aminoguanidine (pre-mix)
5 mM sodium ascorbate
50 mM Tris HCl buffer pH 8.3
Volume was adjusted to 5 μ L with MilliQ water
Incubated at 42°C for 2 hours

The optimal conditions are given. These conditions were varied in each different optimisation click reaction.

After incubation, the CuAAC components were removed from the sample by the previously described ethanol precipitation. The resulting product-containing pellet was resuspended in 5 μ M fluorescent oligo in water (FAM-GS3-an2.R36; 5'-/56-FAM/TTCCGCCCCCGTCCTAGCT) (IDT DNA, USA) and incubated at 42°C for 10 minutes. The CuAAC reactions on translated peptide were analysed by means of 8% urea-0.05% SDS-PAGE gels, as previously described²⁴.

In short, the clicked product, annealed to fluorescent oligo was added to 9 μ L loading buffer (34.5% formamide; 62.5 mM Tris HCl pH 8.3; 10 mM EDTA; 5 mM DTT; 0.05% SDS; trace BPB) and heated at 95°C for 2 minutes. Urea was washed out of the gel wells prior to sample loading. The gel was run at 200 V for 70 minutes in 1x running buffer. The bands were visualised on a c300 gel imaging system (Azure Biosystems, USA) by absorbance of FAM-oligo at 472 nm. In case of click substrate iFluor™ 647 azide (AAT Bioquest, USA), the clicked product bands were visualised on a Sapphire biomolecular imager (Azure Biosystems, USA) by absorbance at 658 nm.

Running buffer preparation:

1x running buffer
25 mM Tris
192 mM glycine
0.05% SDS
pH was adjusted to 8.3 with HCl

Gel preparation:

Separating layer pH 8.8 (5 mL)	Stacking layer pH 6.8 (1 mL)
8M urea	8M urea
8% acrylamide	5% acrylamide
25% gel buffer pH 8.8 (final [SDS] 0.05%, [Tris] 0.375 M)	25% gel buffer pH 6.8 (final [SDS] 0.05%, [Tris] 0.125 M)
0.1% APS	0.1% APS
0.02% TEMED	0.02% TEMED

5. Heparanase biotinylation

38.8 µg HPSE (2 mg/mL) was incubated with 8 eq of Biotin-PEG4-NHS at 4°C over weekend in 50 mM phosphate pH 6.5, 100 mM NaCl buffer. After the reaction, the biotinylated protein was purified by six serial dilutions with 500 µL 20 mM HEPES pH 7.4, 50 mM NaCl buffer on spin filtration using a 10 K MWCO, 0.5 mL filter (Pierce™ protein concentrators PES, ThermoFisher scientific, USA). The resulting protein concentration was determined by absorbance at 280 nm, using MW: 52250 g/mol and extinction coefficient: 59820 M⁻¹ cm⁻¹.

6. Assessment of biotinylated protein binding capacity on beads

20 µL Dynabeads™ M-280 Streptavidin (ThermoFisher scientific, USA) were washed three times with 40 µL 1x PBS-T buffer. A series of 0.5; 1; 2; 5 and 10 µL beads was aliquoted into low binding tubes. The supernatant was removed before adding the protein. 0.2 µg biotinylated HPSE (0.25 µg/ µL stock) and 9 µL 1x PBS-T was added to each amount of beads. The beads and HPSE were incubated at 4°C for 30 minutes, while mixing. The supernatants were reserved and the beads were washed three times with 20 µL 1x PBS-T. Finally, the beads were resuspended in 10 µL 1x PBS-T and 2 µL 6x SDS loading buffer was added to each sample (5 supernatants, 5 beads volumes). The samples were heated at 95°C for 5 minutes, and the beads were removed prior to loading on a 10% SDS-PAGE gel. The gel was run at 120 V for 2 hours. The proteins were visualised by silver stain, as previously described⁴³. The percentage of biotinylation was estimated by quantification of the protein bands in ImageJ.

7. Colorimetric activity assays

HPSE activity assays were carried out as described before⁴⁴. Briefly, 50 nM of (biotinylated) HPSE was incubated with 100 μ M substrate (Fondaparinux) in 40 mM sodium acetate, 100 mM sodium chloride buffer pH 5.2 in 50 μ L reaction volume at 37°C for 45 minutes. After 45 minutes the reaction was stopped by the addition of 50 μ L 1 mg/mL 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1) (Serva Electrophoresis GmbH, Germany) in 0.1 M NaOH and the solutions incubated at 60°C for 1 hour. For each test condition, a similar reaction was setup and directly stopped at t = 0 hr. The final solutions were transferred to a 96 well, PS, F-bottom, clear microplate (Greiner Bio-One, Austria), and the absorbance was measured at 584 nm (POLARstar Omega, BMG labtech, Germany). A dilution series of 0.2 μ M – 0.25 mM D-glucose in 50 μ L 40 mM sodium acetate, 100 mM sodium chloride buffer pH 5.2 was treated with 50 μ L 1 mg/mL WST-1 in 0.1 M NaOH under identical conditions to obtain a standard curve. All reactions / conditions in the activity assays were executed in duplicate.

9. Selection protocol RaPID system

The selection using the RaPID system was executed based on a previous report, with additional click reaction and purification steps⁴⁶. The Puromycin-linked mRNA library was *in vitro* translated using NEB PURExpress solution ΔB (deprived of release factors) and Homemade solution A. Two libraries were screened for HPSE inhibitors, one being a macrocyclic library initiated with ClAc-D-Tyr (Homemade solution A Fd-, all natural amino acids mix -Met), the other being a linear library initiated with Homopropargylglycine (Homemade solution A Fd+, all natural amino acids mix -Met). After translation, the libraries were reverse transcribed using the CGS3an13.R39 reverse primer by reverse transcriptase for 1 hour at 42°C. The reverse transcribed libraries (2 μM) were purified by phenol/chloroform extraction and ethanol precipitation and 'clicked' at 5 μL scale by CuAAC assembled under N₂ using 1 mM heparan-sulfate disaccharide (HS-DS), 500 μM CuSO₄, 2.5 mM THPTA, 5 mM aminoguanidine, 5 mM sodium ascorbate and 50 mM Tris HCl buffer pH 8.3 at 42°C for 2 hours in sealed tubes. The click components and excess of HS-DS were removed by ethanol precipitation, after which the libraries were incubated three times with 1 μL magnetic Dynabeads™ M-280 Streptavidin (ThermoFisher scientific, USA) at 4°C, with constant inversion, to remove bead-binding peptides (negative selection). For positive selection, 4.88 μL M-280 Streptavidin beads were incubated with 291 ng biotinylated HPSE at 4°C for 20 minutes. All negative and positive beads were washed three times with 20 μL ABS-T (40 mM sodium acetate, 100 mM sodium chloride buffer pH 5.2 with 0.01% Tween) before incubations. Finally the library was transferred from negative beads to positive beads, and incubated at 4°C for 30 minutes, while mixing. The positive and third negative beads were washed three times with 20 μL ABS-T, and resuspended in 50 μL MilliQ water. The peptides-mRNA/cDNA conjugates were eluted from the beads by heating at 95°C for 5 minutes and the hot supernatants were transferred to a fresh tube. The recovery of the selected libraries was analysed through qPCR, by comparison to NNK15 cDNA standards with known concentrations. A round of selection ended with amplification of the positives by PCR using the primers: T7g10M.F48 and CGS3an13.R39. PCR amplification was analysed by means of 3% agarose gel with SYBR SAFE (ThermoFisher scientific, USA). The pooled PCR product was precipitated by the addition of 1/10x volume of 3 M NaCl and 2.2x volume of 100% EtOH, followed by centrifugation at 15000 rcf for 15 minutes. The supernatant was discarded and the pellet was air dried. The purified PCR product was transcribed *in vitro* to its corresponding mRNA using T7 RNA polymerase at 37°C overnight. The resulting mRNA was purified, quantified by absorbance at 260 nm and used in the Puromycin ligation reaction at the beginning of a next round of selection. Per library, 2 μM of Puromycin-linked mRNA was used in a translation to start the next round.

9.1. RNA purification in between selection rounds

After round 4 and 8, *in vitro* transcription product mRNA was separated from truncated mRNA by means of a preparative 8% denaturing urea-PAGE gel. Briefly, precipitated air dried pellets were resuspended in MilliQ water, 2x RNA loading buffer and BPB. The mixtures were heated at 95°C for 2 minutes, prior to loading. The gel was run at 250 V for 2 – 3 hours. The gel was transferred to a TLC plate covered with plastic wrap. The RNA bands were carefully visualised by UV light, the product mRNA bands were marked. The marked gel was cut out and transferred to 2 mL microcentrifuge tubes. The gel pieces were crushed with pipette tips and 1 mL 0.3 M NaCl was added, then the samples were mixed for 45 minutes at room temperature. The tubes were centrifuged at 6000 rcf for 5 minutes and the supernatant was collected in separate tubes. Three more 0.3 M NaCl washing, mixing and centrifuging steps were executed. The collected supernatants were filtered with 0.45 µm filters. Twice the volume of 100% EtOH was added to precipitate the product. The samples were vortexed and centrifuged at 15000 rcf for 15 minutes, after which the supernatants were discarded. The pellets were washed with 0.5 mL 70% EtOH, vortexed and centrifuged at 15000 rcf for 3 minutes. The supernatants were discarded and the resulting pellets were air dried. The dried pellets were resuspended in MilliQ and the concentration was determined by absorbance at 260 nm on Nanodrop. The products were diluted with MilliQ to a 20 µM solution, which was used for puromycin ligation reaction at the start of the next round.

9.2. DNA purification in between selection rounds

After round 7, the desired DNA was purified from the PCR product by using the Nucleospin Gel and PCR Clean-up kit for DNA extraction from agarose gels (Macherey-Nagel, Germany). In short, after PCR, precipitated DNA was separated on a 3% agarose gel. The desired DNA fragments were excised from the agarose gel, using a gel scanner to visualize the DNA bands. The weight of the gel slices was determined, 400 µL buffer NT1 was added for each 100 mg of gel. The gel pieces were completely dissolved by incubating at 50°C for 10 minutes, with briefly vortexing every 2 minutes. The DNA was bound to a Nucleospin Gel and PCR clean-up column by loading 700 µL sample on the column. The column was centrifuged at 11000 rcf for 30 seconds, the flow-through was discarded. This was repeated to load the remaining sample. The silica membrane was washed two times with 700 µL buffer NT3, followed by centrifuging at 11000 rcf for 30 seconds, after which the flow-through was discarded. The silica membrane was centrifuged at 11000 rcf for 1 minute to remove all buffer NT3. The column was incubated at 70°C for 5 minutes to fully dry the membrane. The DNA was eluted by addition of 22.5 µL buffer NE, followed by incubation at room temperature for 1 minute and centrifugation at 11000 rcf for 1 minute. The elution step was repeated once. The resulting DNA was precipitated by addition of 1/10x volume of 3 M NaCl and 2.2x volume of 100% EtOH, followed by centrifugation at 15000 rcf for 15 minutes. The supernatant was discarded and the pellet was air dried. The concentration of DNA was determined by absorbance at 260 nm on Nanodrop. The DNA was amplified again by PCR to yield enough product. The product was precipitated and used for *in vitro* transcription to yield the mRNA product that will be used in the puromycin ligation at the start of the next round.

Acknowledgements

Seino, thank you for allowing me to do my major internship in your group. In the end, it was a tough project and it would not have been possible without your support and supervision. Despite me being not always positive about my own work and achievements, when looking back, I really think I have achieved and learned a lot. You have not only taught me a lot about heparanase and mRNA display, but also about science, life as an academic, what it takes to be a scientist and how to set expectations and limits. All this knowledge and information is just as valuable as the practical skills I have learned in the past nine months. The combination of your sense of humor and professional attitude and experience create a perfect working environment for students, where everyone can be themselves and find their own interests. Don't forget your own work and deadlines (dare to say no), bully Minglong sometimes on my behalf and maybe our paths will cross again in the future. Thank you!

Minglong, we have made fun of each other a lot, but I enjoyed working with you every day. I can only admire your working ethics, motivation and knowledge. Thank you for assisting me in the lab and answering all my questions in the past nine months. Good luck with finishing your postdoc and your future career in China. When you are done with academic research, you can always consider a career as a comedian. I think the pigeon joke will do well in theatres. Keep in mind that you can always give me a call when you need advice from your vice manager.

I would like to thank Vito for passing on all your knowledge regarding the heparanase project. Good luck with finishing your PhD thesis and defence and the start of your career at Pepscan. Finally I would like to thank Ryoji, Twan, Helena, Stein and Lin for helping me in and outside the lab, for sharing protocols and materials and for all the nice meetings, lab outings, potlucks and conversations we had. Good luck in the future!

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Supplementary Information

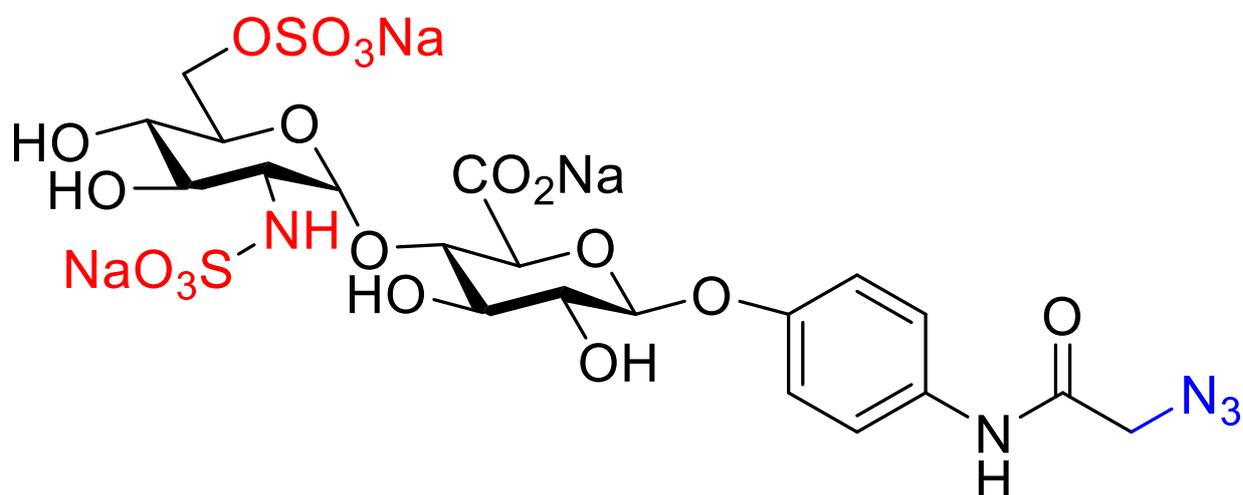


Figure S 1: Heparan-sulfate disaccharide (HS-DS). The sulfated disaccharide that will be clicked on the peptide libraries to target HPSE's active site. MW: 755 g/mol. Sulfate groups of the disaccharide are shown in red. The azide, used in CuAAC, is shown in blue.

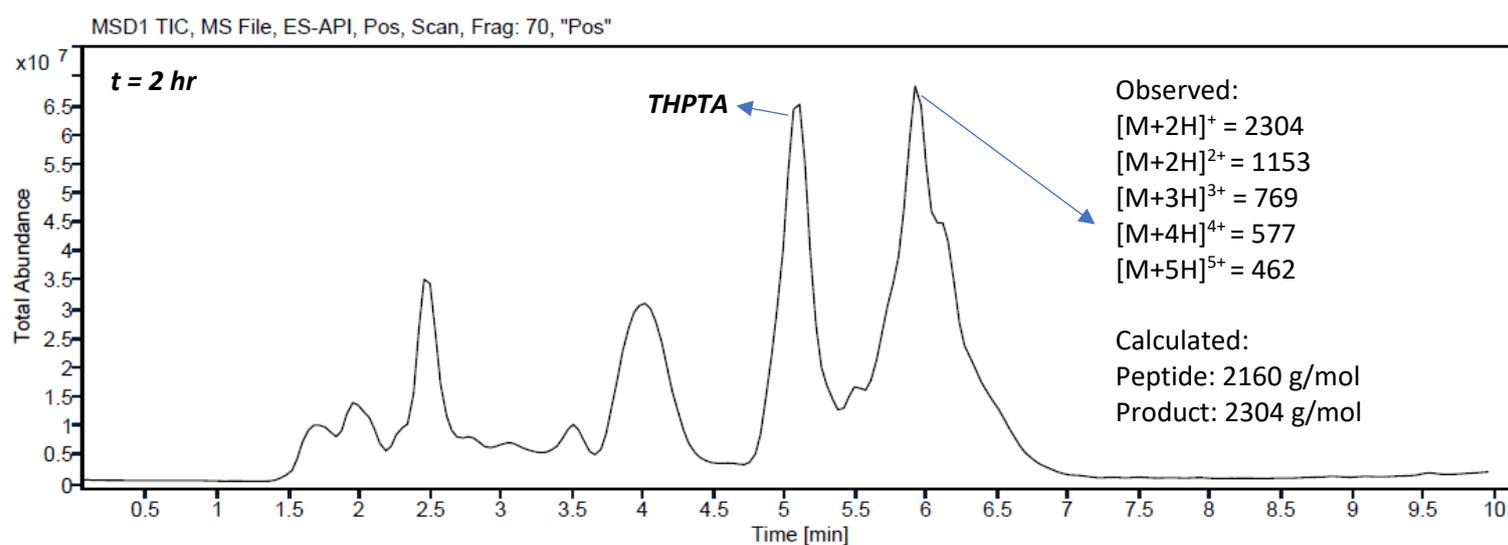
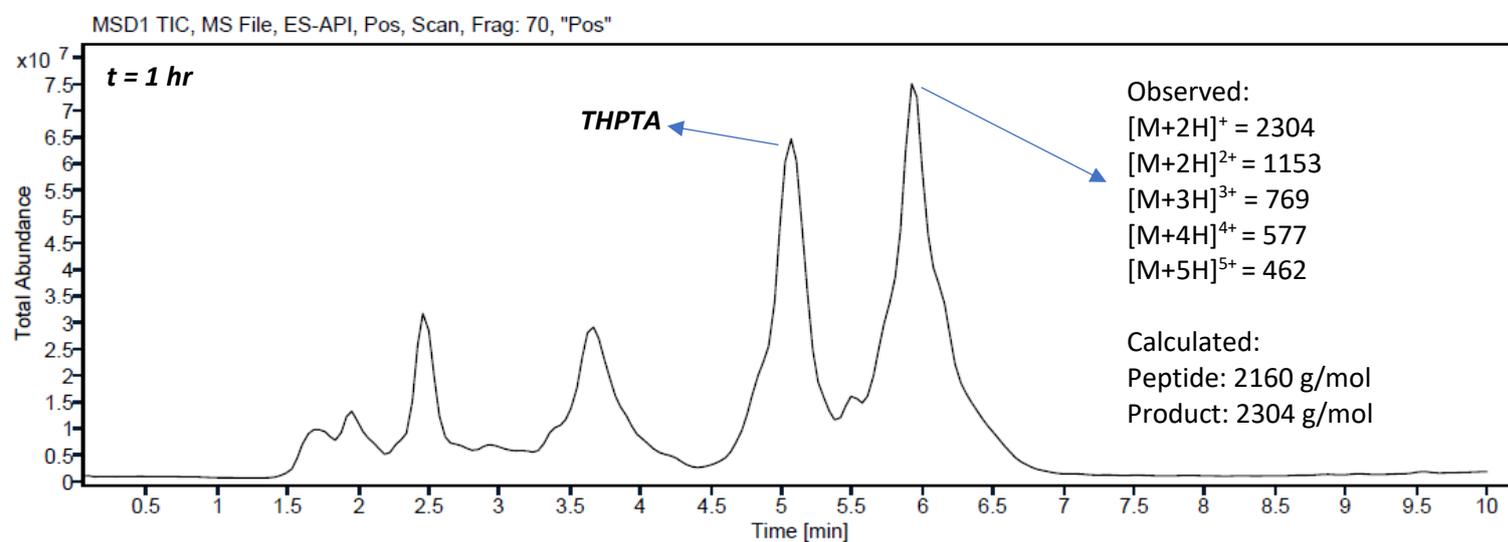
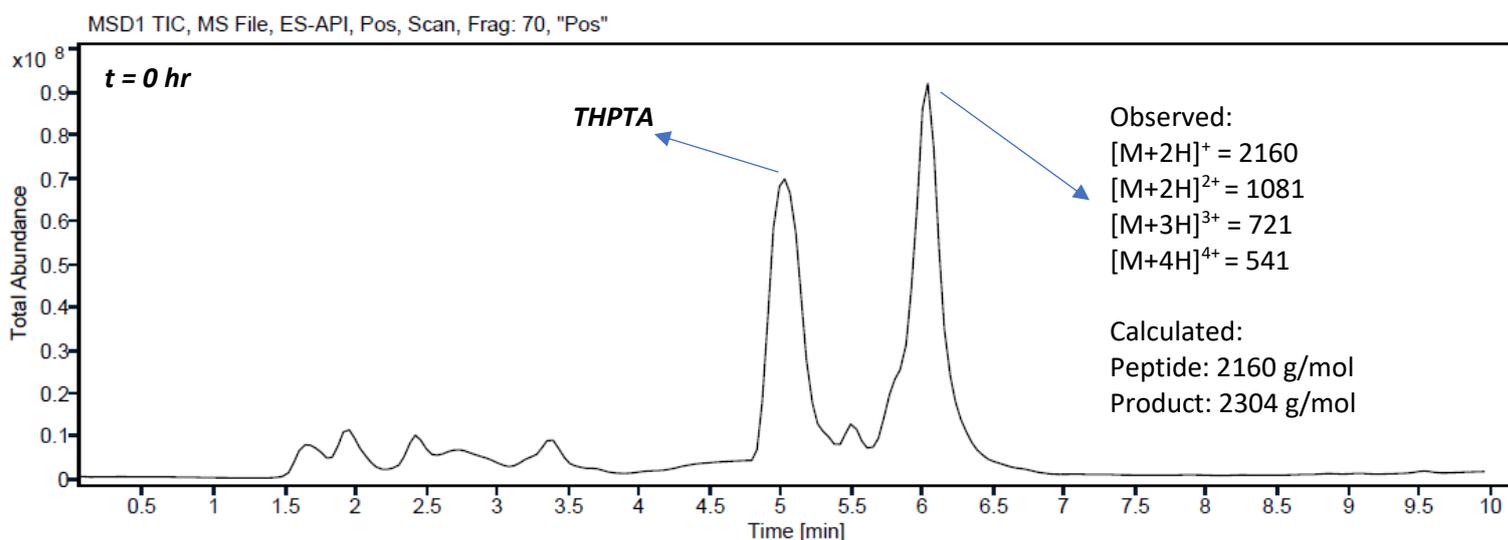


