Acid labile solid phase peptide synthesis for semiconductor oxides and metal oxides with commercially available protected amino-acids.

Semiconductor oxides and metal oxides represent an important class of substrates to detect peptide-protein interactions using biosensors. Silicon dioxide (glass) is probably the most accessible substrate as it is the main component of microscope slides. Techniques such micro spotting use these substrates to immobilize peptide sequences to detect then the interactions with proteins by fluorescence. Silicon-dioxide and metal oxides such as Hf_2O_3 or TiO_2 are used as the interface of biosensors such as field effect transistors (FET), which can be used to detect the interactions of peptides with proteins with lower quantities than fluorescence using miniaturized biosensors. In ElectroMed we have developed a method able to locally produce acidity, which can be integrated with FET sensors that use a semiconductor oxide or a metal oxide interface between the liquid medium and the solid-state transducer. This is why we developed a compatible acid labile solid-phase synthesis.

To optimize resources, we have based our chemistry in commercially available protected amino-acids. We have also investigated the synthesis with raw amino-acids for the ones where the protection of side-chains were not available, being able to obtain reasonable purities of crude amino-acids (up to 70%), for relevant sequences as DYK peptide, which can be used in combination with a flag antibody for a selective immunorecognition that detects only the presence of this sequence.

The first step was the selection of a correct substrate that would allow us to produce larger quantities than those than will be used in the sensors in order to control the synthesis products with mass spectrometry. To this end we employed a Controlled Porous Glass functionalized with 6-*N*-(9-Fluorenylmethoxycarbonyl)aminohexyl succinate and Fmoc-rink amide (from here in we will just refer to the substrate as CPG). The control porous glass is similar to the SiO_2 dielectric interface of the sensors, but as silicon and metal oxides both exhibit oxygen radicals with similar electroaffiity, the chemistry that we will developed with this method will be compatible for these materials.

The next step of the design was to select a model sequence to test the synthesis process that included the different amino acids. This sequence was FP-X-AG (using standard letter notation for the aminoacids), where the middle position X can be variated to test different amino acids. For this position we studied three different classes of amino acids attending to its complexity for the synthesis:

1. Bifunctional amino acids, in our study represented by valine, leucine and isoleucine.
2. Trifunctional protected amino acids, represented by lysine, aspartic acid and glutamic acid, glutamine and asparagine.
3. Trifunctional non-protected amino acids, where we used serine, tyrosine, tryptophan and histidine and threonine.

We studied different coupling conditions in terms of reagents optimising the coupling times (between 30 minutes and 1 hour for our experiments with beads of CPG contained in a syringe), and the relative concentration of amino acids (4, 10 and 20 equivalents). We also explored different solvents (DMF and acetonitrile) and different deprotecting acid agents (TFA and HCl), that in our final platform will be substituted by electrochemically generated acid.

Following the studies of the 5 amino-acid peptide modelled we also studied the extension o 8-mer and 10-mer peptide sequences. And we also studied variations of the DYK flag sequence with extra peptide sequences: DYKDD, DYKK and DYKGGSG.

