A fishing technology of glycated peptides

F. Nuti[1,2*], M. Marino[1,2], P. Ledwon,[1,3,4] R. Latajka [4], A. Lapolla [5], M. Chorev [6], P. Rovero [1,3], A.M. Papini[1,2]

e-mail: francesca.nuti@unifi.it

https://doi.org/10.17952/37EPS.2024.P2186

[1]Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, University of Florence, Sesto Fiorentino, Italy
[2]Department of Chemistry "Ugo Schiff", University of Florence, Sesto Fiorentino, Italy
[3]Department of Neurosciences, Psychology, Drug Research and Child Health, University of Florence, Sesto Fiorentino, Italy
[4]Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Science and Technology, Wrocław, Poland
[5]Diabetology and Dietetics, Department of Medicine (DIMED), University of Padova, Padova, Italy
[6]Laboratory for Translational Research, Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, U.S.A.

Introduction

Glycation is a non enzymatic post-translational reaction between glucose and amino functions in proteins at *N*-terminus and/or on Lys or Arg side-chains. Amadori products are well known in food chemistry, but also as intermediates of Advanced Glycation End Products (AGEs) involved in complications of Diabetes and other neurodegenerative diseases [1]. The non enzymatic glycation of peptides and proteins in human beings occurs spontaneously in vivo but is highly pronounced when persistent and excessive levels of glucose are present in blood as in the case of diabetes: the sugar-modified peptides may modify their functional conformation and lead to lose of their normal activity. Thus, detection, isolation and sequencing of glycated peptides is crucial. Solid supports modified with a phenyl boronate (PhB) moiety can be useful tools to fish out peptide-sugar conjugates in biological fluids.

In our previous work ChemMatrix® Rink (CMRR) resin modified with a lysine substituted two PhB units, PhB-Lys(PhB)-CMRR was found to be able to capture selectively peptides containing deoxyfructosyl-lysine moieties, which then can be efficiently and specifically detected by MS experiments [2]. In order to optimize this "fishing" methodology, in terms of efficiency and selectivity, we studied the capturing of deoxyfructosylated peptides (**1-8**) by (i) comparing the above mentioned the ChemMatrix® Rink resin (CMRR) with ω-Aminohexyl–Agarose resin (ω-AHA) and by (ii) comparing the same two resins, CMRR and ω-AHA, modified with mono- or di-PhB substituted Lys moieties.

Resin Synthesis

To compare the different capture properties of both resins, the ω-AHA was functionalized on the amino function with the Rink Linker present in the commercially available ChemMatrix® Rink resin and called this new solid support Agarose Rink Resin (AGRR).

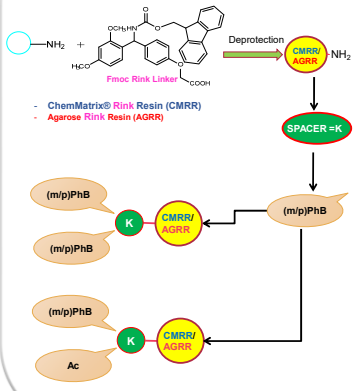


Table 1 Functionalized resins to capture glycated peptides.

CMRR - ChemMatrix® Rink resin; AGRR - Agarose Rink resin

No.		Resin		Loading (mmol/g)	Quantity (g)
1	CMRR	di-modified	*p*-PhB-Lys(*p*-PhB)-CMRR	0.462	0.961
2			*m*-PhB-Lys(*m*-PhB)-CMRR	0.354	0.533
3		mono-modified	Ac-Lys(*p*-PhB)-CMRR	0.298	0.549
4			Ac-Lys(*m*-PhB)-CMRR	0.151	0.487
5			*p*-PhB-Lys(Ac)-CMRR	0.274	0.478
6	AGRR	di-modified	*p*-PhB-Lys(*p*-PhB)-AGRR	0.524	0.0284
7			*m*-PhB-Lys(*m*-PhB)-AGRR	0.406	0.0698
8		mono-modified	Ac-Lys(*p*-PhB)-AGRR	0.317	0.0252
9			Ac-Lys(*m*-PhB)-AGRR	0.203	0.0702

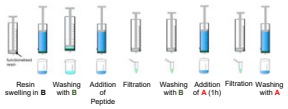
Table 2 List of the Amadori CD59 peptides and the glycosylated peptides to test.