Figure S 2: Total ion chromatograms showing the click reaction of standard conditions with 100 μ M synthetic peptide and 1 mM azidohomoalanine, measured at timepoints 0, 1 and 2 hours.

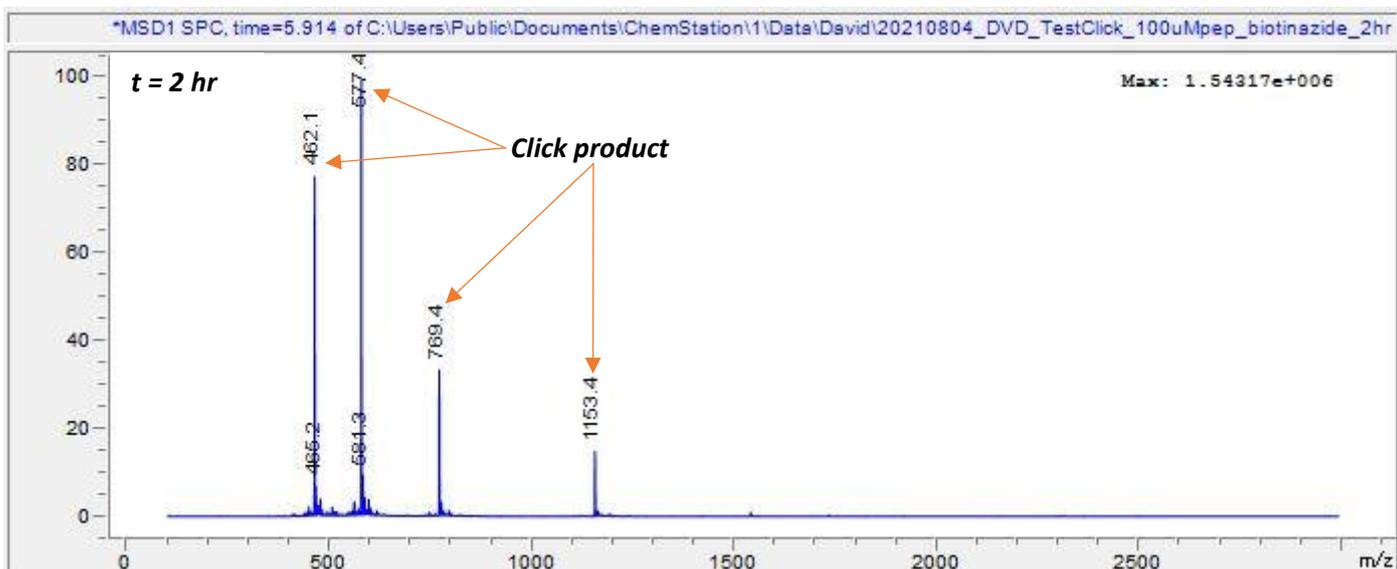
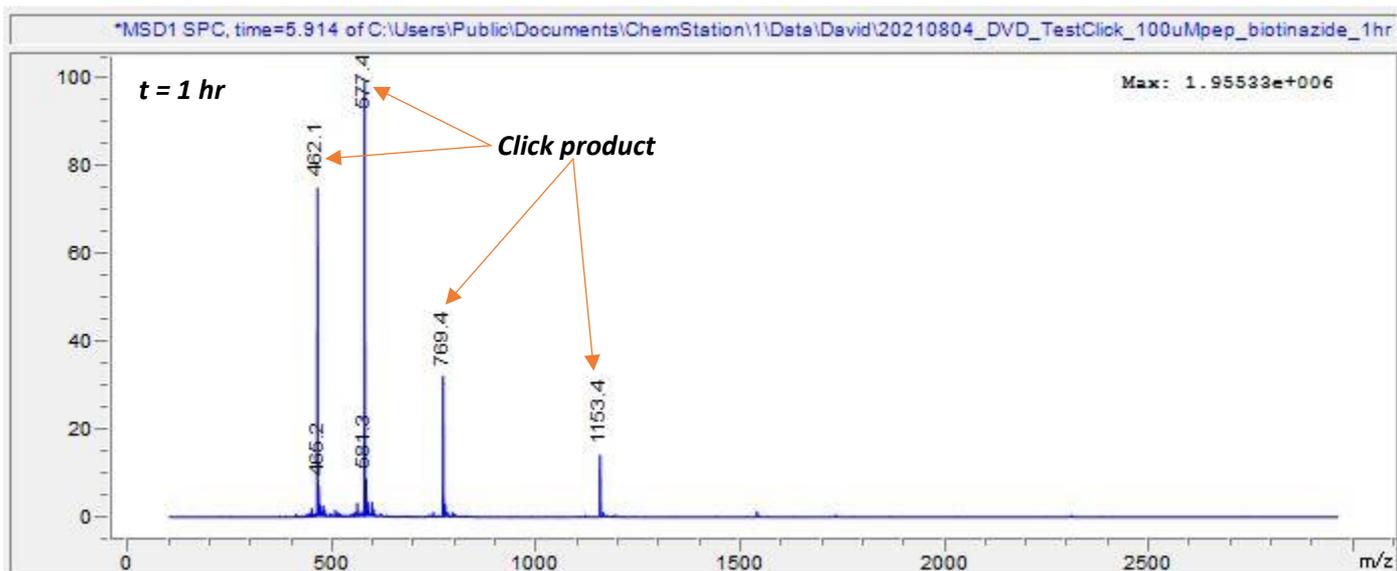
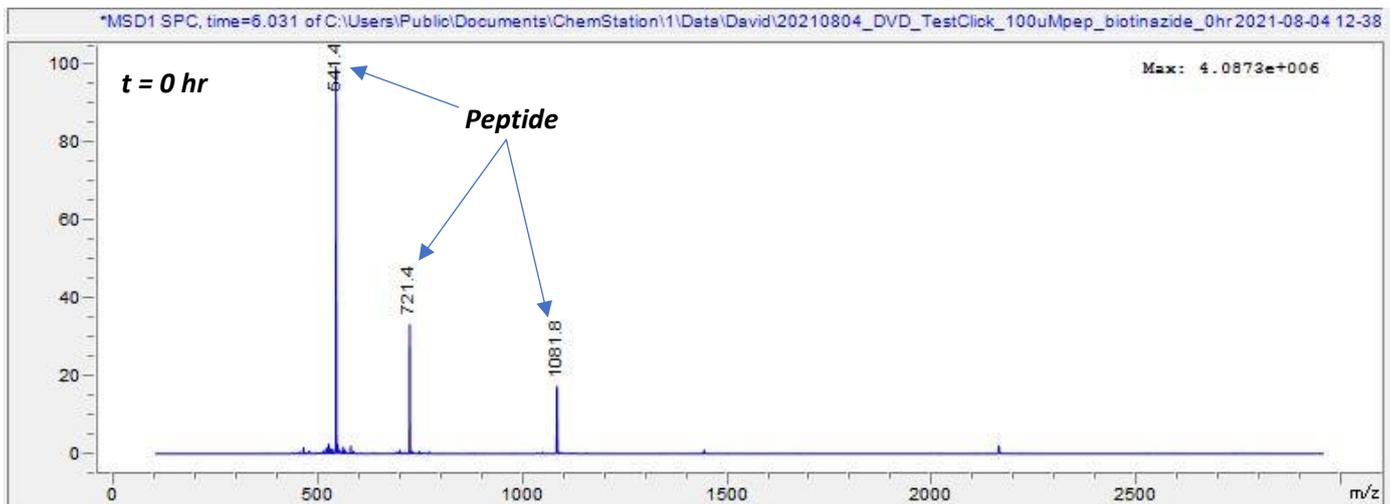


Figure S 3: Extracted mass spectra at retention times 6.0 and 5.9 min (before and after click reaction) shown in figure S 2 at timepoints 0, 1 and 2 hours.

The peak area of the most abundant extracted ion at $t = 0$ (541) was used to roughly estimate the conversion of starting material:

$t = 0$ hr, peak area: 363 million

$t = 1$ hr, peak area: 13 million \rightarrow 97% of starting material converted

$t = 2$ hr, peak area: 11 million \rightarrow 97% of starting material converted

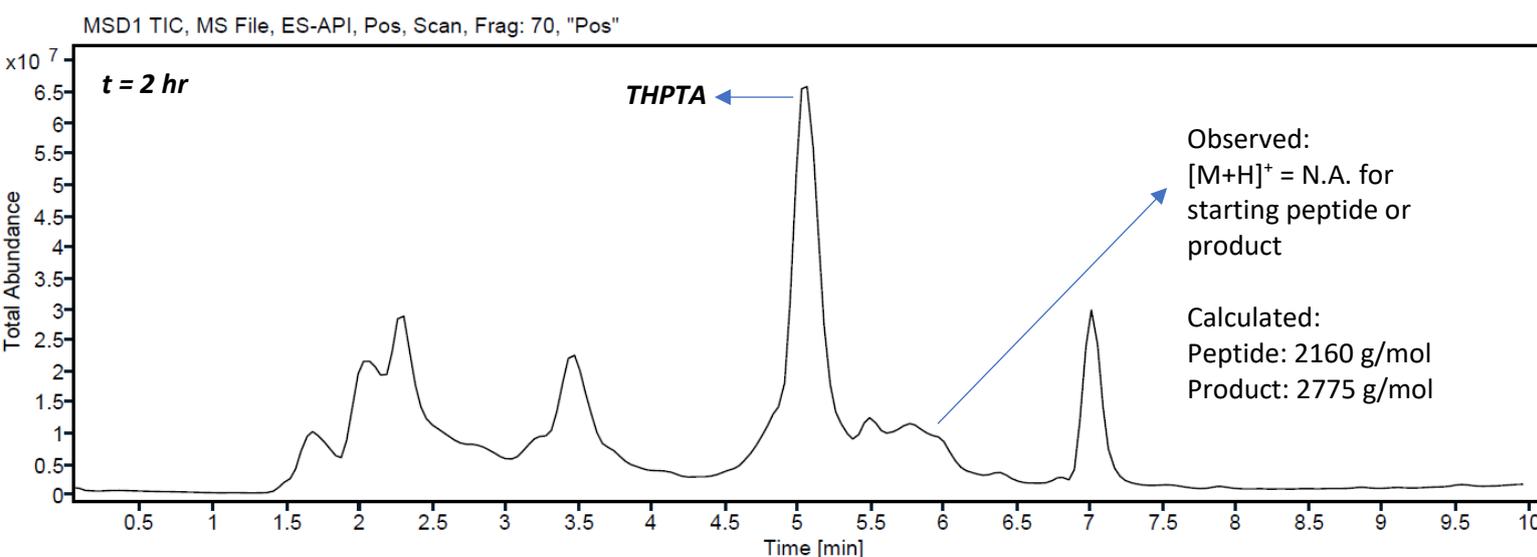
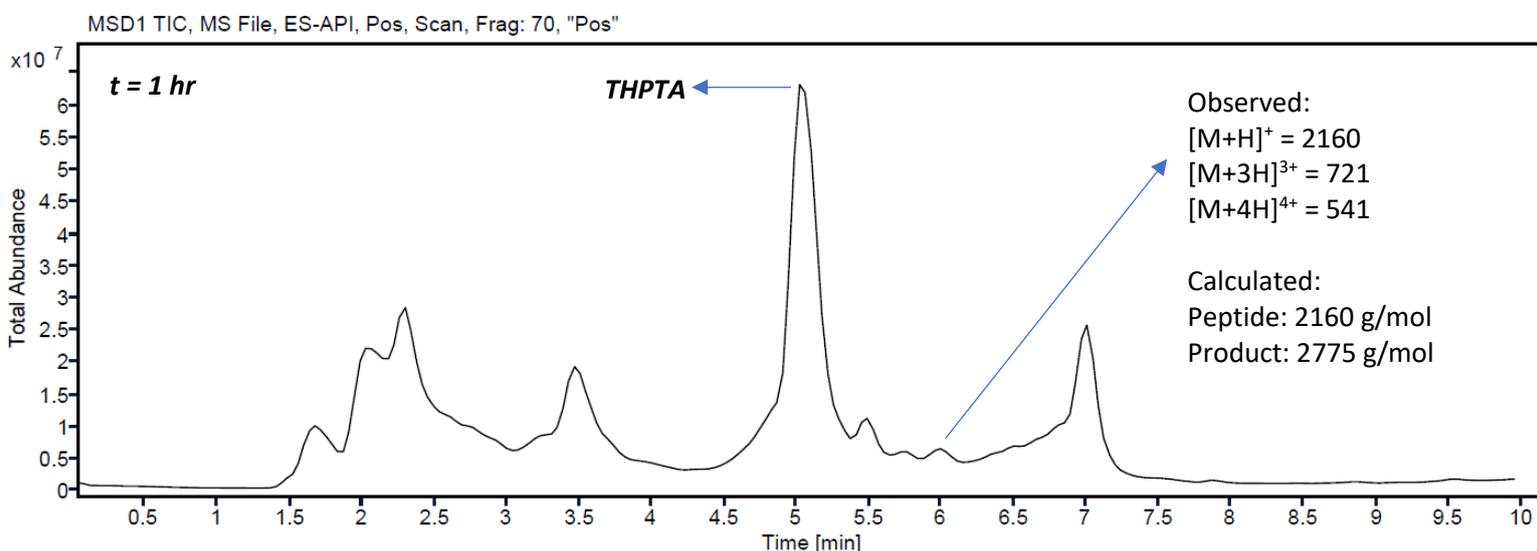
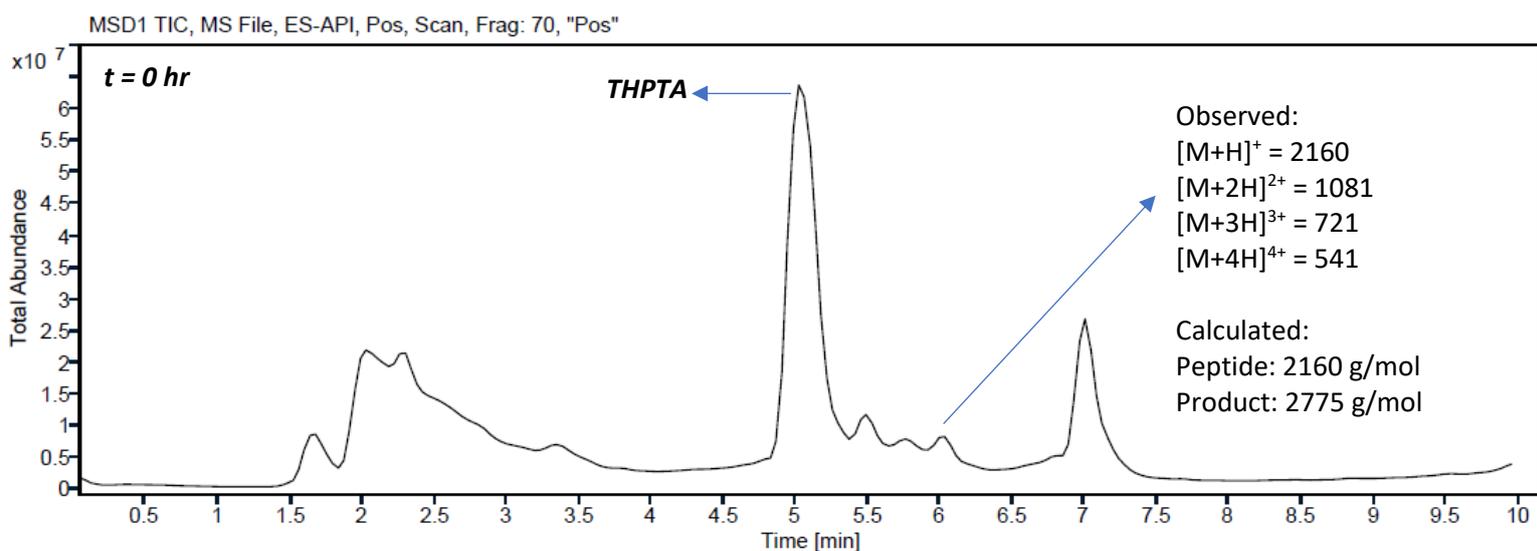


Figure S 4: Total ion chromatograms showing the click reaction of standard conditions with 10 μM synthetic peptide and 100 μM biotin-PEG4-azide, measured at timepoints 0, 1 and 2 hours. The peak at 7 min can not be linked to peptide, product or any of the click components.

