

IDENTIFICATION OF PHOSPHONYLATED PEPTIDES USING A MALDI TARGET FUNCTIONALIZED WITH LANTHANUM STEARATE

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As markers of intoxication, adducts of blood proteins with organophosphorus compounds (OPs) allow establishing the fact of poisoning and, furthermore, enable identification of the OPs by the attached residue. This study aimed to develop a method of specific and selective extraction of blood protein adducts carrying OPs on the surface of a matrix-assisted laser desorption/ionization (MALDI) target functionalized with multimolecular structures based on lanthanum stearate using metal affinity chromatography. We have shown the ability of adsorbent to retain both full-size and dealkylated adducts of blood proteins with OPs. The developed method allowed extraction and identification of peptides of human serum albumin and human butyrylcholinesterase modified with various OPs (after incubation of human blood plasma with OPs in concentrations from 1 to 100 ng/mL), which makes this approach applicable for the purposes of OPs identification in the context of investigation of real cases of intoxication.

Keywords: Lanthanum stearate, Langmuir–Blodgett films, serum albumin adducts, butyrylcholinesterase adducts, organophosphorus compounds

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ИДЕНТИФИКАЦИЯ ФОСФОНИЛИРОВАННЫХ ПЕПТИДОВ С ПРИМЕНЕНИЕМ МАЛДИ-МИШЕНИ, ФУНКЦИОНАЛИЗИРОВАННОЙ СТЕАРАТОМ ЛАНТАНА

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Аддукты белков крови с фосфорорганическими соединениями (ФОС) как маркеры интоксикации позволяют не только установить факт отравления, но и идентифицировать ФОС по присоединившемуся остатку. Целью работы было разработать методику специфичной и селективной экстракции аддуктов белков крови с ФОС на поверхности матрично-активированной лазерной десорбцией/ионизацией (МАЛДИ) мишени, функционализированной мультимолекулярными структурами на основе стеарата лантана, с помощью металл-аффинной хроматографии. Показано, что сорбент способен удерживать как полноразмерные, так и деалкилированные аддукты белков крови с ФОС. С помощью предложенной методики были экстрагированы и идентифицированы пептиды сывороточного альбумина и бутирилхолинэстеразы человека, модифицированные различными ФОС, после инкубации плазмы крови человека с ФОС в диапазоне концентраций 1 × 100 нг/мл, что позволяет использовать этот подход для идентификации ФОС при расследовании реальных случаев интоксикаций.

Ключевые слова: стеарат лантана, пленки Ленгмюра–Блоджетт, фосфорорганические соединения (ФОС), аддукты сывороточного альбумина, аддукты бутирилхолинэстеразы

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Organophosphorus compounds (OPs) form a large class of compounds used in industrial fabrication and agriculture that can inhibit serine esterases and proteases [1]. Highly toxic varieties of OPs are included in Schedule 1 of the Chemical Weapons Convention [2].

OPs adducts to human serum albumin (SA) and human butyrylcholinesterase (BChE) are the key long-lived biomarkers of OPs intoxication. Their half-life is 20–25 days and 11–14 days (SA and BChE, respectively) [3, 4], makes them usable as means of determining the agent in blood samples several weeks after poisoning. The OPs adducts to BChE are the most reliable biomarkers enabling establishing of poisoning with the degree of

enzyme inhibition at less than 1% [5]. There have been developed many approaches to determination of these adducts in complex samples that are based on the targeted analysis performed by means of high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [6–8]. However, MALDI mass spectrometry (MS), although not used as frequently, has certain advantages for screening of blood protein adducts to OPs when the aim is to identify unknown BChE inhibitors. Determination of the mass difference (ΔM) between modified and native peptides allows compiling the gross formula of OPs.

MALDI-MS has grown popular due to its simplicity, reliability, high sensitivity and capability to analyze a wide range of masses

[9–11]. However, its applicability is limited significantly because of the signal suppression effect that emerges when there is a large number of components; it is major hindrance to successful detection of trace amounts of substances. Therefore, this method often requires preliminary sample preparation aimed at either reduction of the number of components in the mixture or analyte enrichment.