No.	Name	Sequence	MW [g/mol]	Quantity available (mg)
1	***"Amadori" Peptide 1***	Ac-YNKSWK(Nε-1-deoxyFru)FEHSNFNDVC-OH	2236	5
2	***Non-modified Peptide 1***	Ac-YNKSWKFEHSNFNDVC-OH	1930	5
3	***Scrambled Peptide 1***	Ac-**VSEND**K(Nε-1-deoxyFru)YNFHKSWNFC-OH	2236	5
4	***Amadori GCD-59 Surrogate Calibrator***	Ac-NKAWK(Nε-1-deoxyFru)FEHANFNDC(OH)-***PEG LINKER***-Ac-ACFNDVTTRLRENELTYYCAAK	5940	5
5	***Glucitol GCD-59 Surrogate Calibrator***	Ac-A[CNFNDVTTRLRENELTYYC]AAK-NH$_2$ (dPEG$_{24}$-Succ) Ac-NKAWK (Glucitol) FEHANFNDC-OH	5944	PCS-32532-PI:5 x 1mg, PCS-30321-PI1 x 5mg
6	**[Ser^7(Glc)]CSF114**	H-TPRVERS^7(β-D-Glc)GHSVFLAPYGWMVK-OH	2579	-
7	**[Asn^7(Gal)]CSF114**	H-TPRVERN^7(β-D-Gal)GHSVFLAPYGWMVK-OH	2606	-
8	**[(Nε-1-deoxyFru)]CSF114**	H-TPRVERK^7(Nε-1-deoxyFru)GHSVFLAPYGWMVK-OH	2621	10

Glycated CD59 peptides(**1-5**) were provided by Prof. Michael Chorev (Mellitus, LLC)

Procedure description:

- Resin swelling in 1 mL of ammonium bicarbonate buffer (**B**) for 1 h, filtration.
- Dissolving a peptide in **B**. Solutions for the capturing will be in saturating concentrations, relatively equal to the amount of dry resin used and calculated taking into account the resin loading value.
- Capturing reaction, shaking continuously (1-1.5 h).
- Filtration.
- Washing the resin with **B** – filtrates should contain the uncoupled peptide.
- Cleavage reaction in 1 mL of acidic buffer **A** (1 h).
- Filtration.
- Washing the resin with buffer **A** – filtrates should contain the captured peptide.
- Lyophilizing both solutions.
- Dissolving the peptide residues in each tube in precise volume of H_2O.
- UV-Vis analysis of both samples – measurement of Abs values, summing them up and calculation of capture percentage. Use of LC-MS analysis in case of peptide mixtures (>1 peptide).



B (basic): 30 min in ammonium bicarbonate buffer (50 mM) at pH 8 in H_2O/MeCN 1:1 (*v/v*)

A (acidic): 0.1% HCOOH in H_2O/MeCN 1:1 (v/v) for 1h

Capturing of naturally occuring sequences of glycated and glycosylated peptides by the new functionalized resin

With the aim to select and optimize a resin to fish out deoxyfructosylated peptides, as early glycation biomarkers of diabetes, in terms of efficiency and selectivity, we selected 8 peptides (**Table 2**): 4 Amadori peptides (**1-4,** and **8**), 1 negative control (**5**) and 2 glycosylated peptides (**6-7**). Herein, we report, the capturing results of glycated peptides by (i) two different resins: the ChemMatrix® Rink resin (CMRR) and the ω-Aminohexyl–Agarose (ω-AHA), and by (ii) the same resins, CMRR and ω-AHA, modified by either mono- or di-PhB substituted Lys moieties (**Table 1**).

mono- or bifunctionality on the capturing ?