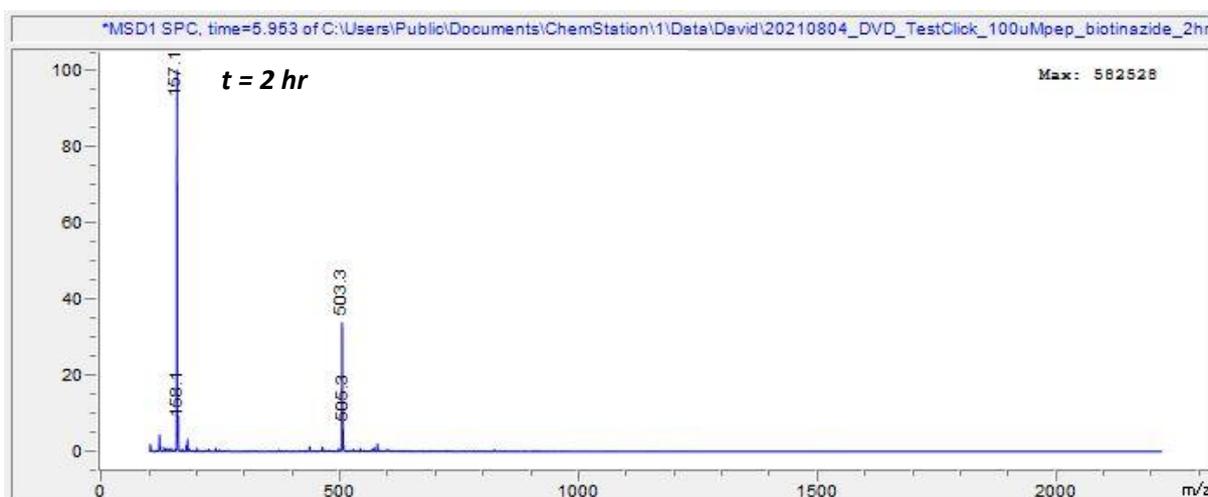
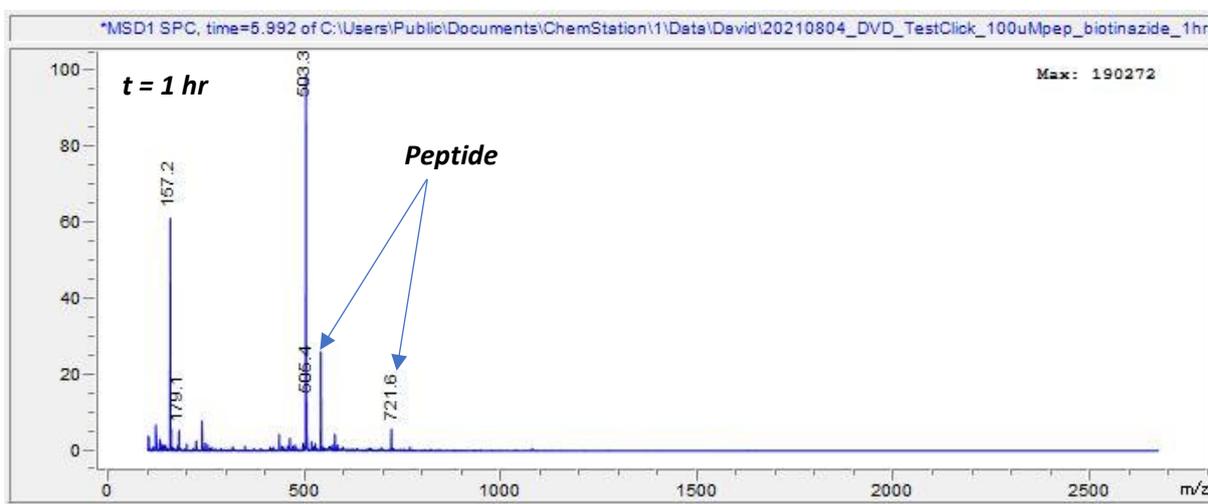
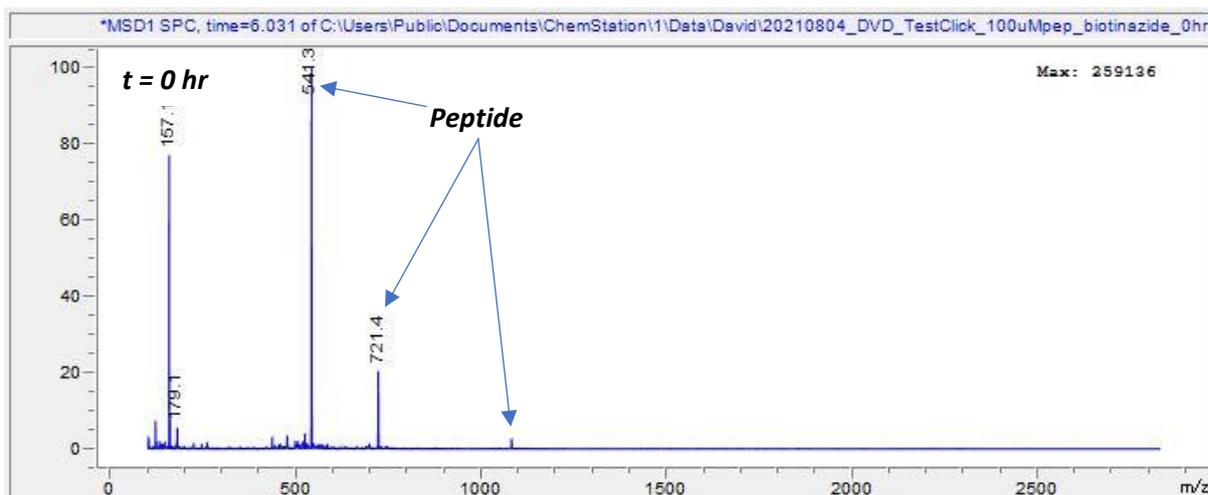


Figure S 5: Extracted mass spectra at retention times 6.0 and 5.9 min (before and after click reaction) shown in figure S 4 at timepoints 0, 1 and 2 hours.

No ions from starting material and clicked product were extracted at t = 2 hours. The conversion could not be calculated (N.A.).

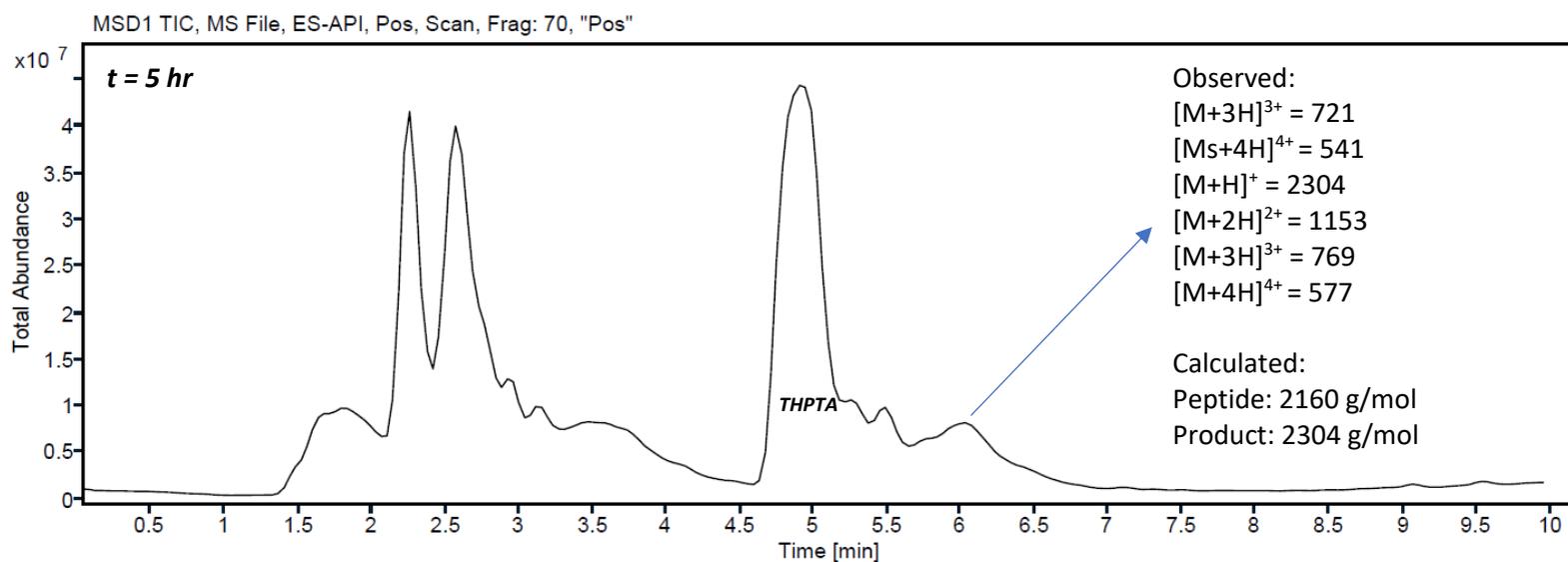
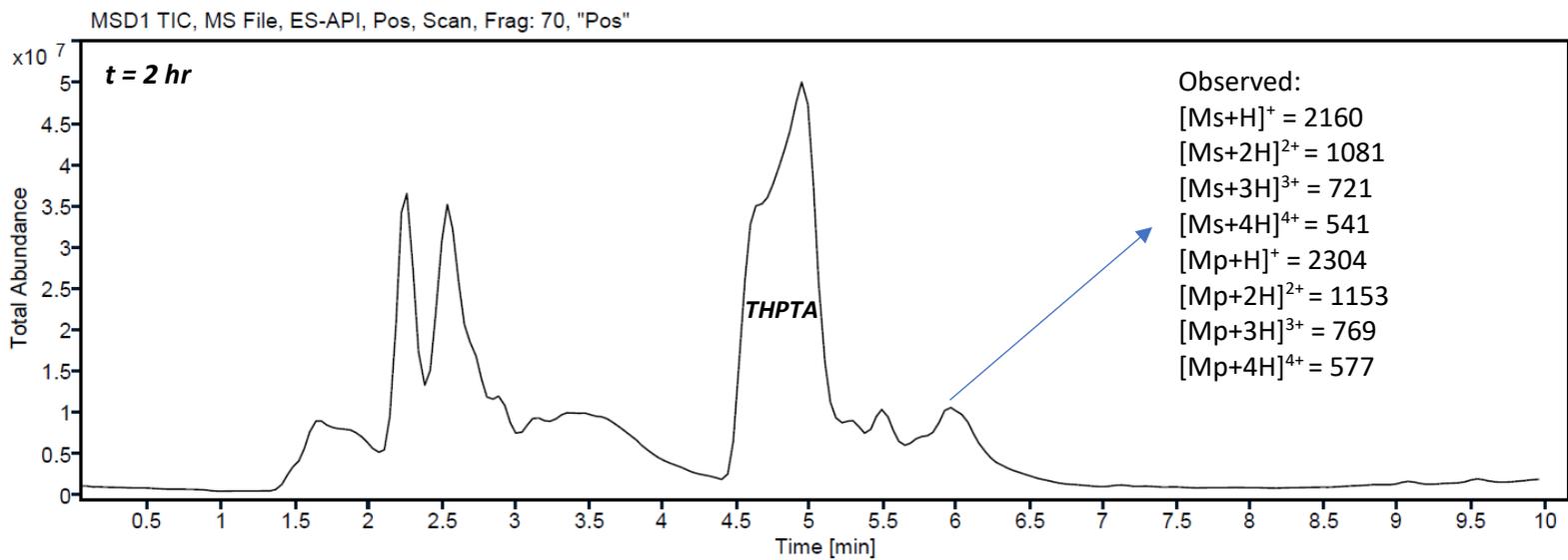
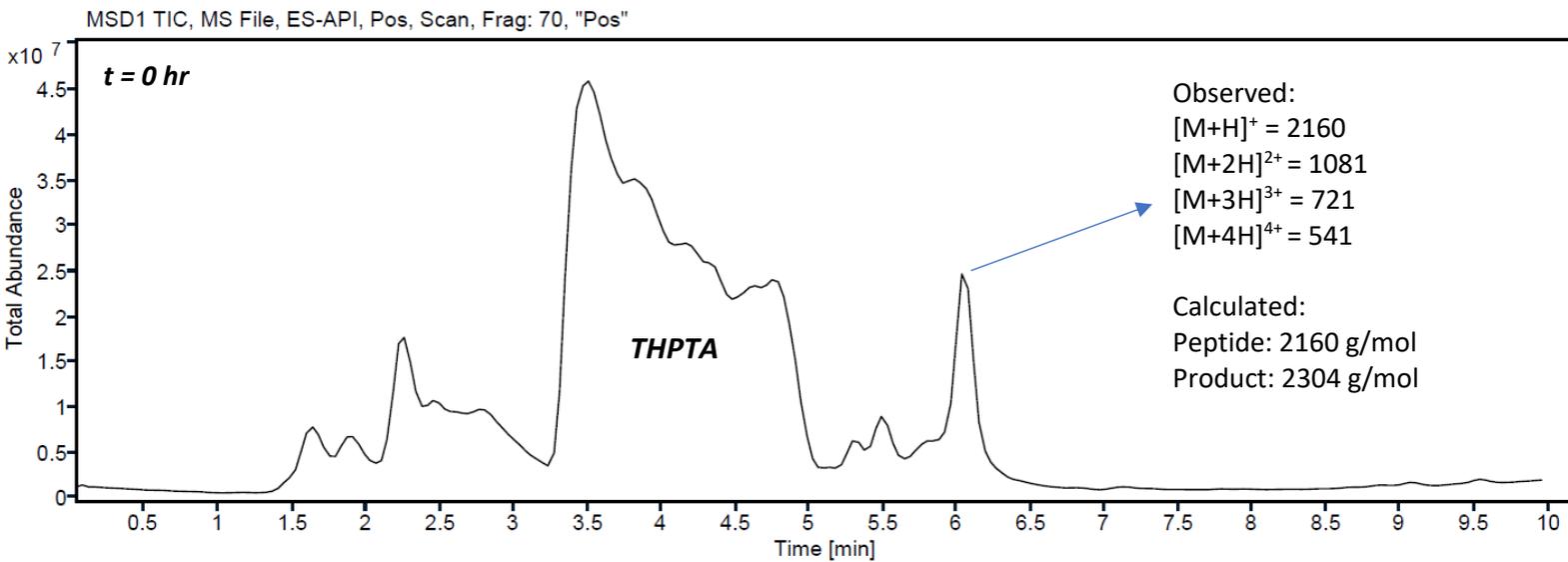


Figure S 6: Total ion chromatograms showing the click reaction of standard conditions with 25 μM synthetic peptide and 100 μM azidohomoalanine, measured at timepoints 0, 2 and 5 hours. $[Ms+H]$ = Mass starting material. $[Mp+H]$ = Mass product. The peaks (duplet) at 2.5 min can not be linked to peptide, product or any of the click components.

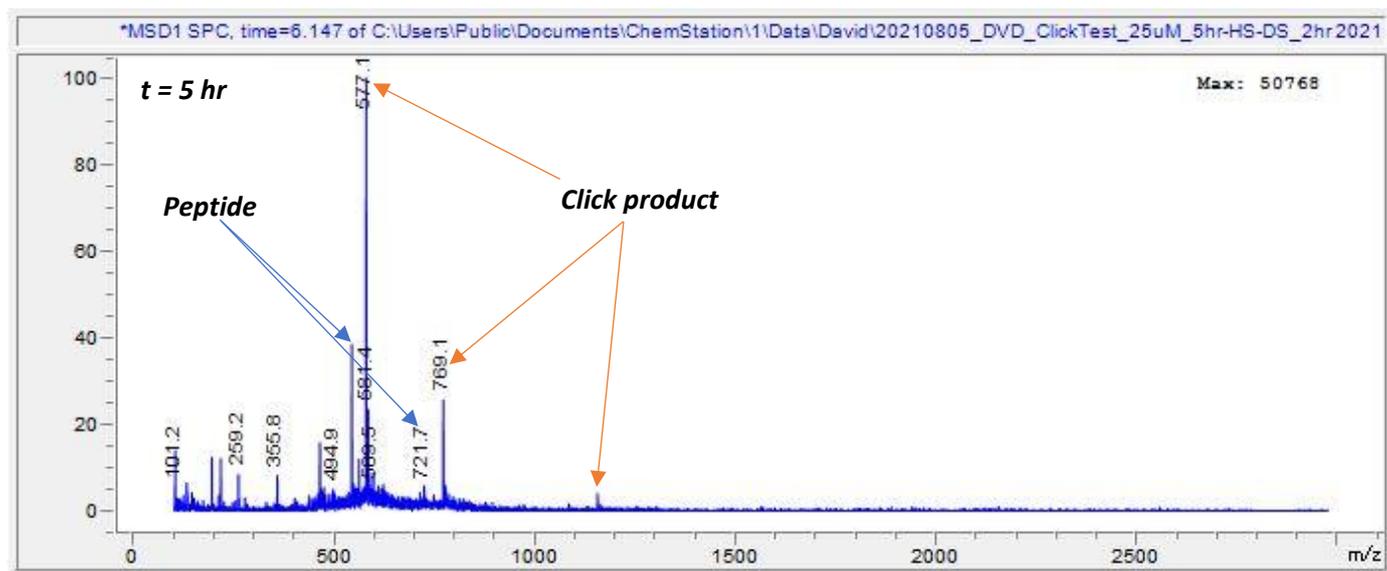
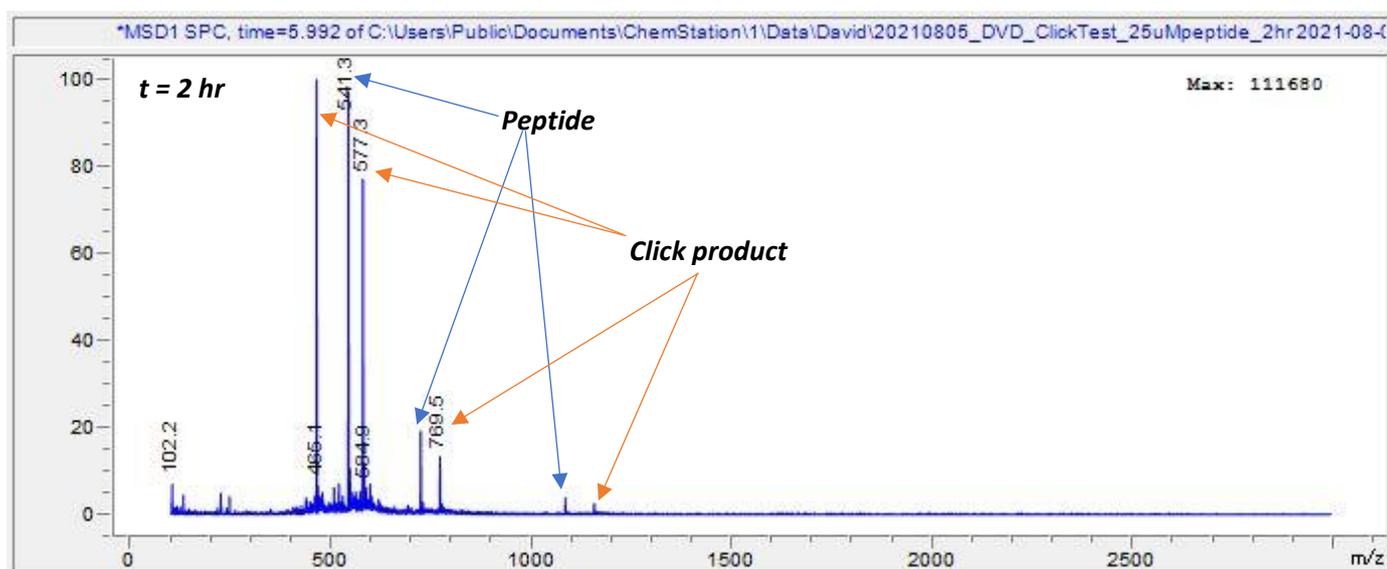
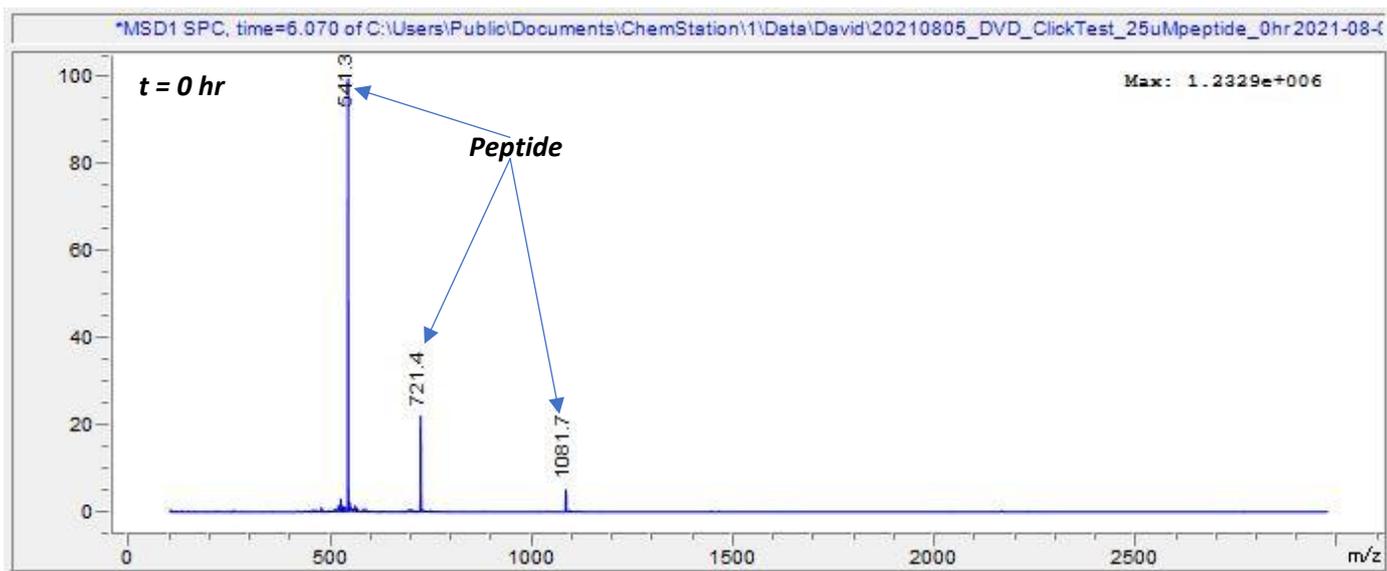


Figure S 7: Extracted mass spectra at retention times 6.0 and 5.9 and 6.1 min (before and after click reaction) shown in figure S 6 timepoints 0, 2 and 5 hours.