Recently, there have been suggested approaches that make the MALDI target not only a sample carrier but also a factor in the analysis protocol, giving it an additional functionality [12]. For this purpose, the surface of the steel substrate is functionalized and thus given the necessary properties [13, 14], which allows conducting various analytical procedures on the target surface before the MS analysis ("lab-on-a-plate"), with the end result being a more effective analysis overall. The "lab-on-a-plate" format reduces the amount of sample needed for the analysis, cuts losses on the adsorbent during extraction, unlocks the possibility of working with very small volume samples and minimizes sample preparation to a few simple steps. A target functionalized with an adsorbent can be used for direct *in situ* enrichment of a sample followed by MALDI-MS.

Collapsed Langmuir monolayers are thin-film adsorbents with unique surface build of metal atoms [15]. They are both hydrophilic and hydrophobic [16] and show sufficient resistance to external influences [17]. Earlier, it was suggested that collapsed monolayers of lanthanum stearate (FLa) may be used in spin columns for extraction of a number of organic and bioorganic compounds [18], including human SA adducts with 2-(fluoromethylphosphoryl)oxy-3,3-dimethylbutane. We have proposed an approach that enables formation of both a collapsed FLA monolayer and its multimolecular structures directly on the MALDI target [19, 20]. The resulting material has a well-developed surface with good adhesion properties. It adheres well to a polished MALDI target and retains its qualities as a metal-affinity adsorbent. The purpose of this study was to investigate the possibility of specific extraction of blood protein adducts with OPs on the surface of a MALDI target functionalized with FLA multimolecular structures in the "lab-on-a-plate" format.

METHODS

Preparation of lanthanum stearate monolayers on a MALDI target

A polished MALDI target (MTP 384 target plate polished steel BC; Bruker Daltonics, Germany) was used as a substrate. We added 0.6 μ l (a drop) of lanthanum (III) salt aqueous solution with a concentration of 1 mM onto the surface of the MALDI target. On top of the drop we added 0.6 μ l of the saturated solution of stearic acid in n-hexane. The procedure was repeated twice, with three monolayers being the result thereof. After that, we removed the remains of the lanthanum salt solution drop. A drop of 0.6 μ l of the lanthanum salt aqueous solution was then again applied to the same target well, followed by three applications of the stearic acid solution. As a result, the MALDI target spot was modified with six FLA monolayers. Seeking to completely remove the lanthanum salt, we washed the applied adsorbent off three times with deionized water by adding 8 μ l of water to the spot, leaving it there for 1 minute and then removing with a dispenser.

Preparation of samples of proteins modified with OPs

To prepare the modified serum albumin samples, within two hours after collection we supplemented samples of donated K2

EDTA human blood plasma with a series of isopropyl alcohol dilutions of 2-(fluoromethylphosphoryl)oxy-3,3-dimethylbutane, CAS № 96-64-0 (hereinafter PFMP) to final concentrations of 1 ng/mL — 1 μ g/mL, as suggested in a previously published article [5]. The first step of the process of producing samples of modified BChE was to isolate the enzyme from the donated human blood plasma by affinity chromatography on procainamide sepharose. Then we added 1 mg/ml of PFMP to the purified enzyme to the final concentration of 100 μ g/ml, which triggered inhibition of over 90% of the enzyme. The incubated and control blood plasma was hydrolyzed with the pepsin, and the incubated and control BChE enzyme was hydrolyzed with trypsin. To assess the specificity and selectivity of adsorption of modified peptides on FLA, we mixed the hydrolyzed incubated and control blood plasma and BChE enzyme in various proportions. With the aim to establish the limits of detection of modified BChE in blood plasma, the enzyme was isolated from 250 μ L of plasma using immunoprecipitation [21]. All the experiments were performed in four replicates.

MALDI mass spectrometry

The mass spectra were acquired by means of an UltrafleXtreme tandem time-of-flight mass spectrometer (Bruker Daltonics; Germany). The mass spectra were registered in the positive reflectron mode in the m/z range of 1000–3200. The number of shots accumulated per one spectrum was 20000, the shot frequency — 2000 Hz. We used the Flex Control 3.4 and Flex Analysis 3.4 software to register and interpret the spectral data. The tandem mass spectra were obtained in the LIFT mode, with precursor ions fragmented under the collision-induced dissociation (CID) conditions.

Sample preparation and MALDI-MS of samples of the Seventh Official OPCW Biomedical Proficiency Test

Samples from the OPCW (pooled human blood plasma) were received in the context of participation of the Research Institute of Hygiene, Occupational Pathology and Human Ecology in the Seventh Official OPCW Biomedical Proficiency Test. VX agent (O-ethyl-S-2-diisopropylaminoethyl methylphosphonothiolate, CAS № 50782-69-9) were added to the samples in the OPCW laboratory to a final concentration of 10 ng/ml, and GE agent (O-isopropyl ethyl phosphonofluoride, CAS № 1189-87-3) — to a final concentration of 20 ng/ml.