Schematic Capturing experiment

Experiment 1. In our previous investigations we tested the *para*-analogues of the CMRR resin (data not shown). Now, a set of 8 peptides (**1-8**) was tested with each *meta*-resin (**Table 1:** Resins **2, 4, 7, 9**) :

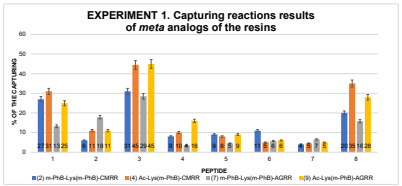
(2) *m*-PhB-Lys(Nε-*m*-PhB)-CMRR (4) Ac-Lys(Nε-*m*-PhB)-CMRR (7) *m*-PhB-Lys(Nε-*m*-PhB)-AGRR (9) Ac-Lys(Nε-*m*-PhB)-AGRR

Meta or para PhB orientation on the capturing ?

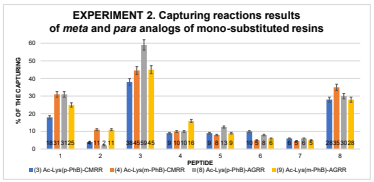
Experiment 2. After completing the **Experiment 1**, a set of 8 peptides (**1-8**), each one separately, was tested with *para*-analogue resin (**Table 1:** Resins **1, 3, 6, 8**) and the most efficient *meta*-analogue resin selected previously.

(3) Ac-Lys(Nε-*p*-PhB)-CMRR (8) Ac-Lys(Nε-*p*-PhB)-AGRR

para analogue of the most efficiently capturing CMRR (**3**) resin
para analogue of the most efficiently capturing AGRR (**8**) resin



("the percentage of capturing was calculated using the sum of peak areas in „captured" and „uncaptured" samples as 100%)



("the percentage of capturing was calculated using the sum of peak areas in „captured" and „uncaptured" samples as 100%)

Conclusions from Experiments 1 and 2.

1. The results suggest **better binding of the Amadori-containing peptides to the mono-substituted CMRR and AGRR resins**
2. Mono-*m*-PhB substituted resins **4-CMRR** and **9-AGRR** demonstrated to be better in terms of efficiency

Comparison of CMRR Resin and AGRR Resin capturing ability once containing only one PhB moiety

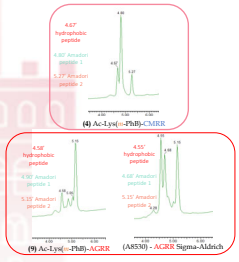
Experiments 3 (A-C) were designed in order to determine the **selectivity of AGRR and CMRR** resins toward the Amadori product when presented in a peptide mixture. Three solid supports were examined: Ac-Lys(*m*-PhB)-CMRR (**4**), Ac-Lys(*m*-PhB)-AGRR (**9**) and the commercially available AGRR (A8530-Sigma Aldrich). Ad hoc aqueous solutions containing the synthetic peptides were prepared as followed: solution of [(Nε-1-deoxyFru)]CSF114 and [(N(Glc)]CSF114 at the same concentration (mg/ml) in the **exp 3A**; the same solution but at 5% of [(Nε-1-deoxyFru)]CSF114 in the **exp 3B**; equimolar solution (mM) of eight synthetic peptides with two Amadori peptides in the **exp 3C**. These peptides were selected for the following characteristics: amino acid sequence, hydrophobicity, different length and bearing post-translational modification (DeoxyFru, Ac, PO_3H_2, and Pam). The mixtures were dissolved in an ammonium bicarbonate buffer solution at pH 8 and then subjected to the capturing reaction conditions described above