The peak area of the most abundant extracted ion at t = 0 (541) was used to roughly estimate the conversion of starting material:

t = 0 hr, peak area: 99 million

t = 2 hr, peak area: 14 million → 85% of starting material converted

t = 5 hr, peak area: 6 million → 94% of starting material converted

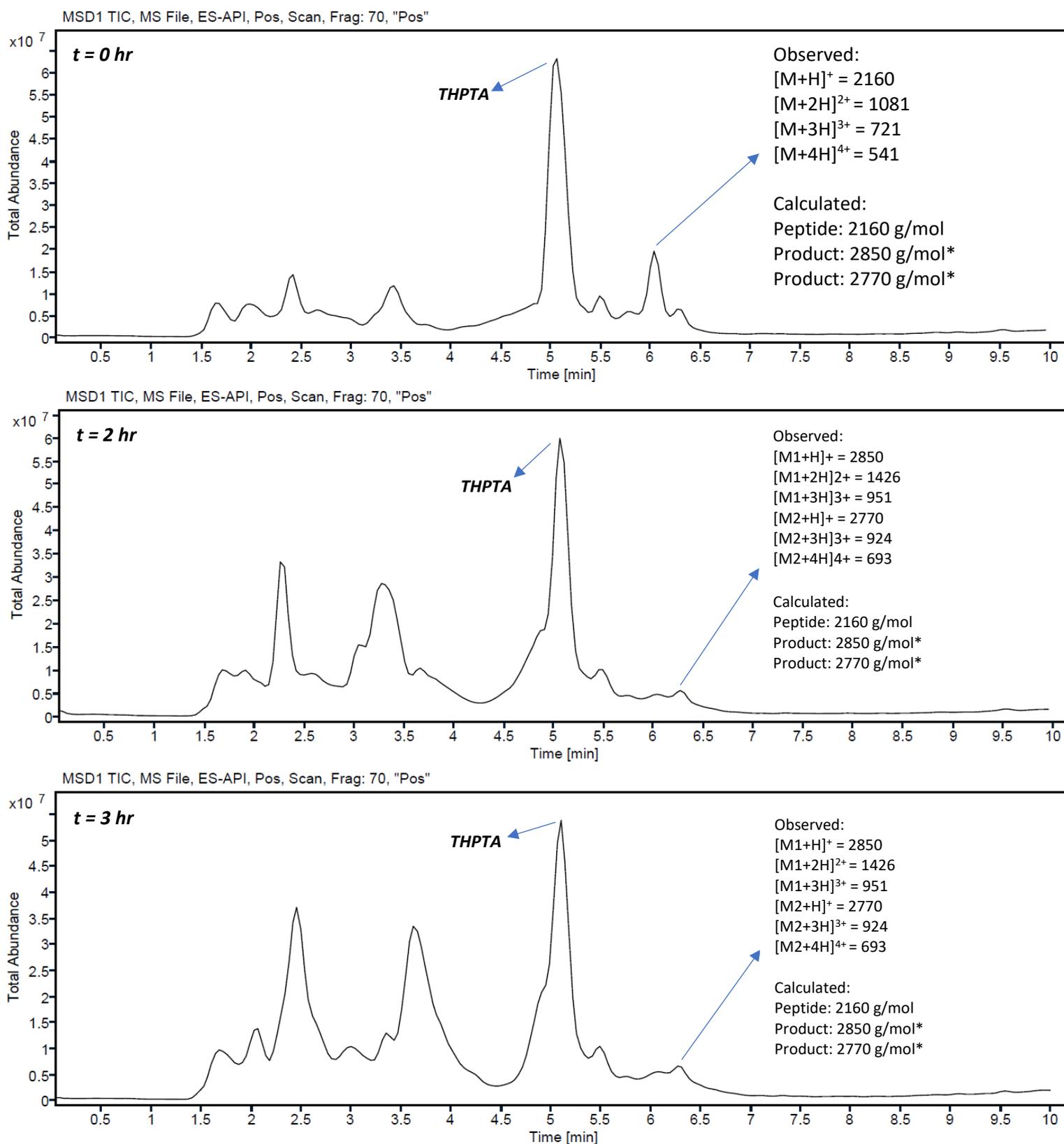


Figure S 8: Total ion chromatograms showing the click reaction of standard conditions with 25 μ M synthetic peptide and 100 μ M HS-DS measured at timepoints 0, 2 and 3 hours. The peaks at 2.5 and 3.7 min can not be linked to peptide, product or any of the click components.

* Calculated MW clicked product: 2850 (Protons instead of Sodiums on disaccharide) and 2770 (desulfation of O).

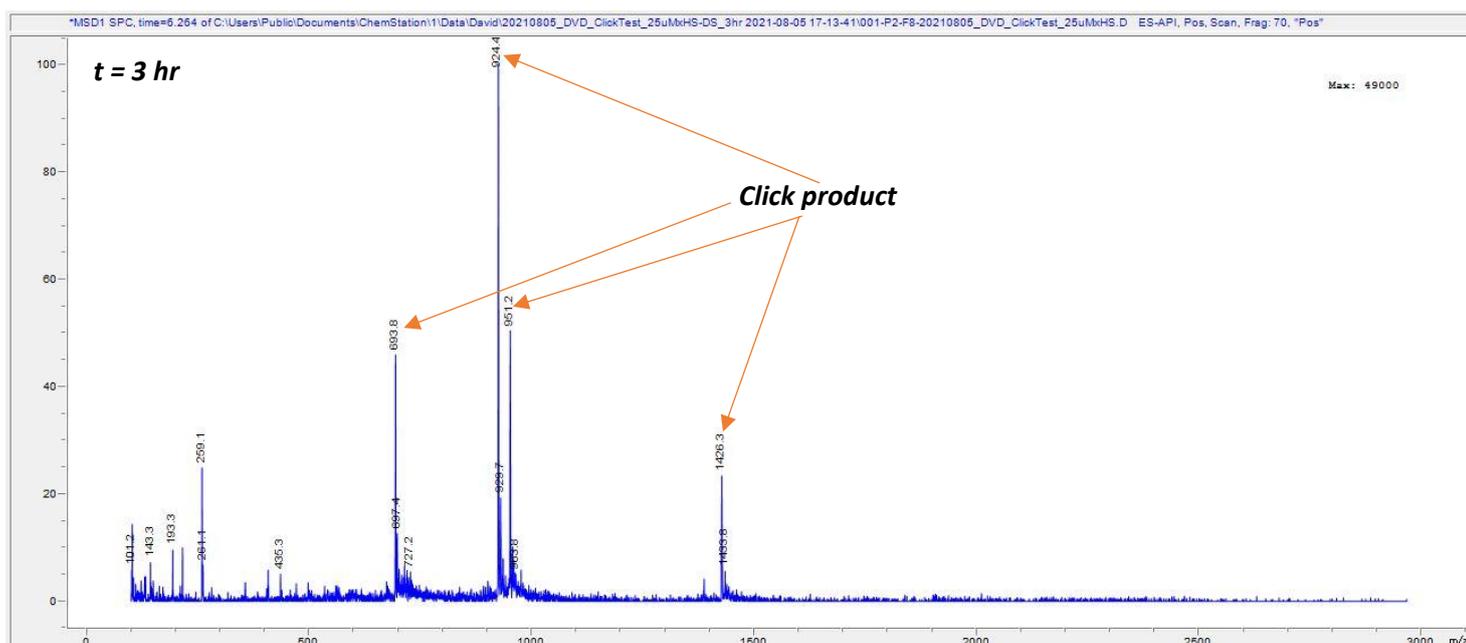
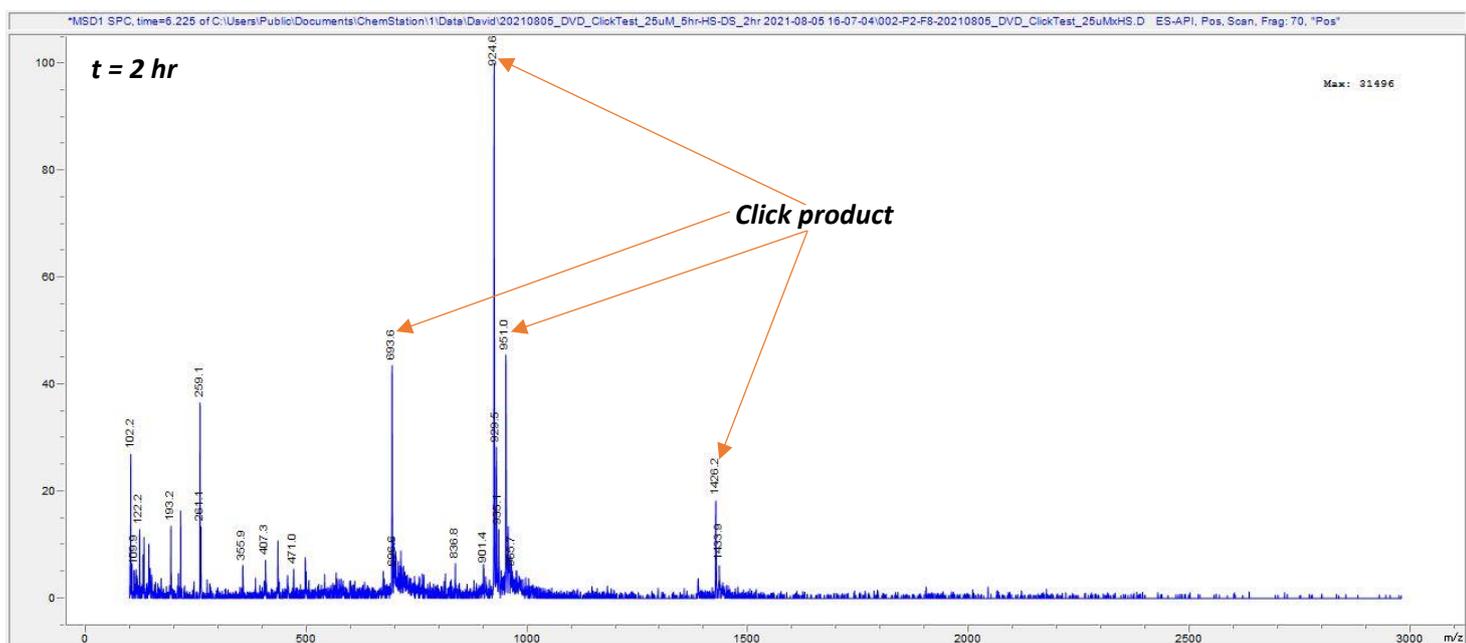
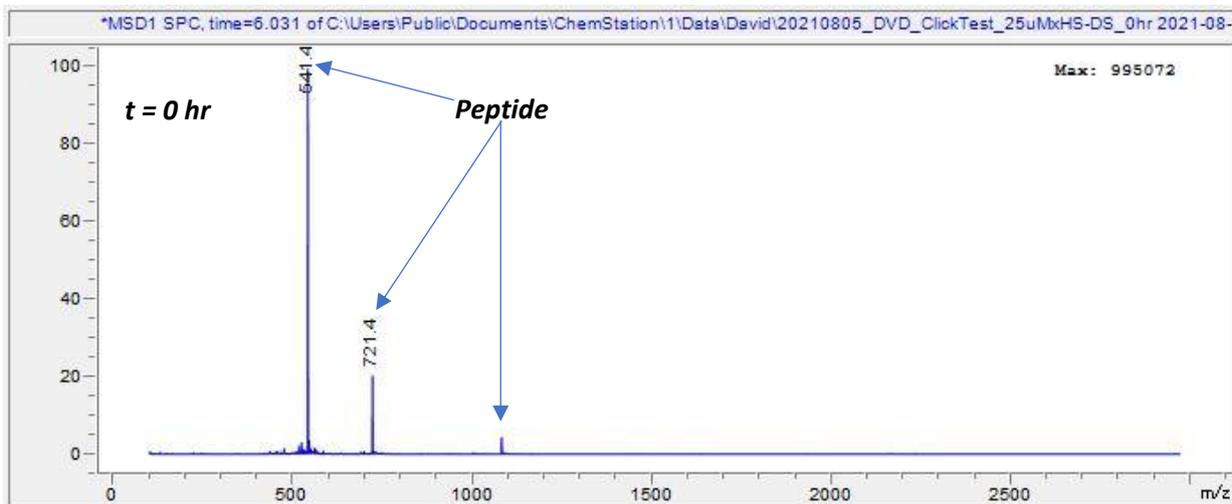


Figure S 9 Extracted mass spectra at retention times 6.0 and 6.2 min (before and after click reaction) shown in figure S 8 at timepoints 0, 2 and 3 hours.

The peak area of the most abundant extracted ion at t = 0 (541) was used to roughly estimate the conversion of starting material:

t = 0 hr, peak area: 75 million

t = 2 hr, peak area: 2 million → 98% of starting material converted

t = 3 hr, peak area: 1.5 million → 98% of starting material converted

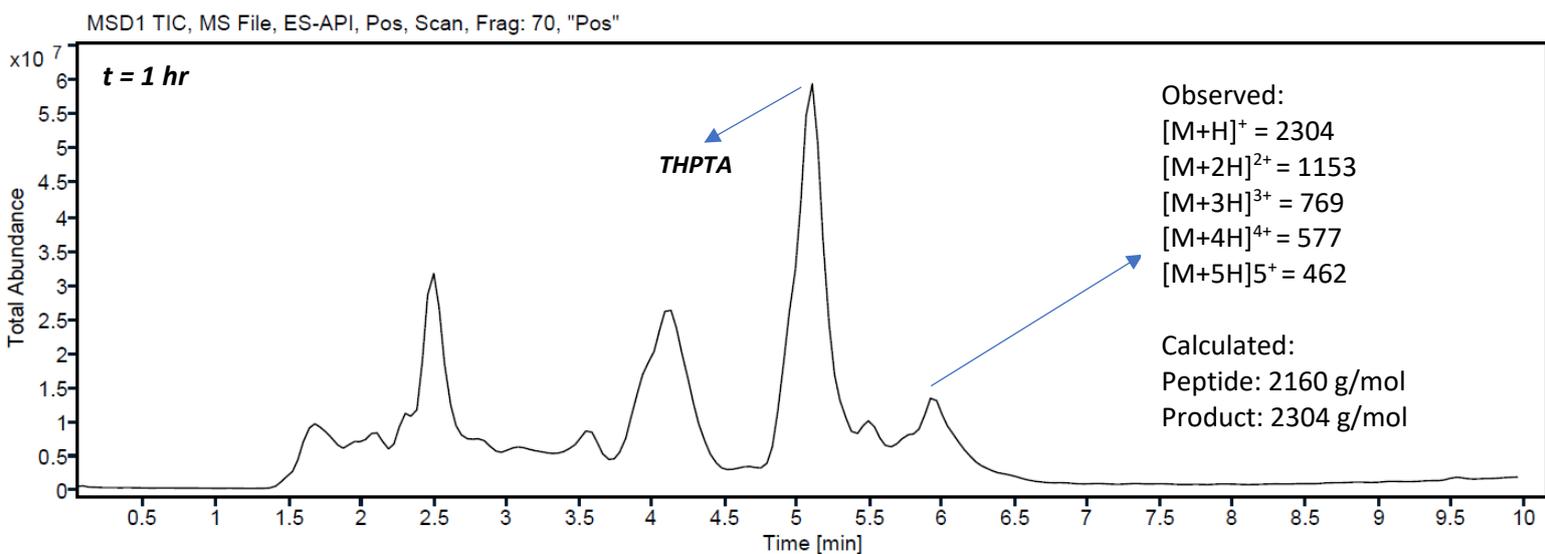
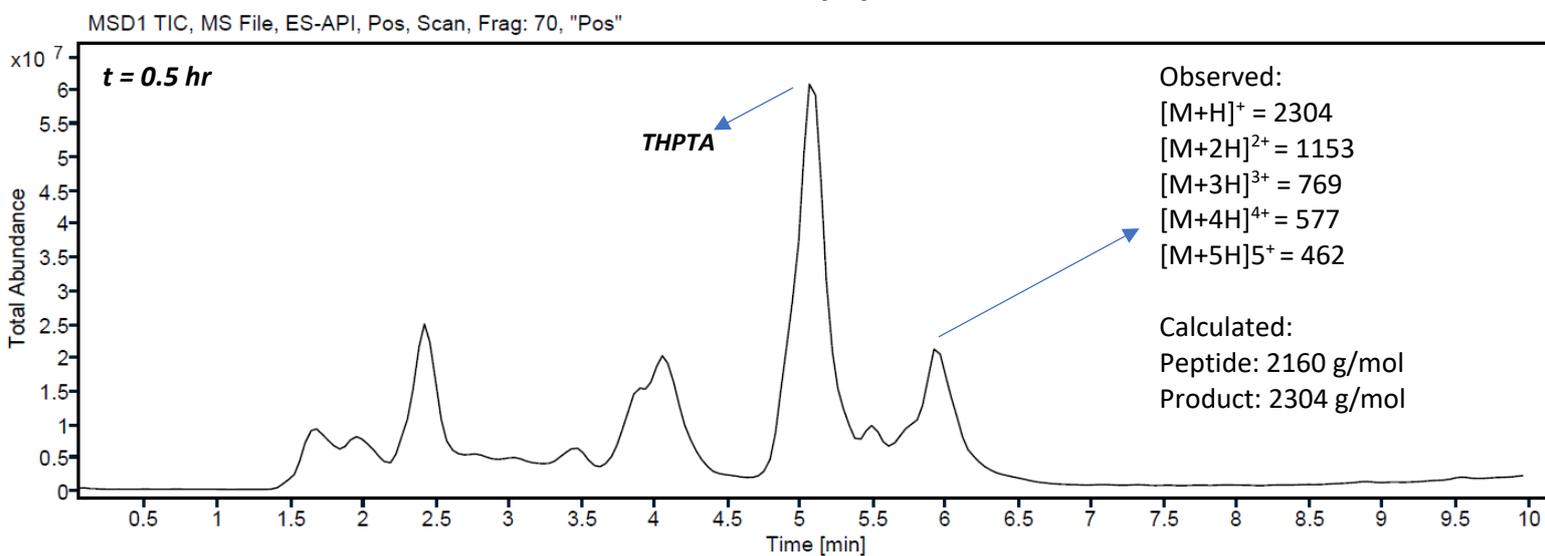
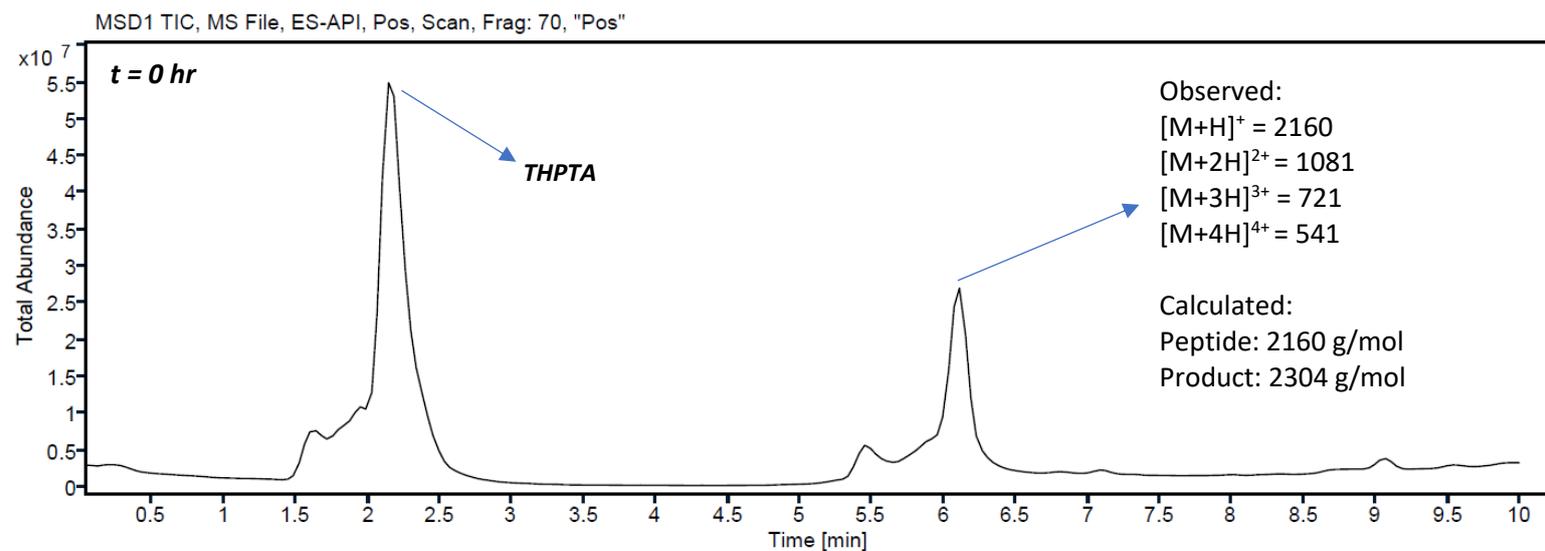