BChE was isolated from the samples by immunoprecipitation, with the help of magnetic microspheres of the Dynabeads Antibody Coupling Kit (143.11D; Invitrogen, USA) that were covalently coupled with BChE antibodies (Pierce BChE monoclonal mouse antibody HAH 0020102; Thermo, USA). The microspheres were prepared according to the Dynabeads Antibody Coupling Kit manufacturer's protocol. An earlier described technique enabled isolation of BChE from plasma [21].

Trypsin (Sigma-Aldrich; USA) was used for enzymatic hydrolysis of BChE. We washed 100 μ l of BChE-containing eluate with water three times in the Amicon Ultra-0.5, 30K centrifuge filters (UFC5030BK; Merck Millipore, USA). Next, the volume of the sample was adjusted to 100 μ l with 25 mM of aqueous ammonium bicarbonate solution, then supplemented with 5 μ l of trypsin (0.1 mg/ml) and incubated at 37 °C for 4 hours, then we added another 5 μ l of trypsin solution (0.1 mg/ml) and incubated the sample at 37°C for 16 hours.

We performed metal affinity chromatography (IMAC) on the FLA-containing adsorbent directly on the MALDI target with deposited monolayers.

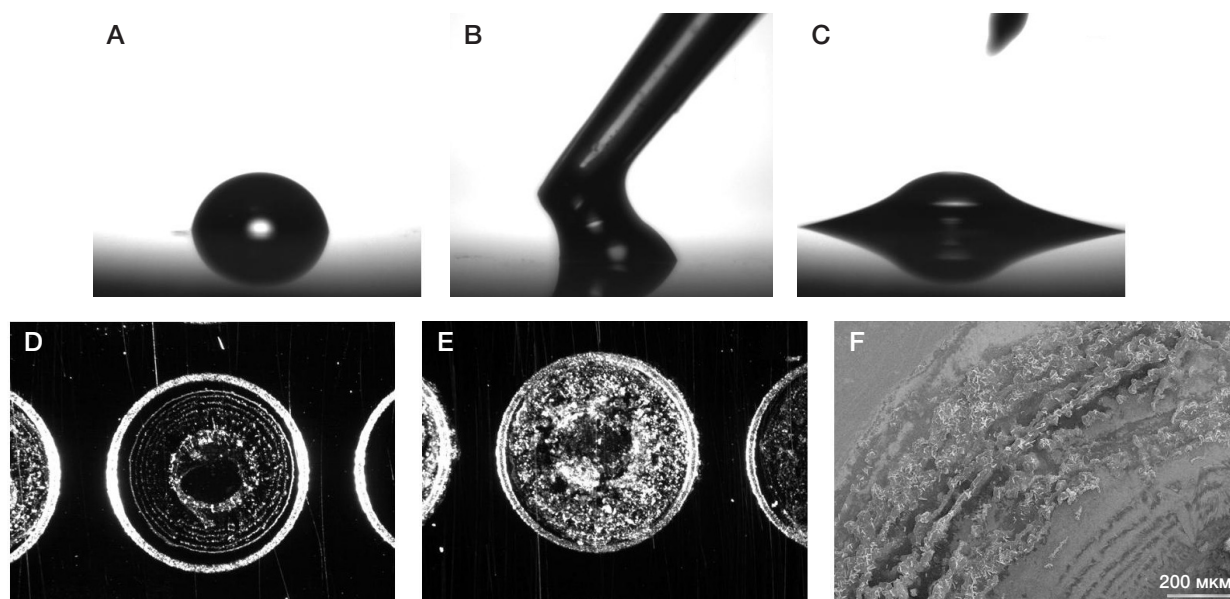


Fig. 1. **A.** Drop of an aqueous subphase. **B.** Application of the n-hexane stearic acid solution. **C.** Distribution of the organic phase over the substrate. **D, E.** Image of MALDI target spots after deposition of one and six FLA layers (SMZ 1500 stereomicroscope with a DS-2MBWc digital camera (Nikon; Japan)). **F.** SEM image of six FLA layers formed on the MALDI target (scanning electron microscope S-3400N; Hitachi, Japan)

Samples of BChE tryptic hydrolyzate modified with OPs were adsorbed in 2.5% aqueous ammonia solution. The duration of this step was 20 minutes. To remove the unbound fraction, we washed the adsorbent twice with 5 μ l of 2.5% aqueous ammonia solution and 8 μ l of water. To improve desorption of phosphonylated peptides, we added 3 μ l of 30% acetonitrile with 0.1% trifluoroacetic acid (TFA), and after that 2 μ l of CHCA matrix (5 mg/ml in 70% aqueous acetonitrile) was added to the target. After IMAC, the FLA monolayers retained adsorbed phosphonylated peptides of blood proteins.