Experiments 3A and 3B (n=2)		
	RESINS	**PEPTIDES**
A	(**4**) Ac-Lys(*m*-PhB)-CMRR (**9**) Ac-Lys(*m*-PhB)-AGRR *m*-Aminophenylboronic acid–Agarose (A8530)	**Nε-1-deoxyFru *vs* Glc (1:1)** [Lys^7(Nε-1-deoxyFru)]CSF114 *vs* Asn^7(Glc)CSF114
B	(**4**) Ac-Lys(*m*-PhB)-CMRR (**9**) Ac-Lys(*m*-PhB)-AGRR *m*-Aminophenylboronic acid–Agarose (A8530)	**Nε-1-deoxyFru *vs* Glc (0.1 :1)** [Lys^7(Nε-1-deoxyFru)]CSF114 *vs* Asn^7(Glc)CSF114

RESIN	Amount	Captured peptide	CONCLUSION
(**4**) Ac-Lys(*m*-PhB)-CMRR **Captured**	1:1	**Nε-1-deoxyFru**	**Resin (4) is able to capture selectively the glycated peptide, even at low concentrations**
	1:0.1	**Nε-1-deoxyFru**	
(**9**) Ac-Lys(*m*-PhB)-AGRR **Captured**	1:1	**Nε-1-deoxyFru**	Resin (**9**) is able to capture selectively the glycated peptide only when the peptide is present in high excess
	1:0.1	**Nε-1-deoxyFru** & Glc	
m-Aminophenylboronic acid–Agarose (A8530) **Captured**	1:1	**Nε-1-deoxyFru**	Commercial agarose resin (A8530) is able to capture selectively the glycated peptide only when the peptide is present in high excess
	1:0.1	**Nε-1-deoxyFru** & Glc	

Experiment 3C (n=2)	
RESINS	**PEPTIDES**
(**4**) Ac-Lys(*m*-PhB)-CMRR (**9**) Ac-Lys(*m*-PhB)-AGRR *m*-Aminophenylboronic acid–Agarose (A8530)	equimolar amounts (1:1) **in a peptide mixture**

RESIN	CONCLUSION
(**4**) Ac-Lys(*m*-PhB)-CMRR	**Only these three peptides are captured by Resin (4):** hydrofobic peptide, Amadori peptide 1, and amadori peptide 2
(**9**) Ac-Lys(*m*-PhB)-AGRR	Resin (**9**) captures hydrophobic peptide, Amadori peptide 1, and Amadori peptide 2, but also other non-Amadori peptides **Resin (9) is not selective when other, unmodified sugars are present in the MIX**
m-Aminophenylboronic acid–Agarose (A8530)	Agarose resin (A8530) resin captures hydrophobic peptide, Amadori peptide 1, and Amadori peptide 2, but also other, non-Amadori peptides. **Commercial AGRR resin is not selective when other, glycosylated peptides are present in the MIXTURE.**



Conclusions

•Monofunctionalized (mono-PhB) resins more than bifunctionalized (di-PhB) resins are capable to capture the tested peptides;
•Among CMRR resins, *meta* analogue (*m*-PhB) is more efficient and more selective than *para* (*p*-PhB) analogue;
•Among all the monosubstituted resins, **(4)** Ac-Lys(*m*-PhB)-CMRR seems to present the consensus between the efficacy AND the selectivity.
•AGRR – solid support containing –OH groups. CMRR– a polymer non containing –OH groups. In case of CMRR, the presence of the –OHs may affect specificity of capturing that depends only on the interaction with the PhB groups. AGRR beads may interact non-specifically → lower specificity.

Perspectives

•Optimization work will look at the further modifications probing the spatial relationship between the two *m*-PhB substituting the Lys.
•Ac-Lys(*m*-PhB)-CMRR resin **(4)** will be tested in fishing the Amadori-modified peptide fragments from an enzymatic digest of a glycated protein

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