Figure S 10: Total ion chromatograms showing the click reaction of standard conditions with 25 μM synthetic peptide and 100 μM azidohomoalanine, measured at timepoints 0, 0.5 and 1 hour.
 The peaks at retention time 2.4 and 4 can not be linked to peptide, product or any click component.

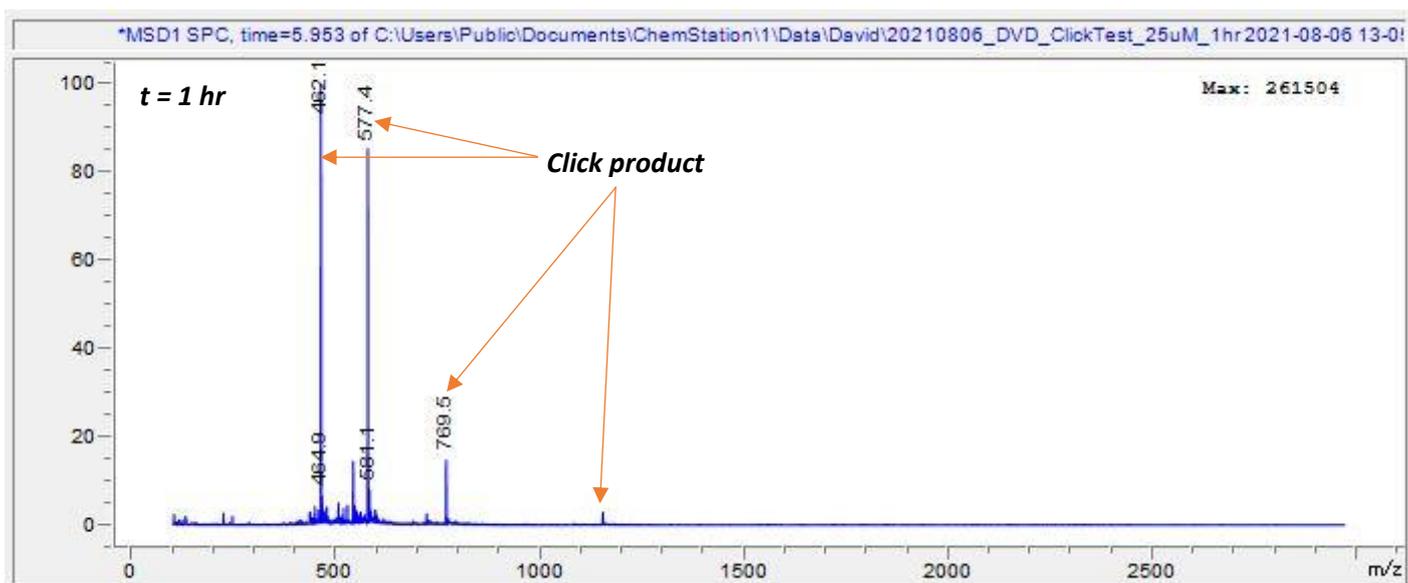
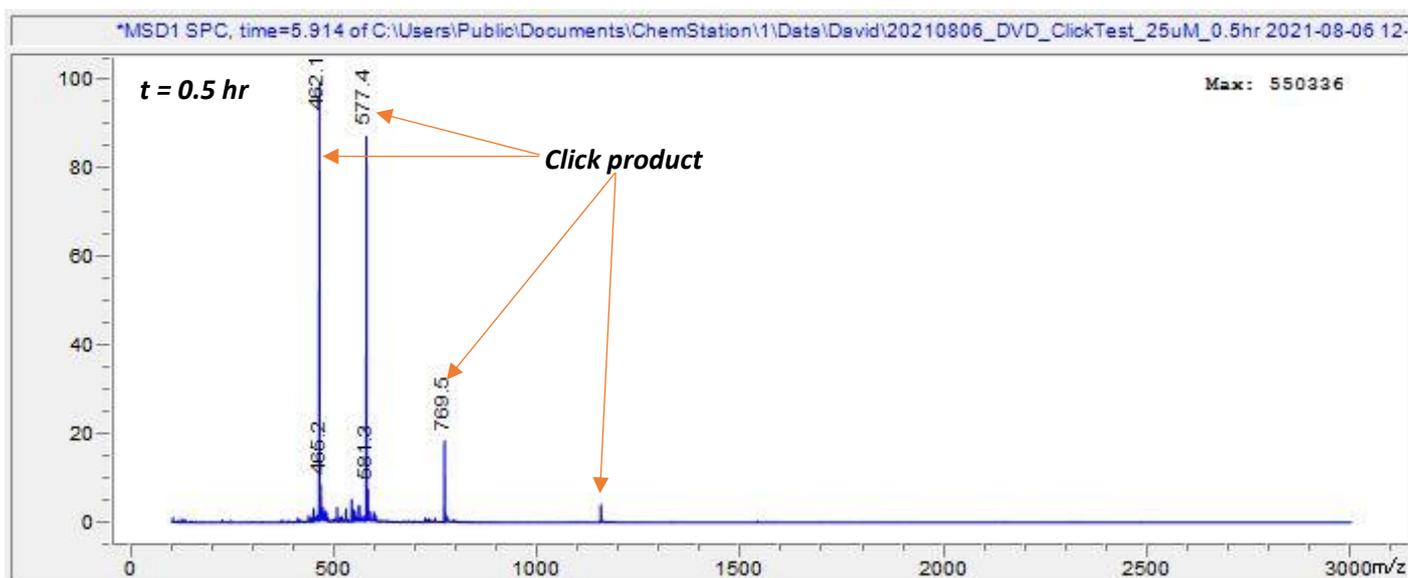
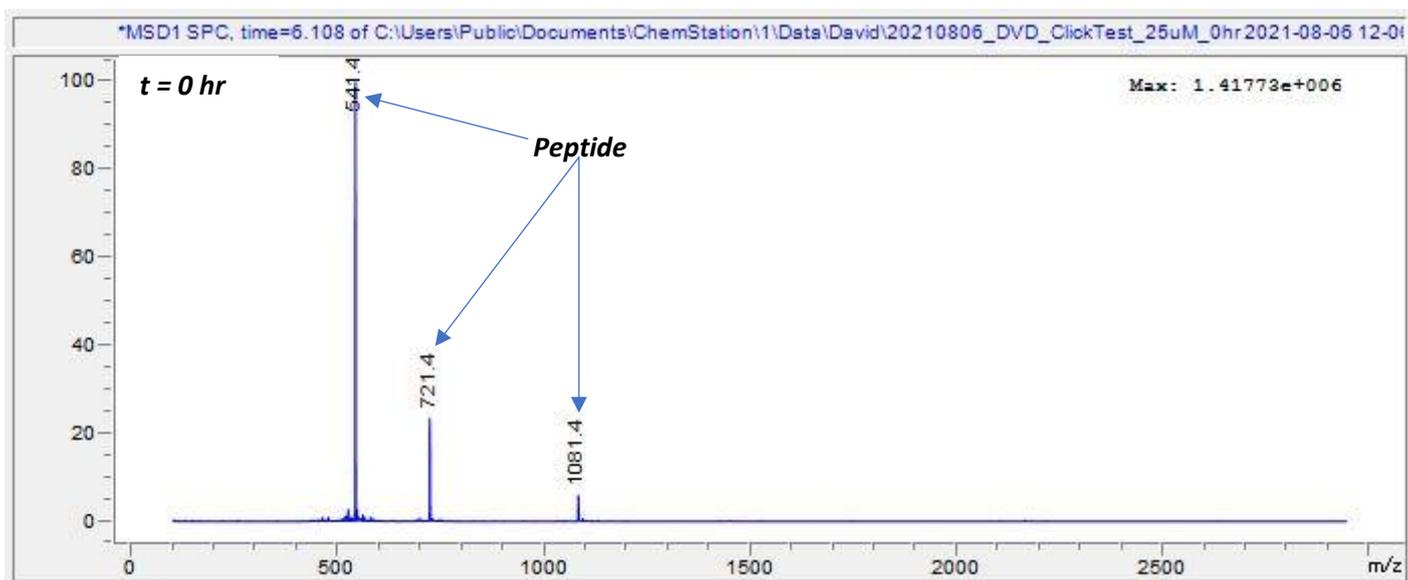


Figure S 11: Extracted mass spectra at retention times 6.1 and 5.9 min (before and after click reaction) shown in figure S 10 at timepoints 0, 0.5 and 1 hours.

The peak area of the most abundant extracted ion at t = 0 (541) was used to roughly estimate the conversion of starting material:

t = 0 hr, peak area: 109 million

t = 0.5 hr, peak area: 27 million → 75% of starting material converted

t = 1 hr, peak area: 10 million → 91% of starting material converted

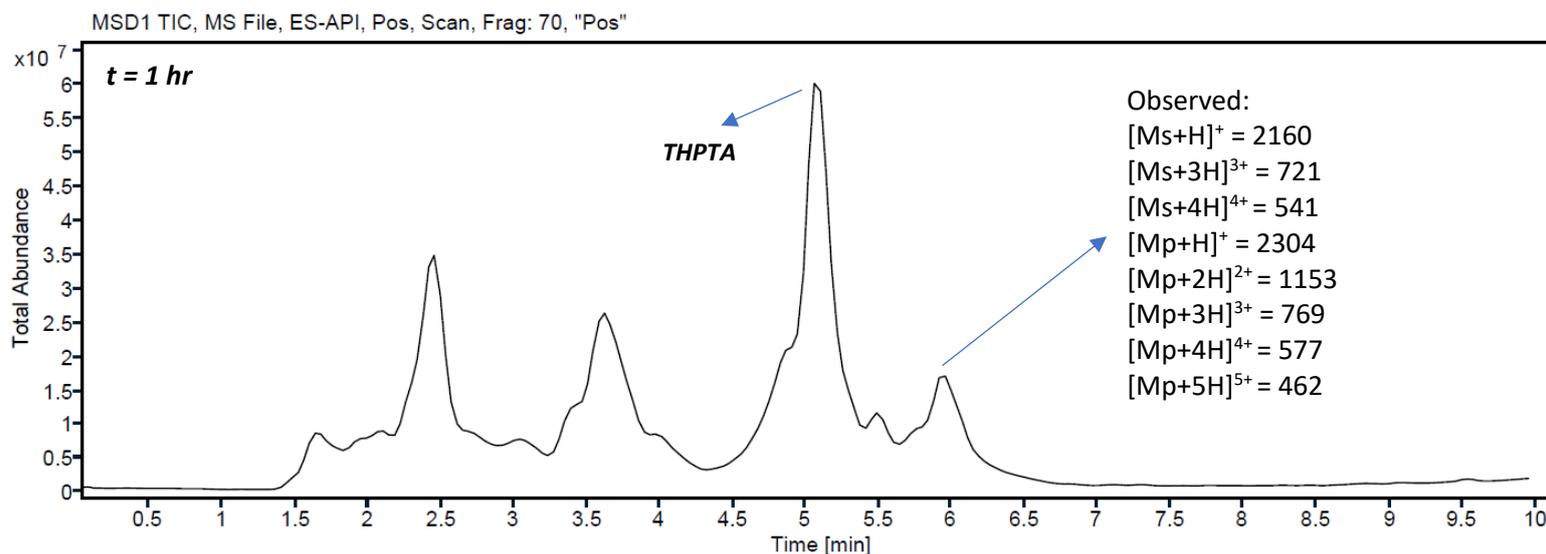
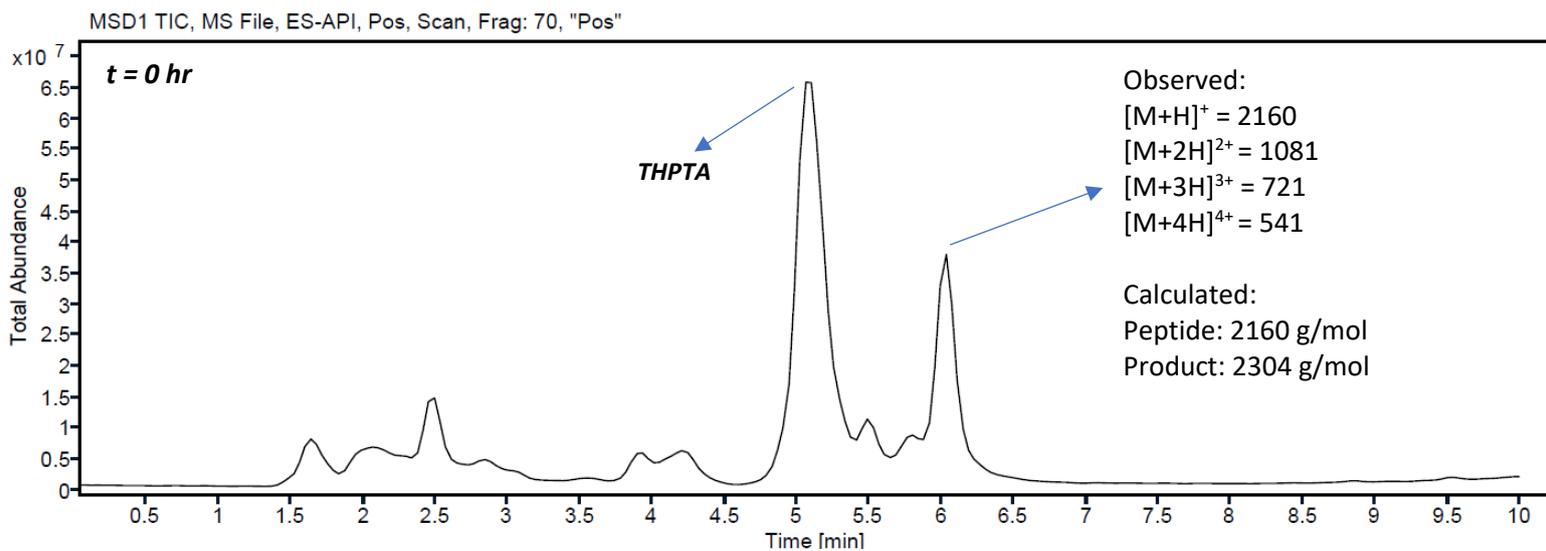


Figure S 12: Total ion chromatograms showing the click reaction of standard conditions with 25 μ M synthetic peptide and 100 μ M azidohomoalanine, measured at timepoints 0 and 1 hours. 50% Na Ascorbate was added at $t = 0$, the remaining 50% was added after 30 minutes. [Ms+H] Mass starting material. [Mp+H] Mass product.