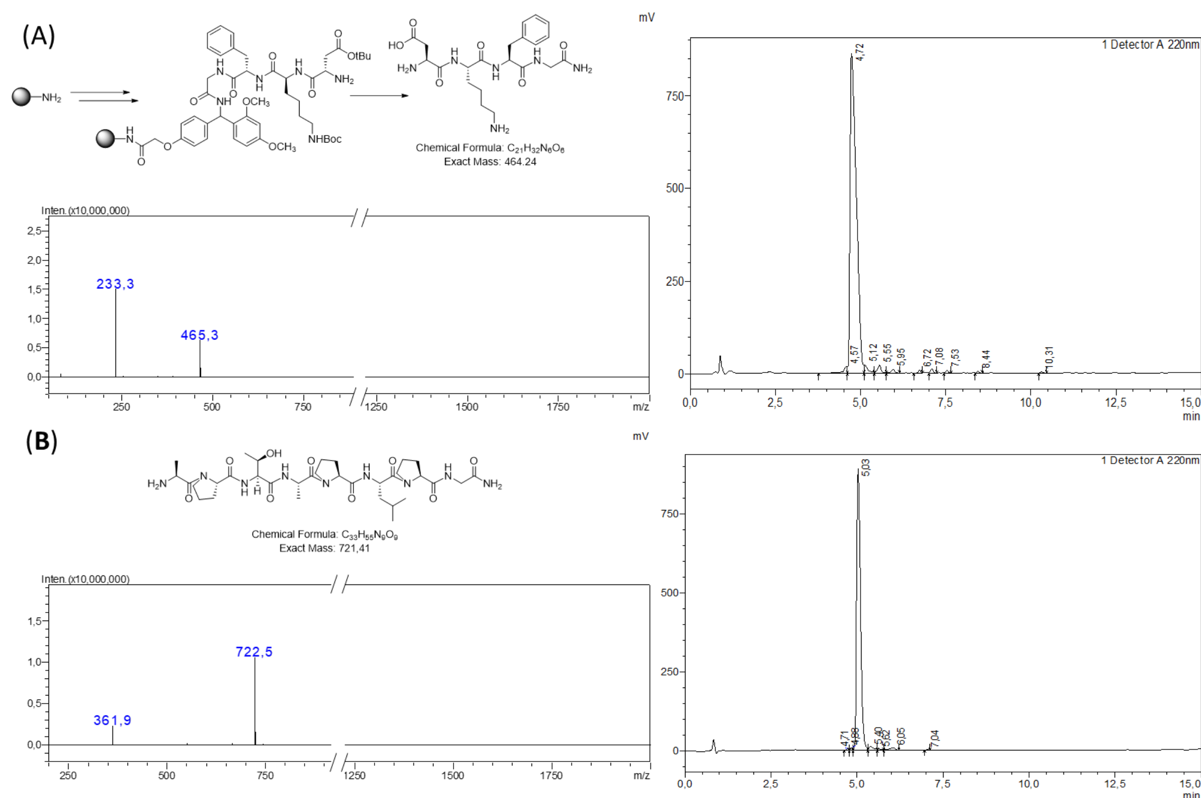
The amino acids were cleaved from the porous glass and then studied by mass spectrometry in tandem with high-performance liquid chromatography (HPLC-MS).

First results summary.

Following the selection of CPG several approaches were studied in detail to choose the best option for the glass beads as a surface resembling the material of FET sensors. Controlled pore glass (CPG) was stablished as the final choice as can be purchased already functionalized as long chain alkylamine (Biosearch Technologies) and therefore represents a better starting point.

After the selection of CPG, several linkers were tested (6-N-(9-Fluorenylmethoxycarbonyl)aminohexyl succinate and Fmoc-rink amide) using an standard basis labile Fmoc/tBu strategy for the preparation of 4-mer and 8-mer peptides. After obtaining high purities (95%), it was concluded that these linkers attached to a glass support worked smoothly, and remaining attached after an standard conditions for basic deprotections (piperdine/DMF). The final peptide was isolated by the cleavage of the linker in acid media where side-chain of amino acids were also removed satisfactory.

Figure 1 shows the chromatogramsof the High PerformanceLiquid Chromatography-mass spectrometry (HPLC-MS) for the synthesis for the 4-mer peptide (fig.1(A)) and the 8-mer peptide (Fig.1(B)). In all the chromatograms the main peaks correspond to the counts of the targeted sequence, showing the high purity achieved. **Figure 1 (A)** shows, in addition, the chemical structure of the rink-amide linker (acid sensitive) used for the synthesis and the 4-mer sequence. The coupling conditions of the 4mer peptide were achieved using 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 2h. The Fmoc removal was carried out using piperidine/DMF (2:8) for 15min. High crude puriy (94%). **Figure 1 (B)** shows the HPLC-MS results for the 8-mer peptide APTAPLPG (Strong HHV5 epitope) that was synthesised with the following coupling conditions: 20 eq aa, 20 eq HBTU, 40 eq DIPEA in DMF for 1h. The Fmoc removal was carried out with Piperidine/DMF (2:8) for 15min. High crude purity (96%).



Following the selection of the substrate we moved to validate a proof of concept for the synthesis protocols compatible with acid labile groups as electrochemically generated acid is the final aim for local deprotection. To implement the use of acid labile chemistry, tert-Butyloxycarbonyl (Boc) was selected as a deprotection methodology for the synthesis of peptides (Boc-Aa's). Then, a base labile linker and base labile protection of the side chains were required to have an orthogonal method respect to the Boc protection used for the synthesis. The base labile linkers will not be used in the *in-situ* synthesis on the final configuration with sensors as solid phase support, but it is intended for the cleaving of the peptides to monitor the quality of the synthesis with MS. Hidroxybenzoic acid (HMBA) was studied as a linker for one of the sequences reported in the first deliverable APTAPLG, to be selective to HHV5 epitopes. HMBA can also be used employing Fmoc chemistry using milder basic conditions for the synthesis deprotection while stronger basic conditions for the peptide cleavage of the linker.

In the following step we used smaller trial peptides testing Boc-APFG on CPG for the initial optimisation of the conditions with acid deprotection. We tested different acidic deprotections and after the cleavage in basic conditions (NaOH 0.1M in dioxane for 2h) the tested sequences were successfully detected by HPLC showing that HMBA was appropriate for the Boc synthesis.

We tested combinations of acid/solvents for Boc deprotection obtaining the following preliminary results:

Acid	**Solvent**	**Time**	**Yield**
50% TFA	DCM	5 min	78% Boc removal
4M HCl	Dioxane	5 min	88% Boc removal
1M HCl	Ac.	5 min	No Boc removal
Citric acid buffer pH 3.5	Ac.	5 min	No Boc removal

Extended results.

As it was previously reported, CPG has been stablished as a solid support in our model. The UPF 5-mer model sequence based on the strategy of acid labile groups deprotection (Boc chemistry). The sequence model (FP-X-AG) contains 4 bifunctional amino acids in the edges and a variation of amino acids in the third position of the sequence (X) to test the different behaviour of the different chemical handles. When commercially available, this strategy can use the base-labile Fmoc protection for functional side-chains. Three different groups of amino acids that were studied as candidates for the X position were: 1) Bifunctional, 2) Trifunctional protected and 3) Trifunctional non-protected amino acids.

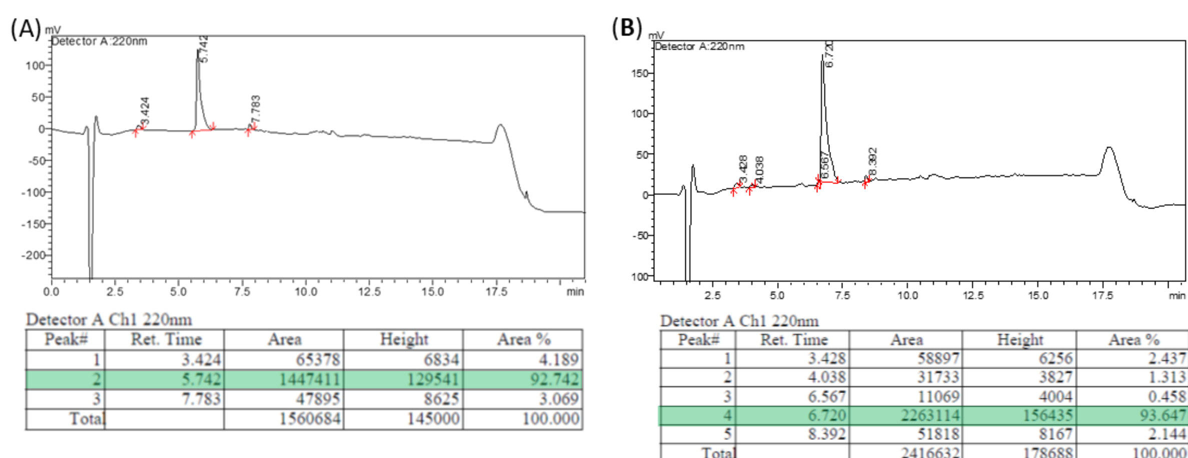
Table 1 Summary of the results obtained with the first trial amino acids:

Sequence	Eq Aa, HBTU;DIPEA	Solvent	Coupling time	Deprotection time	Purity
FPLAG	4eq Aa, 4eq HBTU, 8eq DIPEA	DMF	1h (x2)	45min (x2)	66%
FPLAG	20eq Aa, 20eq HBTU, 40eq DIPEA	DMF	1h (x2)	45min (x2)	93%
FPIAG	4eq Aa, 4eq HBTU, 8eq DIPEA	DMF	1h (x2)	45min (x2)	60%
FPIAG	20eq Aa, 20eq HBTU, 40eq DIPEA	DMF	1h (x2)	45min (x2)	81%
FPEAG	20eq Aa, 20eq HBTU, 40eq DIPEA	DMF	1h (x2)	45min (x2)	95%
FPEAG	20eq Aa, 20eq HBTU, 40eq DIPEA	ACN	1h (x2)	45min (x2)	95%
FPDAG	20eq Aa, 20eq HBTU, 40eq DIPEA	DMF	1h (x2)	45min (x2)	90%
FPEAG	20eq Aa, 20eq HBTU, 40eq DIPEA	Propylene carbonate	1h (x2)	45min (x2)	Low
FPEAG	20eq Aa, 20eq HBTU, 40eq DIPEA	diethyl carbonate	1h (x2)	45min (x2)	Low
FPEAG	20eq Aa, 20eq HBTU, 40eq DIPEA	ethylene carbonate	1h (x2)	45min (x2)	Low
FPKAG	20eq Aa, 20eq HBTU, 40eq DIPEA	DMF	1h (x2)	45min (x2)	
FPKAG	20 (Aa), 20 (OXIMA), 40 (DIC)	DMF	1h (x2)	45min (x2)	
FPKAG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	1h (x2)	45min (x2)	86%
FPYAG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	1h (x2)	45min (x1)	56%
FPTAG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	1h (x2)	45min (x1)	76%
FPSAG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	1h (x2)	45min (x1)	63&
FPYAG	20 (Aa), 20 (HBTU), 40 (DIPEA) + 20 OXIMA, 20 DIC	DMF	1h (x2)	45min (x2)	56%
FPTAG	20 (Aa), 20 (HBTU), 40 (DIPEA) + 20 OXIMA, 20 DIC	DMF	1h (x2)	45min (x2)	68%
FPSAG	20 (Aa), 20 (HBTU), 40 (DIPEA) + 20 OXIMA, 20 DIC)	DMF	1h (x2)	45min (x2)	
FPYAG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	25 min (x2)	45min (x1) + 2min (x1)	56%
FPTAG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	25 min (x2	45min (x1) + 2min (x1)	81%
FPSAG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	25 min (x2)	45min (x1) + 2min (x1)	77%
DYKG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	25 min (x2)	45min (x1) + 2min (x1)	67%
DYKGG	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	25 min (x2)	45min (x1) + 2min (x1)	75%
DYKD	10 (Aa), 10 (HBTU), 20 (DIPEA)	DMF	25 min (x2)	45min (x1) + 2min (x1)	71%

In our next experiments, the chemical part of the project has been focused on exploring the whole range of amino acids into the sequence of the CPG model developed by Pompeu Fabra University (UPF).

Specifically, the scope of the bifunctional Aa was extended to Valine (FPVAG-HMBA-CPG) and the previously optimized conditions were applied using 10eq of the corresponding Aa, 10eq of HBTU and 20eq of DIPEA as coupling reagents. Shorter reaction time was also tested and stablished within 45 min to avoid side reactions. Deprotection of Boc-Aa was carried out with HCl 4M in dioxane for 45min to obtain 94%. The result of the Isoleucine derivative (FPIAG-HMBA-CPG) was improved employing the conditions mentioned above with shorter reactions times reaching 93% of purity.

Figure 2 shows: (A) HPLC-MS of FPVAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 30min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (94%). (B) HPLC-MS of FPIAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 30min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (93%).



(A) Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	3.424	65378	6834	4.189
2	5.742	1447411	129541	92.742
3	7.783	47895	8625	3.069
Total		1560684	145000	100.000

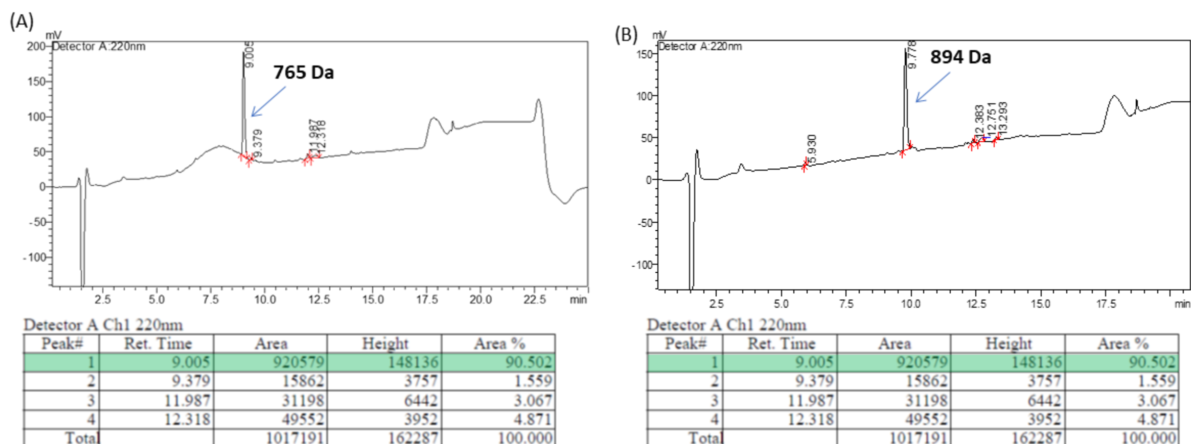
(B) Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	3.428	58897	6256	2.437
2	4.038	31733	3827	1.313
3	6.567	11069	4004	0.458
4	6.720	2263114	156435	93.647
5	8.392	51818	8167	2.144
Total		2416632	178688	100.000