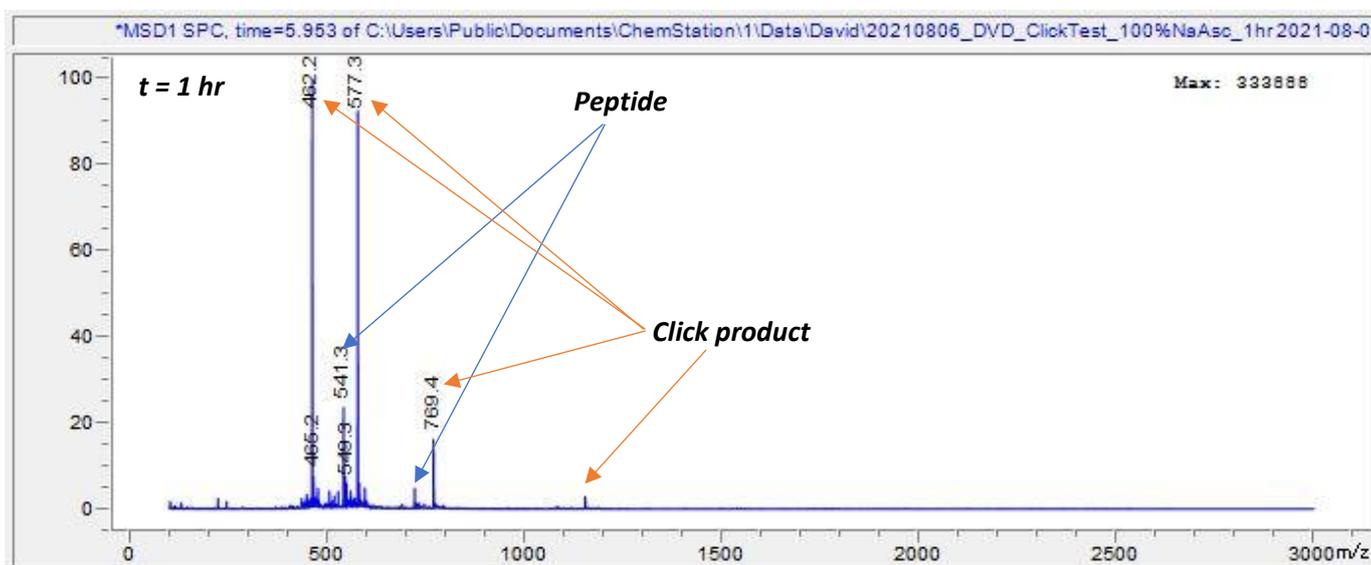
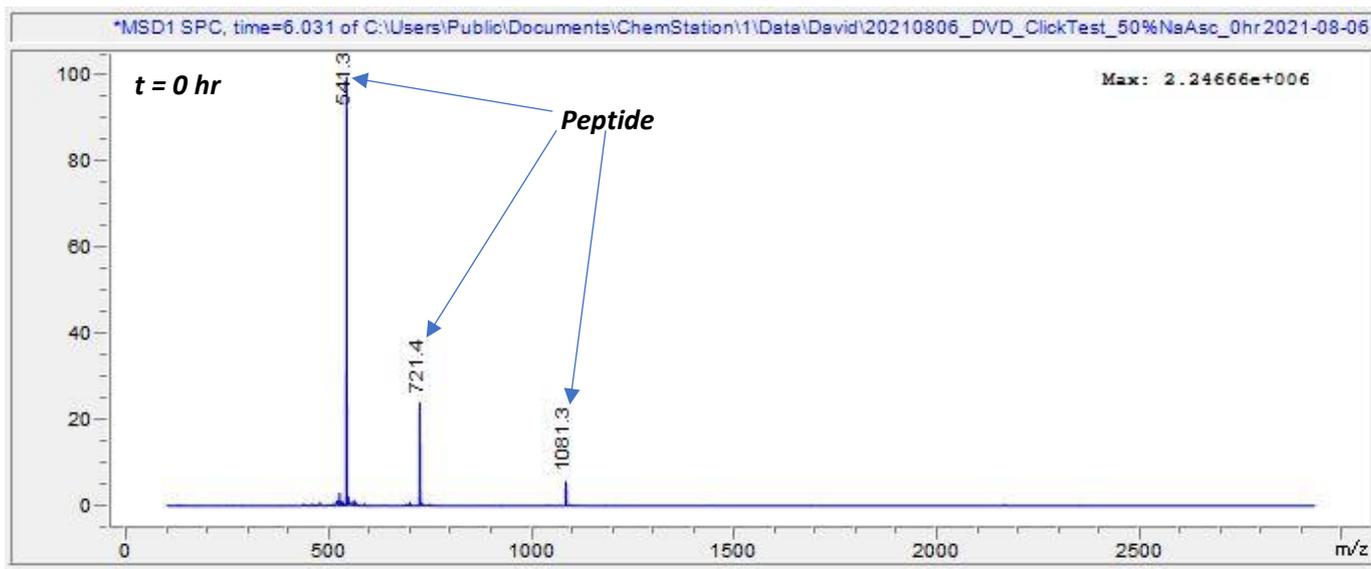


Figure S 23: Extracted mass spectra at retention times 6.0 and 5.9 min (before and after click reaction) shown in figure S 12 at timepoints 0 and 1 hours.

The peak area of the most abundant extracted ion at t = 0 (541) was used to roughly estimate the conversion of starting material:

t = 0 hr, peak area: 160 million

t = 1 hr, peak area: 21 million → 87% of starting material converted

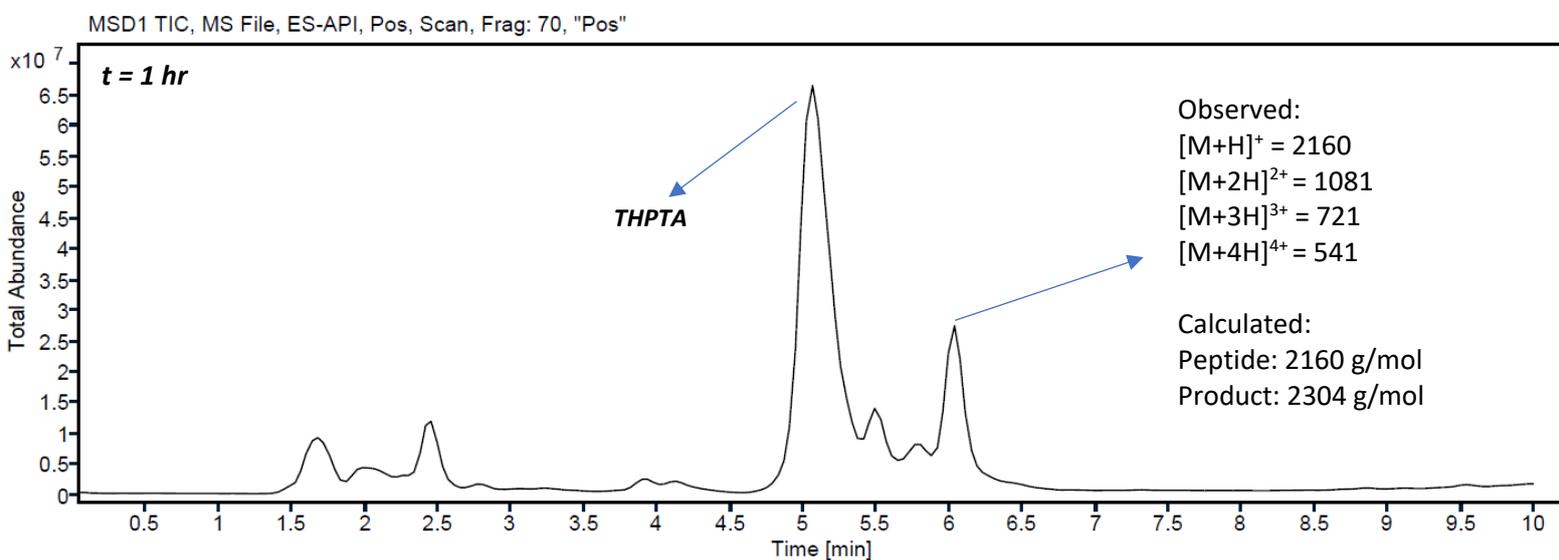
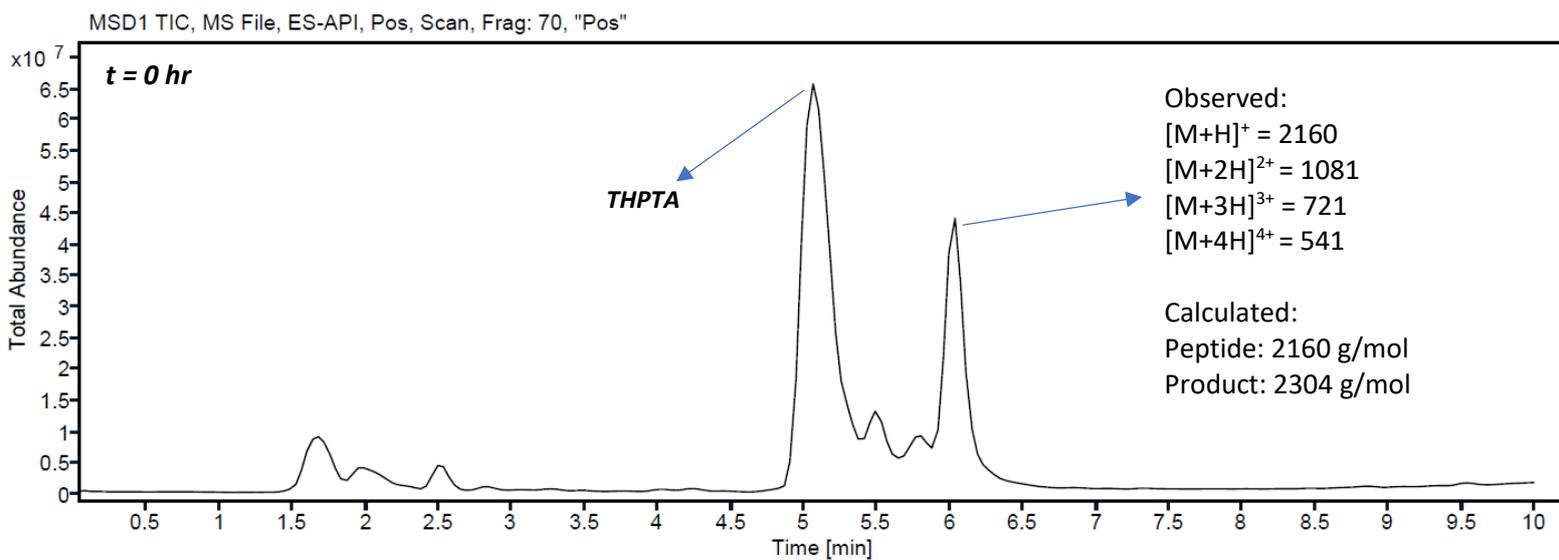


Figure S 14: Total ion chromatograms showing the click reaction of standard conditions with 25 μM synthetic peptide and 100 μM azidohomoalanine, measured at timepoints 0 and 1 hours. *Cul* was used as source of Cu^{1+} instead of CuSO_4 .

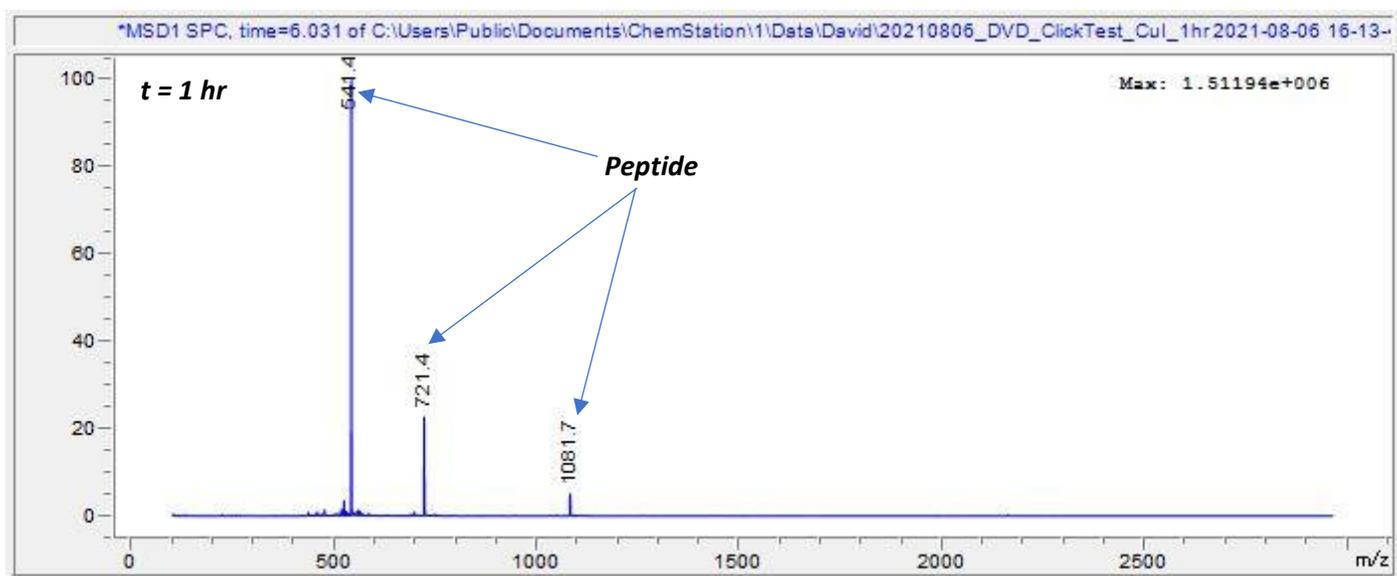
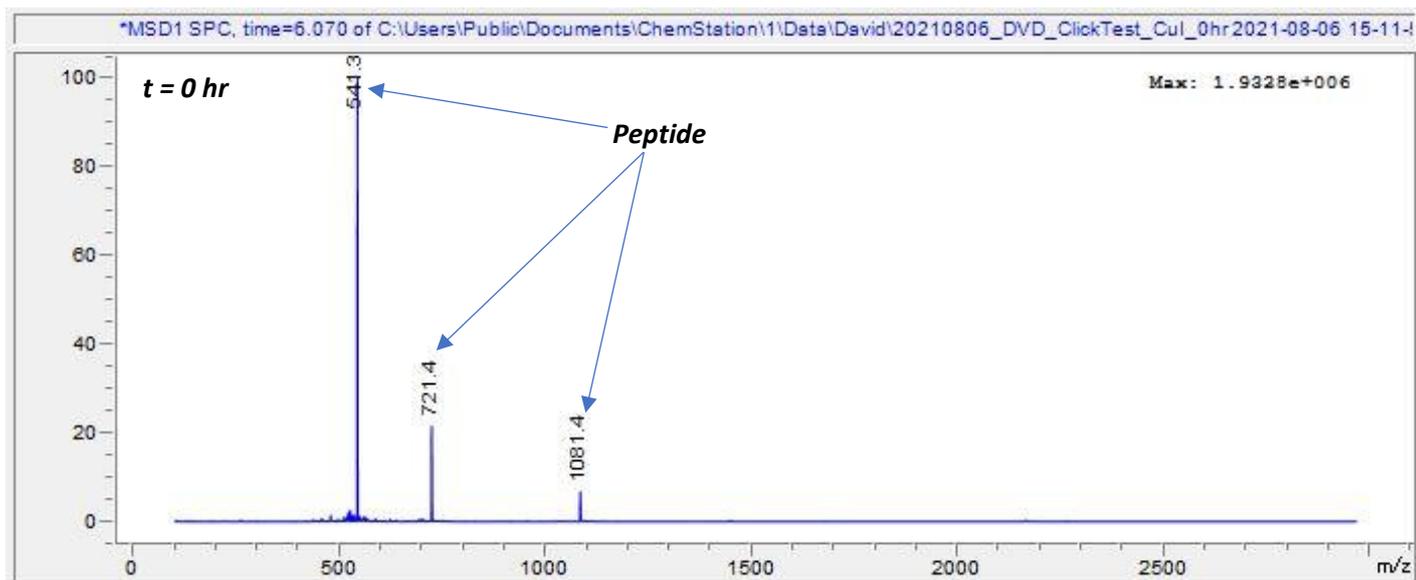


Figure S 15: Extracted mass spectra at retention time 6.0 min (before and after click reaction) shown in figure S 14 at timepoints 0 and 1 hours. The peak area of the most abundant extracted ion at t = 0 (541) was used to roughly estimate the conversion of starting material:

t = 0 hr, peak area: 199 million

t = 0.5 hr, peak area: 140 million → 30% of starting material converted

t = 1 hr, peak area: 113 million → 43% of starting material converted

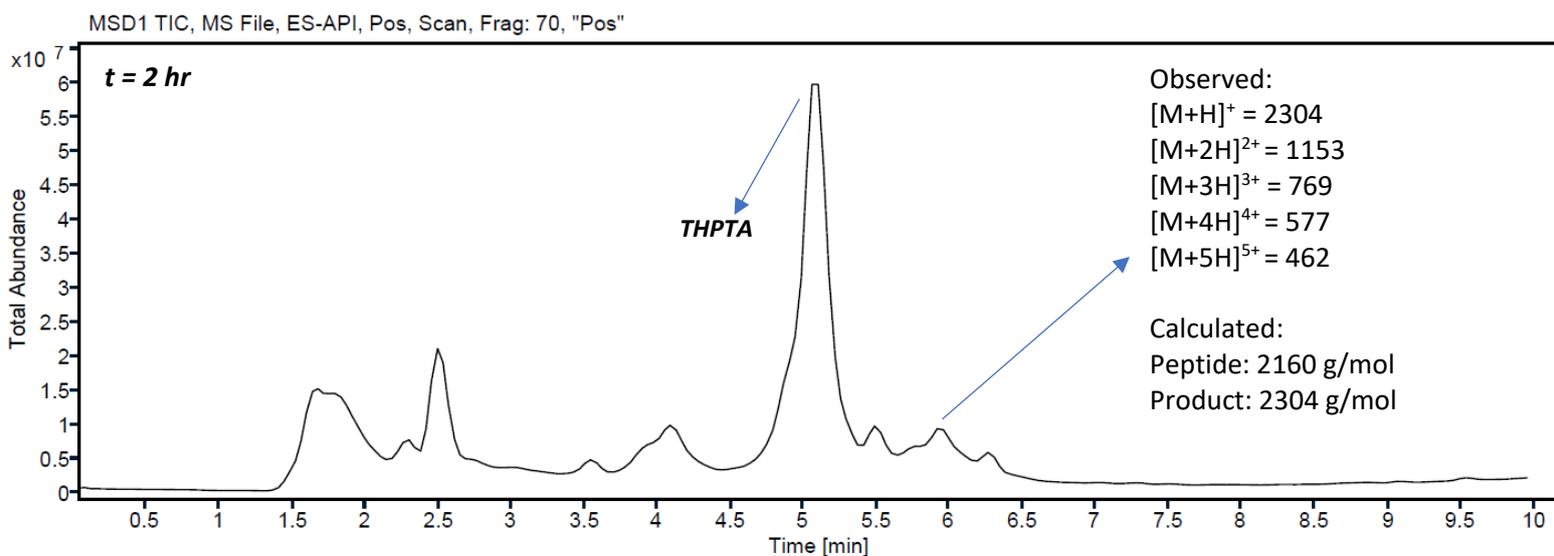
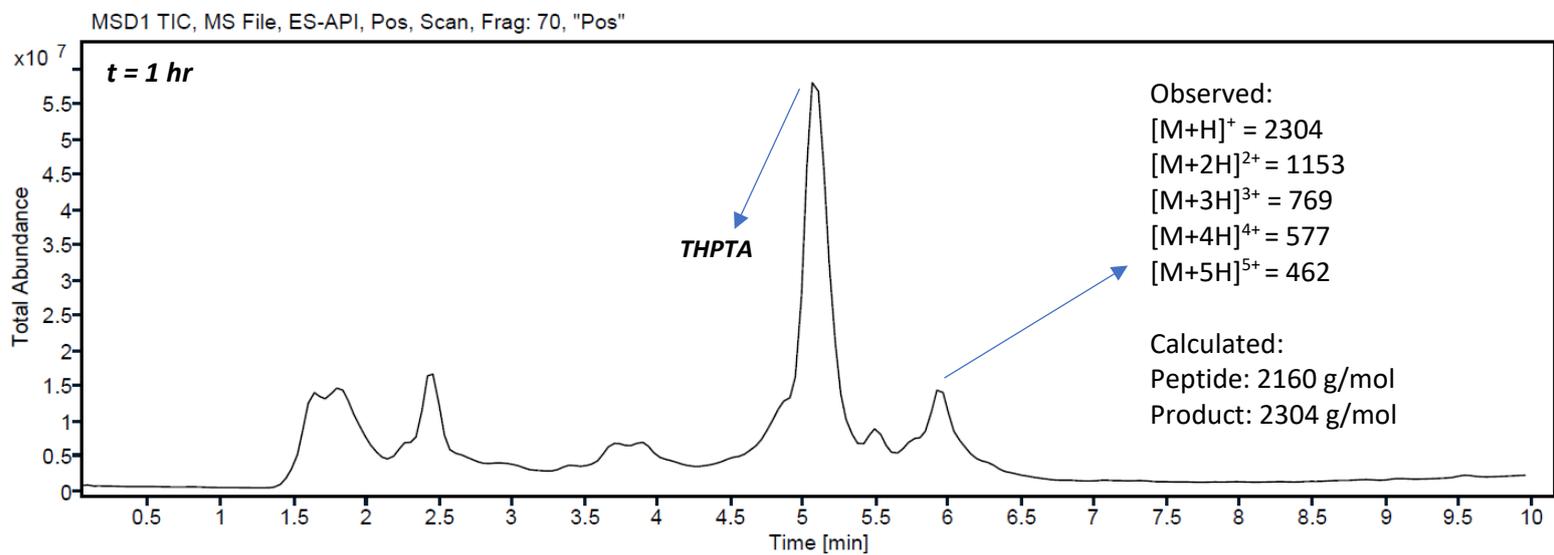
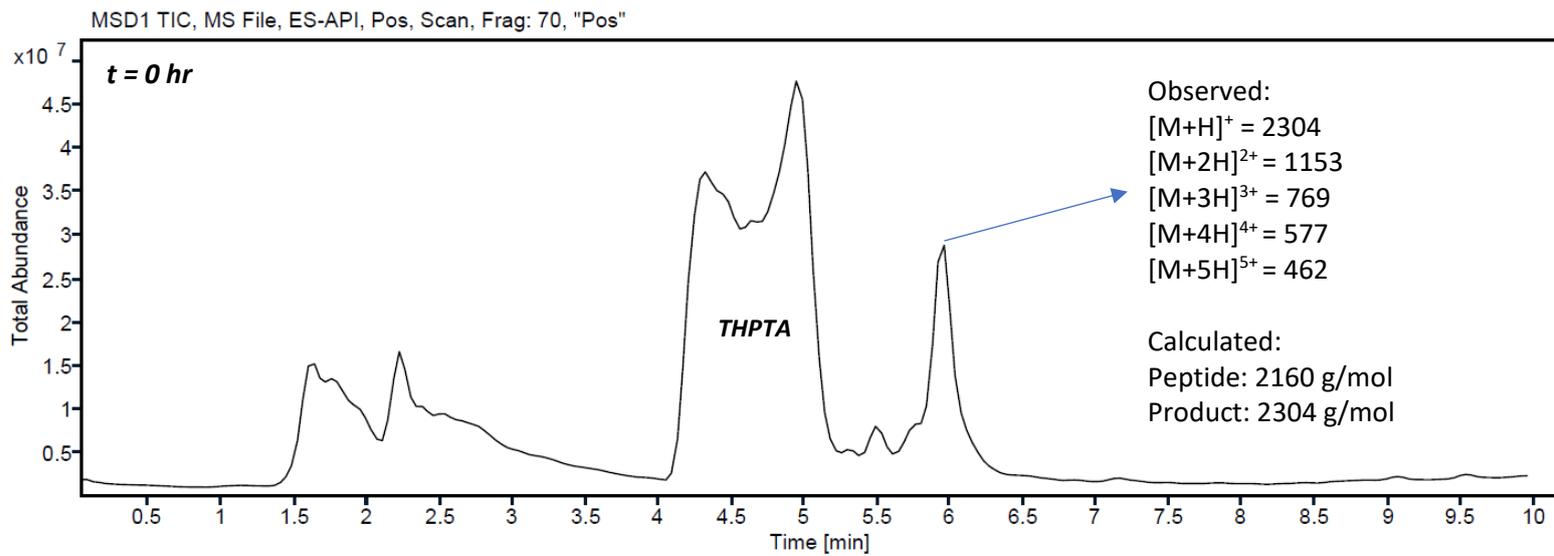