To extend the scope of this proof of concept, longer sequences were synthesized to validate the feasibility of the system.

First, an 8-mer (FGAFPVAG-HMBA-CPG) was made by introducing three bifunctional amino acids into de N-terminal (FGA) showing a 91% of purity without purification. Second, the sequence was elongated by adding two more residues up to 10-mer (GAFGAFPVAG-HMBA-CPG) without affecting the purity of the peptide (90% purity). The reliability of the system is shown by the high purity for these octa and deca-peptides. The combination of a glass solid support employing Boc chemistry is adequate for the modulation of semiconductors and open a new area to explore in this field. The HMBA-CPG model shows the viability to construct sequence up to 20-mer showing high purity without purification.

Figure 3 shows: A) HPLC-MS of FGAFPVAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 30min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (91%). B) HPLC-MS of GAFGAFPVAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 30min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (90%).



Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	9.005	920579	148136	90.502
2	9.379	15862	3757	1.559
3	11.987	31198	6442	3.067
4	12.318	49552	3952	4.871
Total		1017191	162287	100.000

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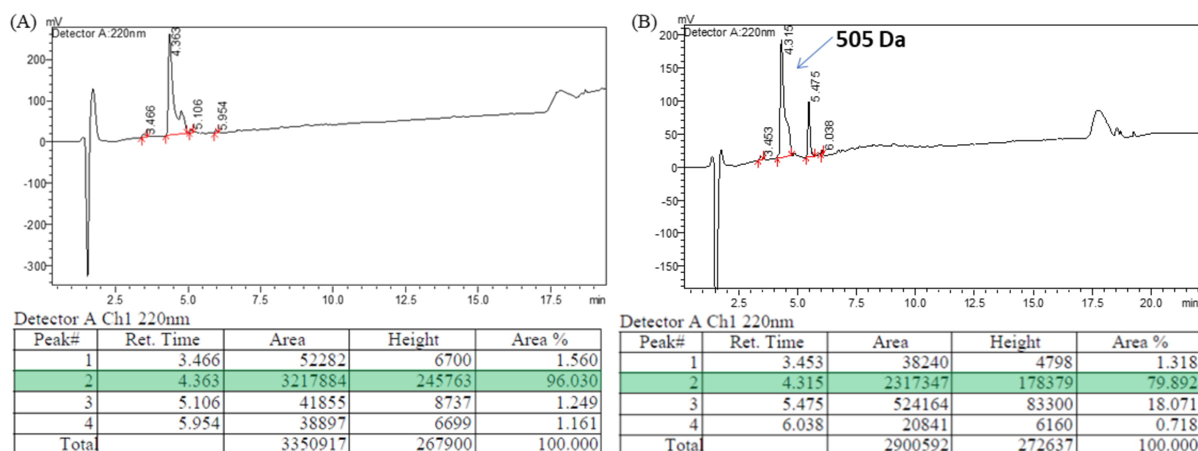
Total		1017191	162287	100.000

To complete our 5-mer model based on CPG, asparagine (N) and glutamine (Q) were also introduced into the sequence (FPNAG-HMBA-CPG and FPQAG-HMBA-CPG) under the stablished conditions. As every amino acid has their own specific characteristics according to the functional group in the side chain, it is remarkable to highlight that amide group can give place to an acid under acidic conditions. In order to overcome this inconvenient, shorter coupling times helped to avoid this transformation in the case of Glutamine an a 96% of purity was achieved. In the case of asparagine, 80% of purity was achieved.

Figure 4 shows: (A) HPLC-MS of FPQAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 25min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (96%). (B) HPLC-MS of FPNAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 25min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (80%).



Detector A Ch1 220nm

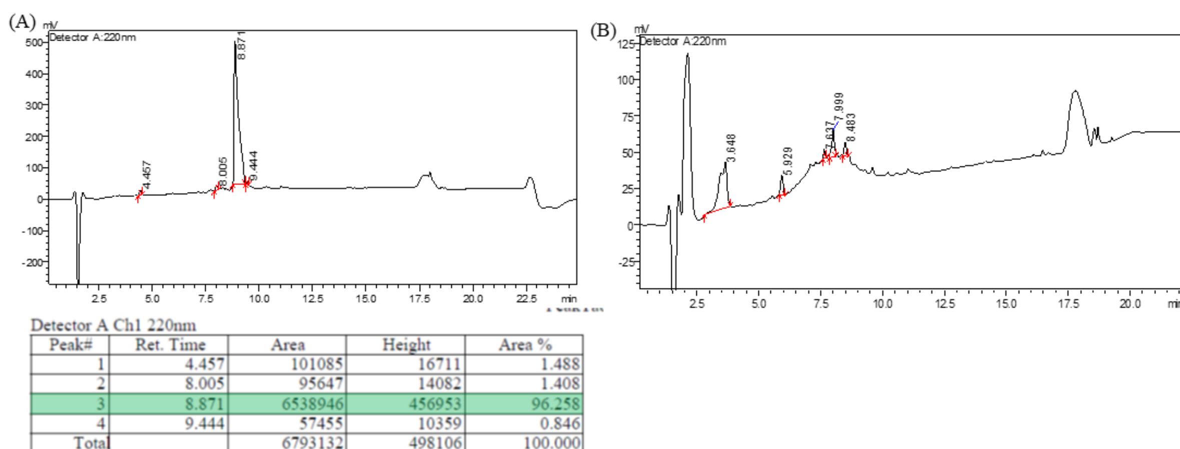
Peak#	Ret. Time	Area	Height	Area %
1	3.466	52282	6700	1.560
2	4.363	3217884	245763	96.030
3	5.106	41855	8737	1.249
4	5.954	38897	6699	1.161
Total		3350917	267900	100.000

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	3.453	38240	4798	1.318
2	4.315	2317347	178379	79.892
3	5.475	524164	83300	18.071
4	6.038	20841	6160	0.718
Total		2900592	272637	100.000

Heterocyclic amino acids are also present in many structures and represents a gold value to introduce it in our structure. For this purpose, the next combination was synthesized (FPWAG-HMBA-CPG) achieving the best purity of all sequences (96% of purity). In the case of the heteroaromatic 5-member ring histidine (FPHAG-HMBA-CPG) was not feasible to achieve the final peptide due to solubility problems of this amino acid.

Figure 5 shows: (A) HPLC-MS of FPWAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 25min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (96%). (B) HPLC-MS of FPHAG-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 25min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (0%).