Figure S 16: Total ion chromatograms showing the click reaction of standard conditions with 25 μ M synthetic peptide and 100 μ M azidohomoalanine, measured at timepoints 0, 1 and 2 hours in Tris HCl buffer pH 8.3 instead of Phosphate buffer pH 6.5.

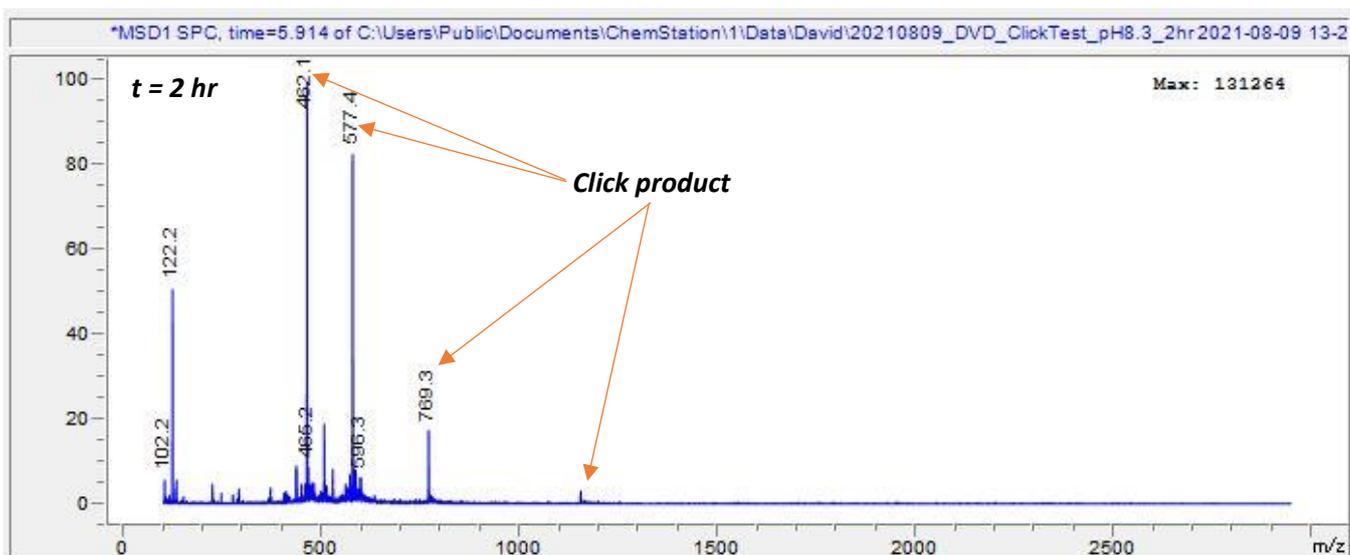
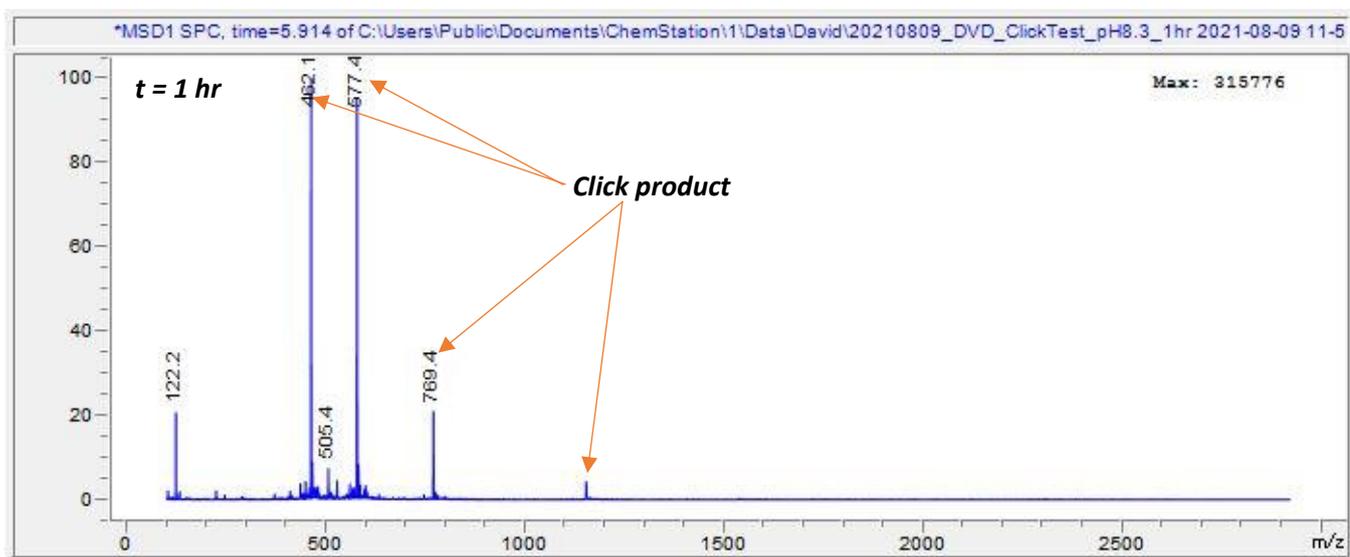
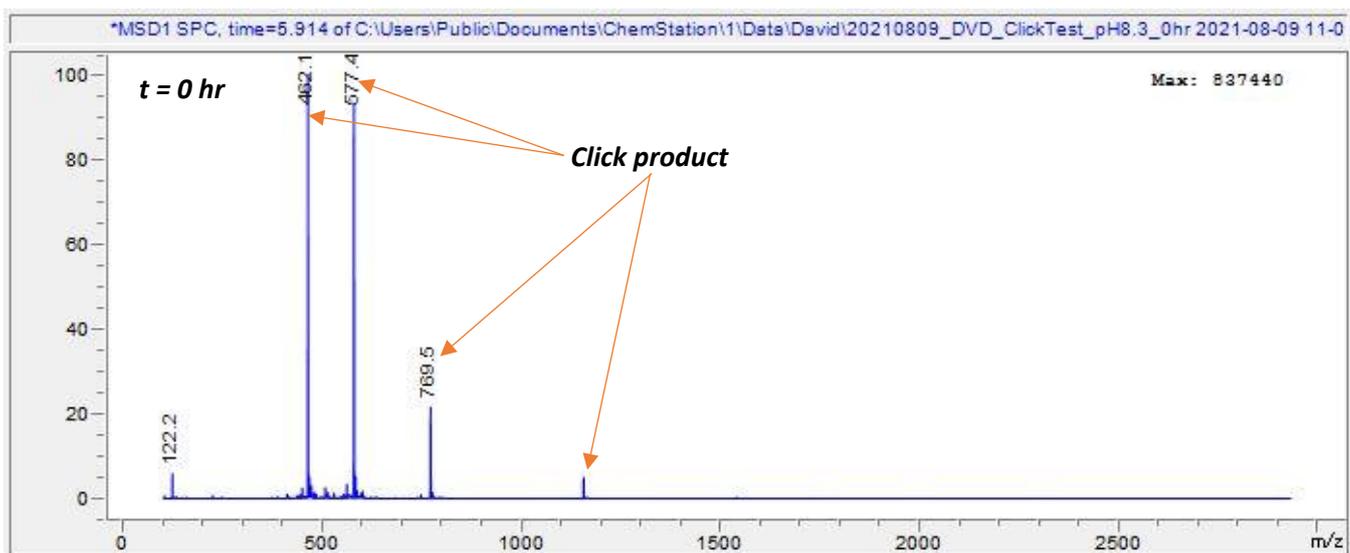


Figure S 17: Extracted mass spectra at retention time 5.9 min (before and after click reaction) shown in figure S 16 timepoints 0, 1 and 2 hours. No ions from starting material were extracted at t = 0, 1 and 2 hours. The conversion of starting material could not be calculated (N.A.).



Figure S 18: 8% urea-PAGE gel. Two click reactions with a 3 kDa azidopeptide to *in vitro* translated peptide (Streptag template). After translation, for one of the samples an ethanol precipitation was done prior to the click reaction (lane 4, 6, 8). Click reaction was executed at 12.5 μ L scale, and incubated 45 minutes at 42°C. 2x RNA loading buffer and traces BPB were added prior to sample loading (total volume 25 μ L, split into three lanes as 3, 6 and 9 μ L).

Lanes:

- 1) 2 μ M mRNA Streptag template
- 2) 2 μ M puro-linked mRNA Streptag template
- 3) -
- 4) 3 μ L of click after EtOH precipitation
- 5) -
- 6) 6 μ L click after EtOH precipitation
- 7) -
- 8) 9 μ L click after EtOH precipitation
- 9) -
- 10) 3 μ L click no EtOH precip.
- 11) -
- 12) 6 μ L click no EtOH precip
- 13) -
- 14) 9 μ L click no EtOH precip

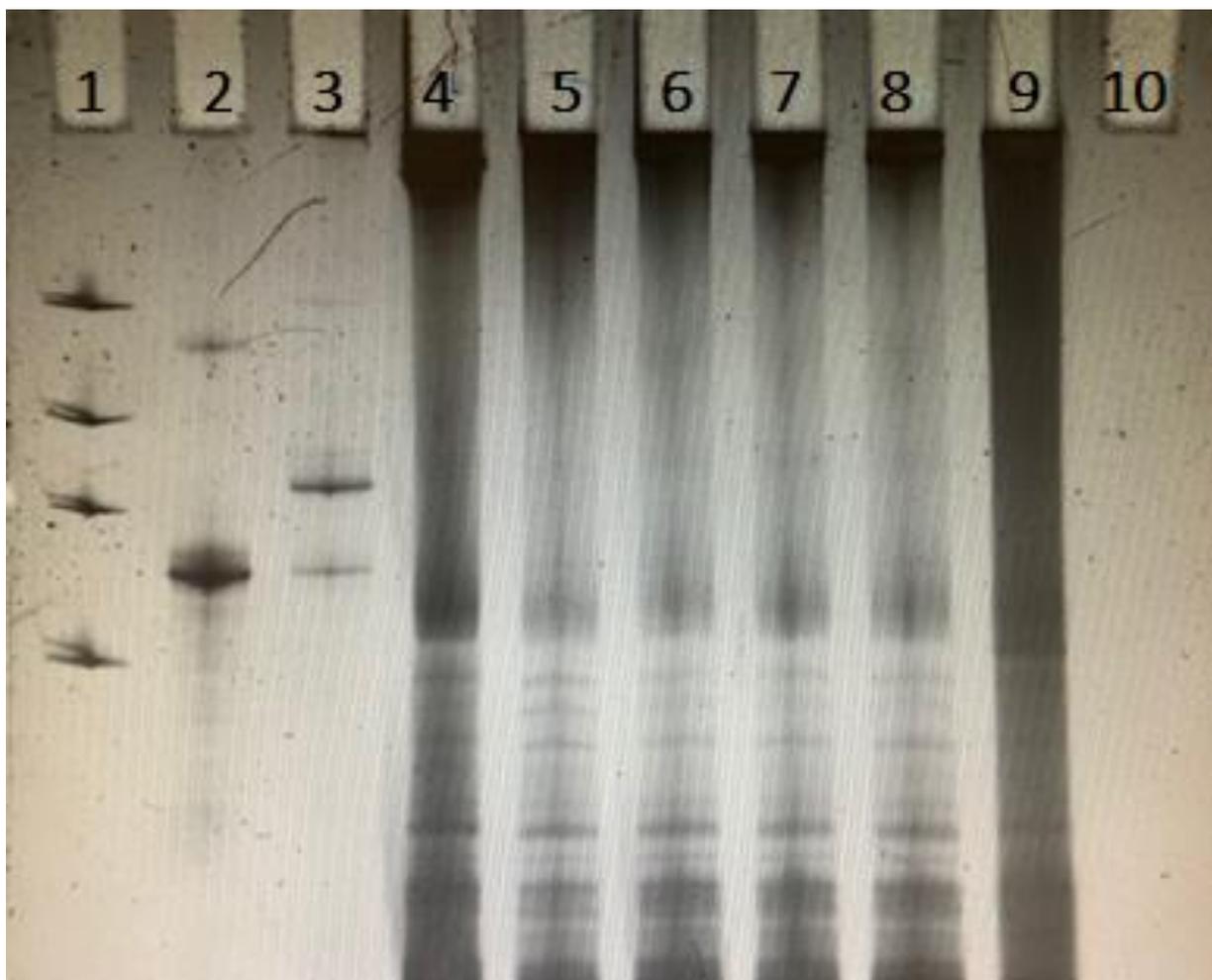


Figure S 19: 8% urea-PAGE gel. 5x scale translation of puromycin-linked Streptag mRNA was executed. The effect of each separate click component to the gel.

Lanes:

- 1) FastRuler Low Range DNA marker;
- 2) 2 μM mRNA Streptag template;
- 3) 2 μM puro-linked mRNA Streptag template;
- 4) 2 μM translated mRNA without EtOH precipitation
- 5) 2 μM translation mix + 0.5 mM CuSO_4
- 6) 2 μM translation mix + 2.5 mM THPTA
- 7) 2 μM translation mix + 5 mM aminoguanidine
- 8) 2 μM translation mix + 5 mM sodium ascorbate
- 9) 2 μM translated peptide clicked to 100 μM azidopeptide
- 10) -

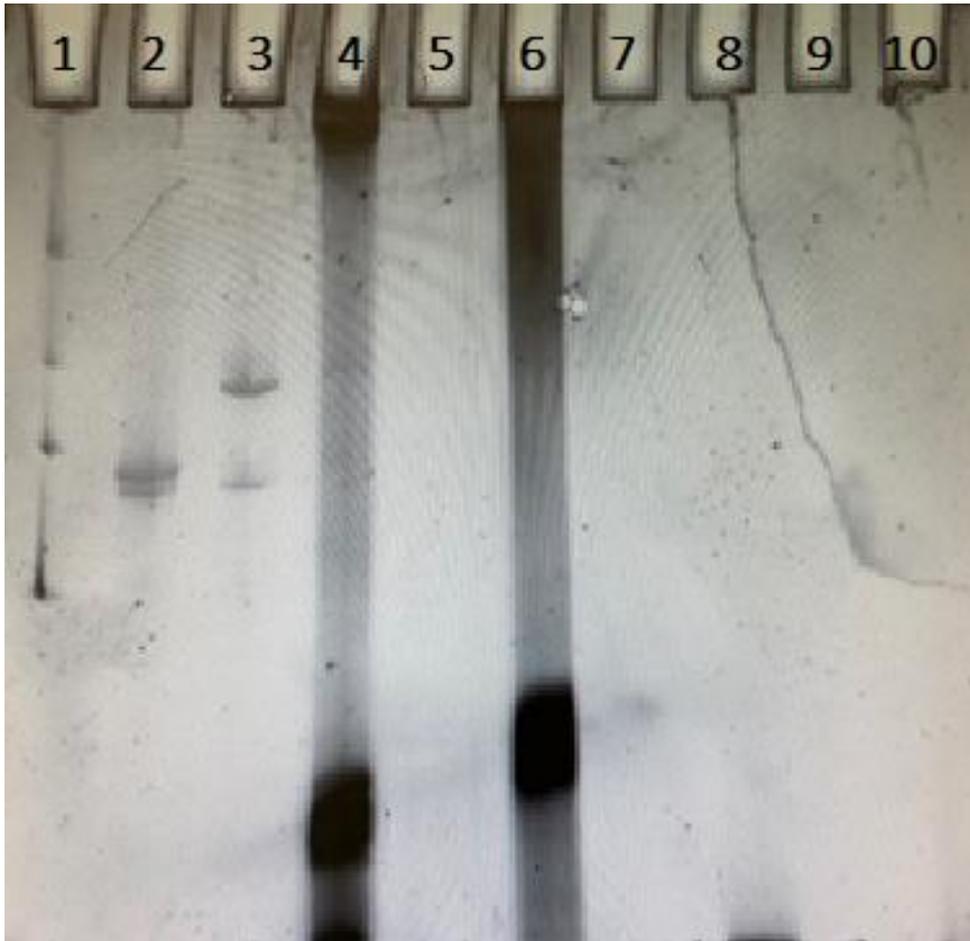


Figure S 20: 8% urea-PAGE gel. After 2x scale translation of the puromycin-linked Streptag mRNA, phenol-chloroform extraction and EtOH precipitation was done for both. 1 translation was directly loaded, the other was first used in a click reaction to 3 kDa azidopeptide, followed by EtOH precipitation.