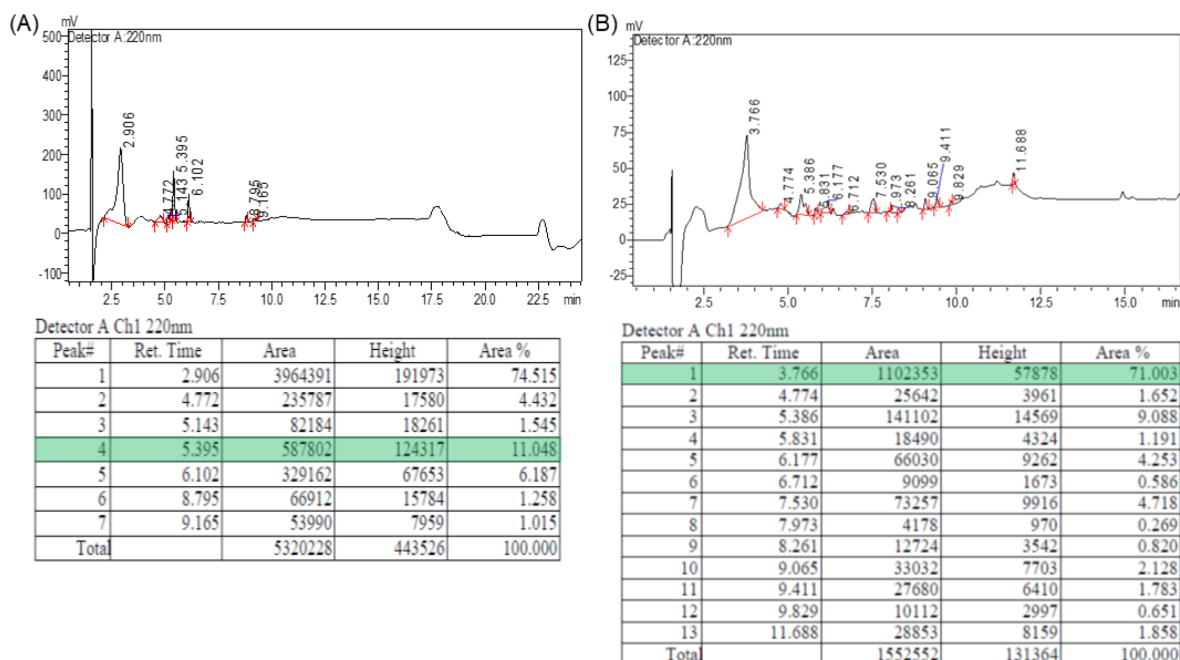


Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	4.457	101085	16711	1.488
2	8.005	95647	14082	1.408
3	8.871	6538946	456953	96.258
4	9.444	57455	10359	0.846
Total		6793132	498106	100.000

Taking into account the previous results, DYK fragments were synthesized based on the specific recognition with a monoclonal antibody. New combinations keeping DYK fragment were also tested. Next synthesis was (DYKDD-HMBA-CPG) employing standard conditions (HBTU, DIPEA and 30 min coupling time) and a moderate purity was obtained reaching 65%. In another sequence (DYKD-HMBA-CPG) the purity was also moderate obtaining 71%.

Figure 6 shows: (A) HPLC-MS of DYKDD-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 25min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (75%). (B) HPLC-MS of DYKD-HMBA-CPG peptide. Coupling conditions: 10 eq aa, 10 eq HBTU, 20 eq DIPEA in DMF for 25min (x2times). Boc removal: HCl/dioxane: 1x45min. Crude purity (71%).



(A) Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	2.906	3964391	191973	74.515
2	4.772	235787	17580	4.432
3	5.143	82184	18261	1.545
4	5.395	587802	124317	11.048
5	6.102	329162	67653	6.187
6	8.795	66912	15784	1.258
7	9.165	53990	7959	1.015
Total		5320228	443526	100.000

(B) Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	3.766	1102353	57878	71.003
2	4.774	25642	3961	1.652
3	5.386	141102	14569	9.088
4	5.831	18490	4324	1.191
5	6.177	66030	9262	4.253
6	6.712	9099	1673	0.586
7	7.530	73257	9916	4.718
8	7.973	4178	970	0.269
9	8.261	12724	3542	0.820
10	9.065	33032	7703	2.128
11	9.411	27680	6410	1.783
12	9.829	10112	2997	0.651
13	11.688	28853	8159	1.858
Total		1552552	131364	100.000

Table 2: Summary of experiments using DMF as solvent with coupling conditions:10eq Aa, 10eq HBTU, 20eq DIPEA.

Entry	**Sequence**	**Coupling time**	**Deprotection time**	**Purity**
1	FPVAG	30min (x2)	45min (x1)	94%
2	FPIAG	30min (x2)	45min (x1)	93%
3	FGAFPVAG	30min (x2)	45min (x2)	91%
4	GAFGAFPVAG	30min (x2)	45min (x2)	90%
5	FPYAG	25min (x2)	25min (x1)	56%
6	DYKGGSG	25 min (x2)	45min (x1)	10%
7	DYKDD	25 min (x2)	45min (x1)	65%
8	DYKKK	25 min (x2)	45min (x1)	57%
9	FPNAG	25 min (x2)	45min (x1)	80%
10	FPQAG	25 min (x2)	45min (x1)	96%
11	FPRAG	25 min (x2)	45min (x1)	NP
12	FPWAG	25 min (x2)	45min (x1)	96%
13	FPHAG	25 min (x2)	45min (x1)	NP

In conclusion, the CPG- based model is the one that most closely resemble the final device. These results show a valid proof of concept to build peptides on a glass surface with moderate-high purity and versatility under mild conditions. Finally, HPLC-MS represents a viable test of the reliability of the results.