Lanes:

- 1) FastRuler Low Range DNA marker
- 2) 2 μ M Streptag mRNA template
- 3) 2 μ M puromycin-linked Streptag mRNA
- 4) 2 μ M translated mRNA
- 5) -
- 6) 2 μ M translated mRNA clicked to 100 μ M azidopeptide
- 7) -
- 8) -
- 9) -
- 10) -

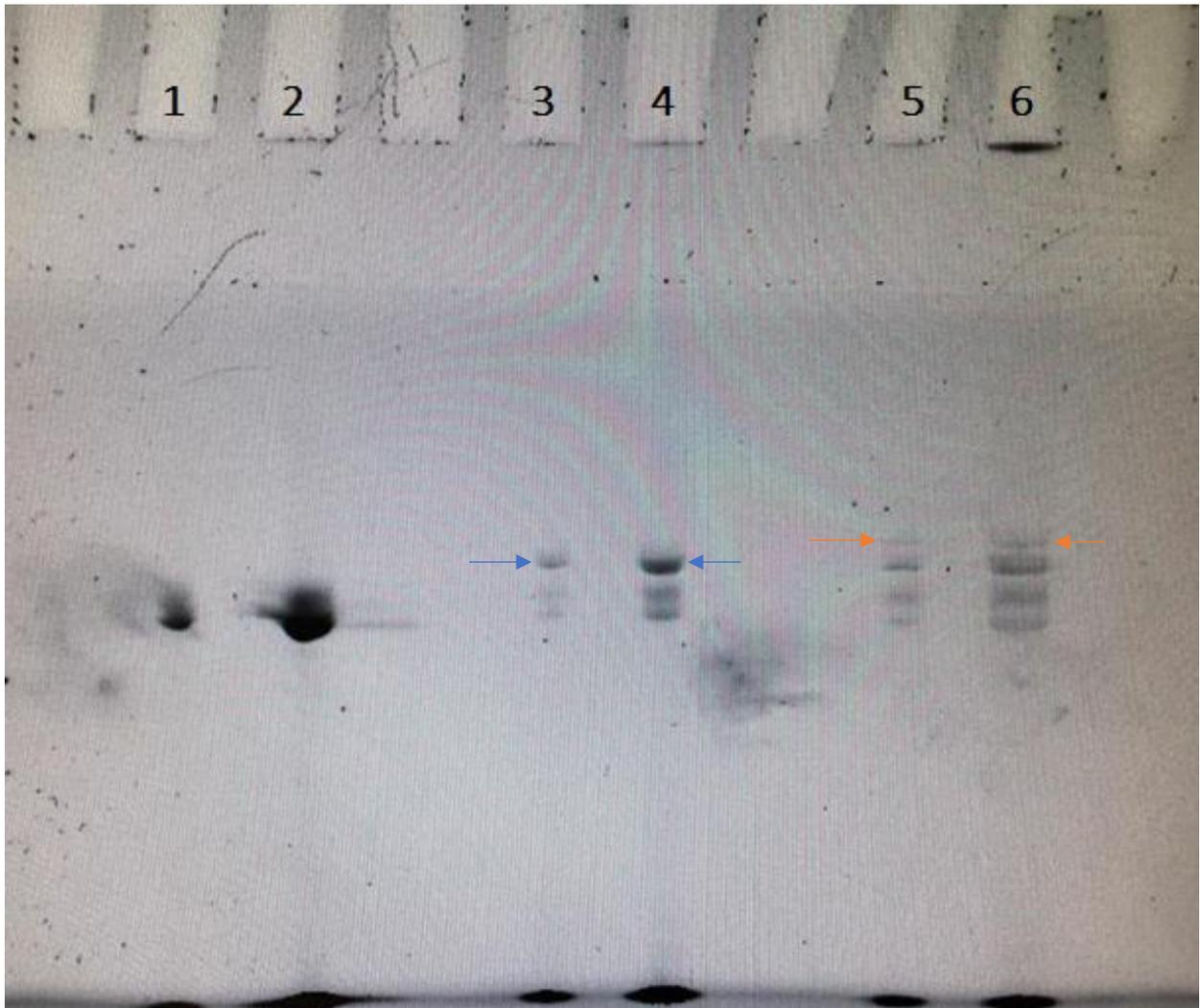


Figure S 21: Raw image of Figure 8. Ligation product indicated with blue arrows, translation product indicated with red arrows.

Lanes:

- 1) 200 nM template mRNA;
- 2) 2 μ M template mRNA;
- 3) 200 nM puromycin-ligated mRNA;
- 4) 2 μ M puromycin-ligated mRNA;
- 5) 200 nM peptide-puromycin-mRNA;
- 6) 2 μ M peptide-puromycin-mRNA.

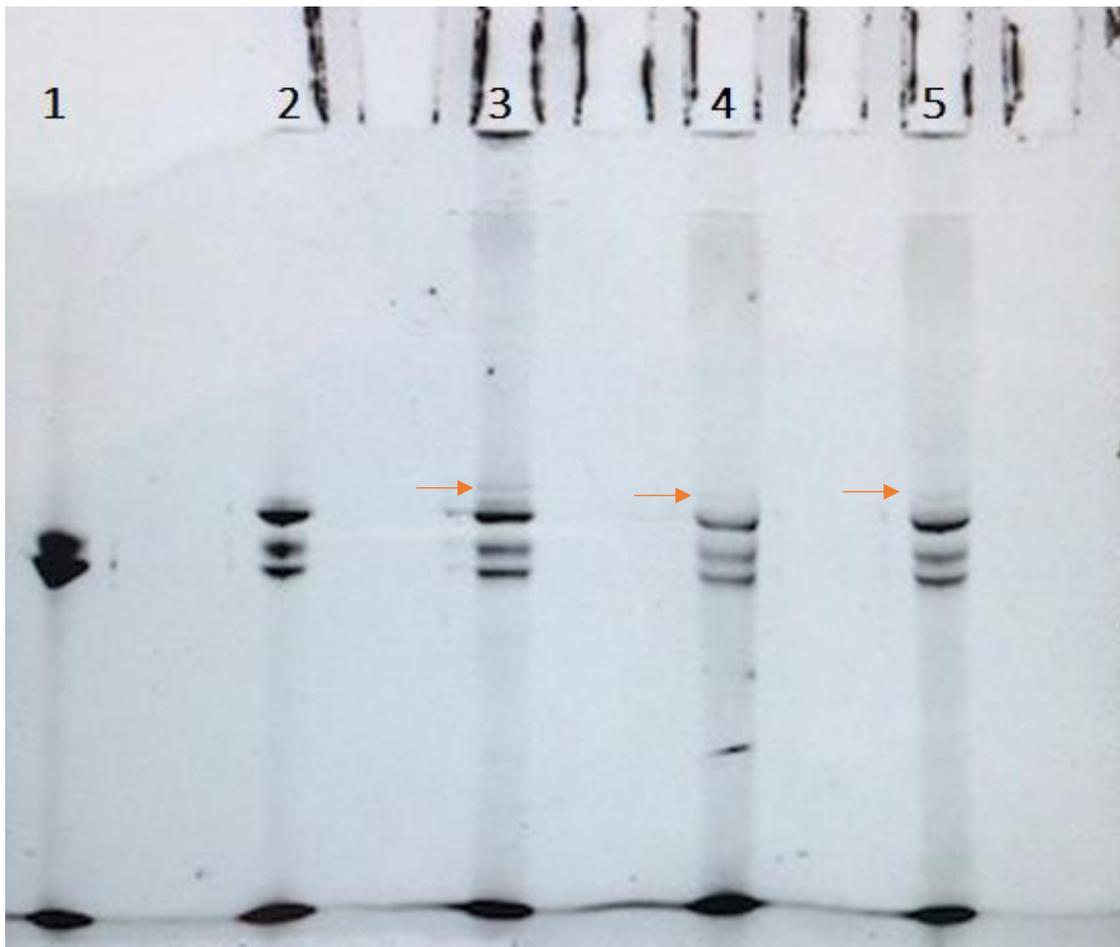


Figure S 22: Raw image of Figure 9. Translation product indicated with red arrows.

Lanes:

- 1) 2 μM Streptag template mRNA;
- 2) 2 μM puromycin-ligated mRNA;
- 3) 2 μM translation product;
- 4) 2 μM translation product clicked to 100 μM 3 kDa azidopeptide;
- 5) 2 μM translation product clicked to 100 μM iFluorTM 647 azide.

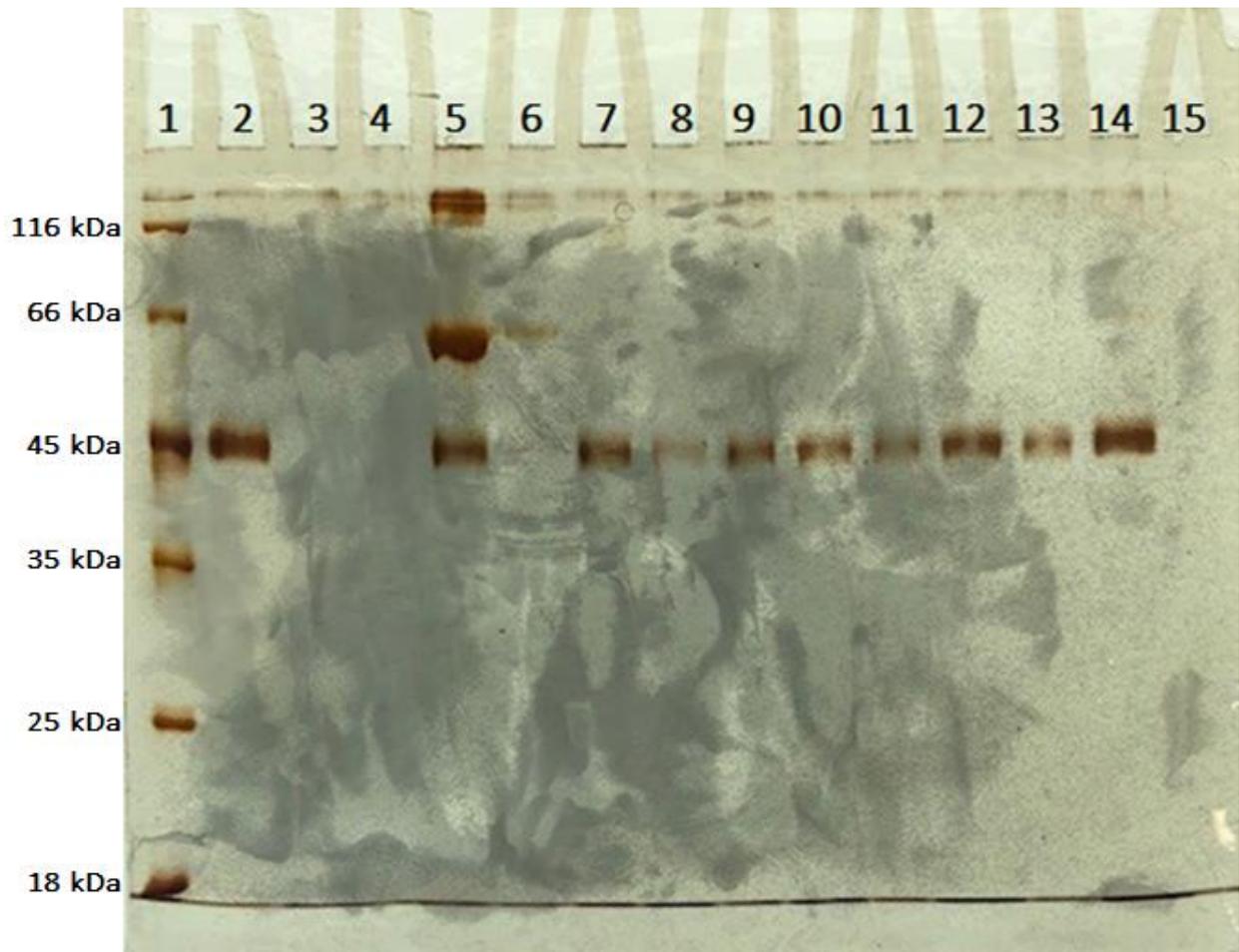


Figure S 23: Optimization of biotinylated HPSE binding to magnetic M-280 Streptavidin beads. The proteins were visualized by silver staining the SDS-PAGE gel. The bands were quantified by ImageJ to yield a biotinylation percentage and an optimal volume of beads to immobilize the protein during selection. Experimental methods section 6.

Lanes:

- 1) Unstained protein MW marker
- 2) 200 ng biotinylated HPSE control
- 3) 1 μ L beads control
- 4) -
- 5) S/N 0.5 μ L beads
- 6) 0.5 μ L beads + 200 ng biotinylated HPSE
- 7) S/N 1 μ L beads
- 8) 1 μ L beads + 200 ng biotinylated HPSE
- 9) S/N 2 μ L beads
- 10) 2 μ L beads + 200 ng biotinylated HPSE
- 11) S/N 5 μ L beads
- 12) 5 μ L beads + 200 ng biotinylated HPSE
- 13) S/N 10 μ L beads
- 14) 10 μ L beads + 200 ng biotinylated HPSE
- 15) -

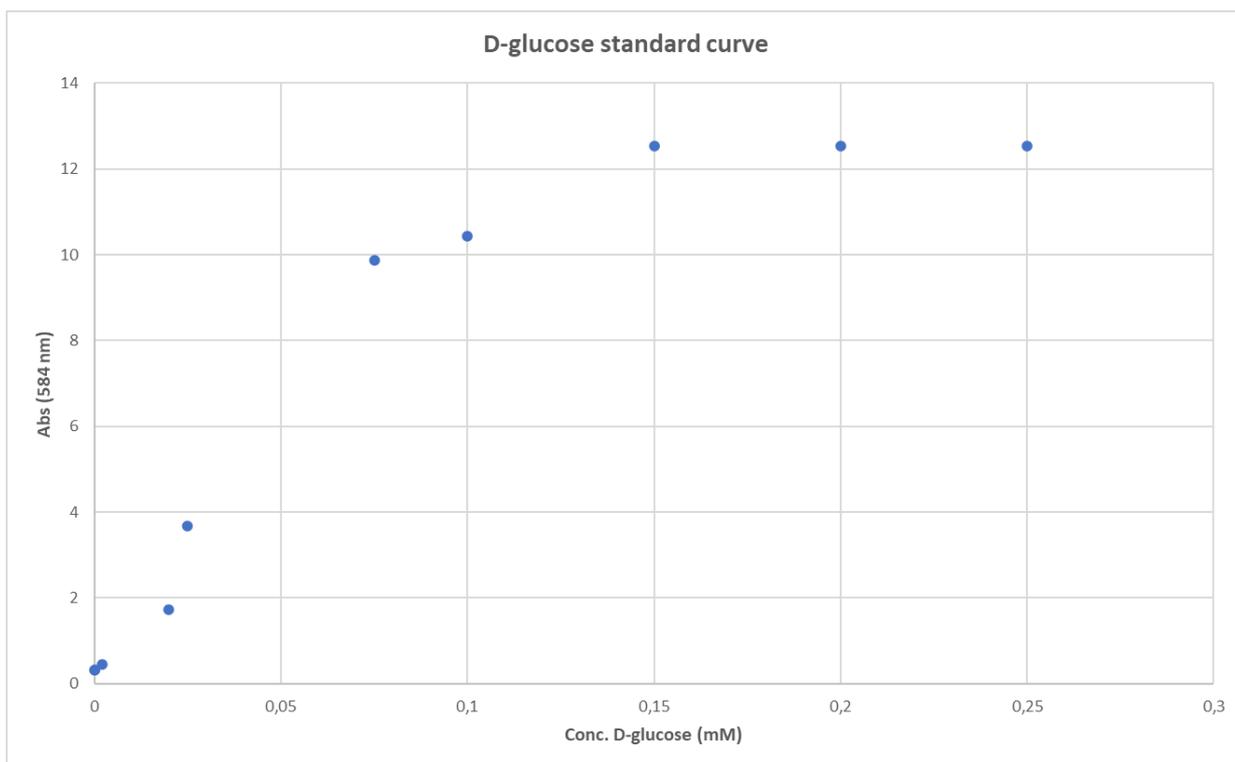


Figure S 24: Colorimetric activity assay standard curve of 0.2 μ M – 0.25 mM D-glucose. The absorbance plateau is just above 12. Experimental methods section 7.

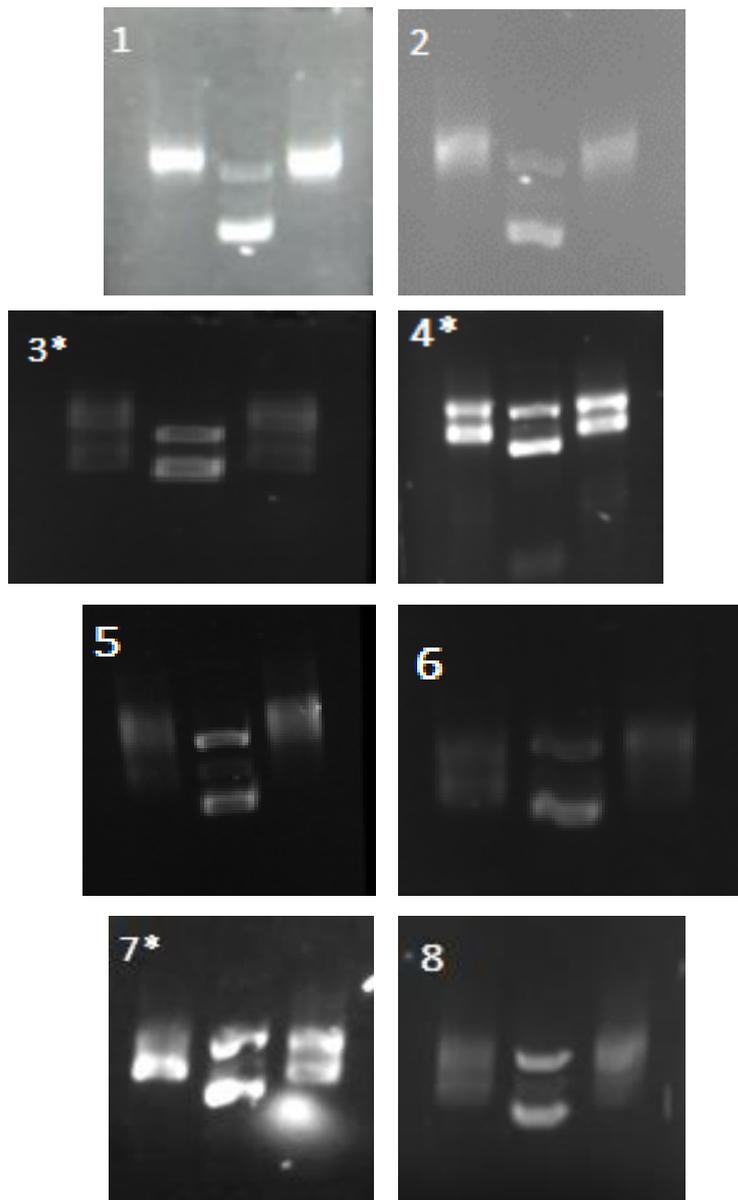


Figure S 25: 3% agarose gels of PCR product after selection round 1 – 8. In every figure, the left bands represent the D-Tyr library, the middle bands represent marker and the right bands represent the Linear library.

* indicates the rounds where purification steps were executed.

3* and 7* RNA purification by denaturing urea-PAGE gel;

4* DNA extraction from agarose gel.

The fact that the truncation already strongly returned in round 4, 5 and 8, directly after the purification steps, indicates the impact of this problem in the selection.