To perform the MALDI-MS analysis of samples of the Seventh Official OPCW Biomedical Proficiency Test, we used Solarix XR 7T (Bruker Daltonics; Germany), a Fourier transform ion-cyclotron resonance mass spectrometer equipped with SmartBeam-II laser (355 nm). The mass spectra were acquired in the positive ion mode in the m/z range of 1000–3200. We used the Data Analysis 5.0 software (Bruker Daltonics; Germany) to process the mass spectral data.

RESULTS

Functionalization of the MALDI target surface

For this work, we selected a polished MALDI target with hydrophobic surface that can carry a drop of water without altering its geometry (Fig. 1A); it was functionalized using the technique published earlier [20]. The volume of solutions allowed keeping n-hexane inside the spot when the solution of stearic acid in n-hexane was applied to the aqueous drop (Fig. 1B), which means the layers formed strictly inside it, too (Fig. 1C–F). We discovered that after successive formation of six monolayers the material no longer stays in the well, and seven or more monolayers adversely affect adhesion of lanthanum stearate films to the MALDI target. The MALDI-MS analysis of the films formed on the target showed that the main structural unit of the adsorbent is the lanthanum distearate ion, as evidenced by the signal with m/z 705.46. The physicochemical parameters of lanthanum stearate films were described earlier [20]. The MALDI target surface was functionalized through formation of adsorbent on the spots based on the multimolecular FLA structures consisting of six collapsed monolayers.

Evaluation of adsorption properties of FLA

Developing a "lab-on-a-plate" approach to metal-affinity extraction, we have shown that drops of widely used IMAC solutions reliably stay on a spot of adsorbent consisting of six collapsed FLA monolayers in the following volumes: 2.5% aqueous ammonia — 7–8 μ l; water — 10–12 μ l; 30% aqueous acetonitrile — 3–5 μ l. A peptic blood plasma hydrolyzate, which has the serum albumin (SA) PFMP-modified by 90% by tyrosine-411, was chosen as a model object. Afterwards, all experiments that included IMAC followed the pattern described below. To equilibrate the phase, we added a drop of 2.5% aqueous ammonia to the adsorbent, kept it there for 5 minutes, then discarded and applied again. Next, we added 1 μ l of the sample to the drop and allowed the adsorption process to progress for 20 minutes. The unbound fraction was transferred to an adjacent spot for further MALDI-MS. FLA was washed successively with 2.5% aqueous ammonia, water and 0.1% aqueous TFA. Then we dried the adsorbent and applied 3 μ l of 30% aqueous acetonitrile and 2 μ l of 5 mg/ml CHCA matrix solution for desorption, which was followed by MALDI mass spectrometry.

To investigate the adsorption capacity of FLA, we diluted the human blood plasma peptic hydrolyzate containing 90% modified serum albumin (initial concentration of SA — 1 mg/ml) with distilled water to the following concentrations: 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 mg/mL. IMAC-MALDI-MS showed that after IMAC the signal with m/z 1567.87, corresponding to the VRY(PFMP)TKKVPQVST adduct, was not detected in the mass spectrum of the unbound fraction after topping the adsorbent with 1 μ l of a 0.5 mg/ml protein solution. The highly intense m/z 1567.87 signal was registered in the mass spectra recorded for all adsorbent spots; fragment analysis confirmed that this signal belongs to the VRY(PFMP)TKKVPQVST adduct. Based on the degree of protein modification, which is 90%, and with hydrolysis complete (as evidenced by the absence of the LVRY(PFMP)TKKVPQVST signal, m/z 1680.89, that indicates incomplete hydrolysis, in all mass spectra), we can conclude that six layers of FLA are capable of retaining 0.012 μ g of the VRY(PFMP)TKKVPQVST adduct.

To assess the specificity and selectivity of the approach, peptic hydrolyzates of modified and unmodified SA were mixed

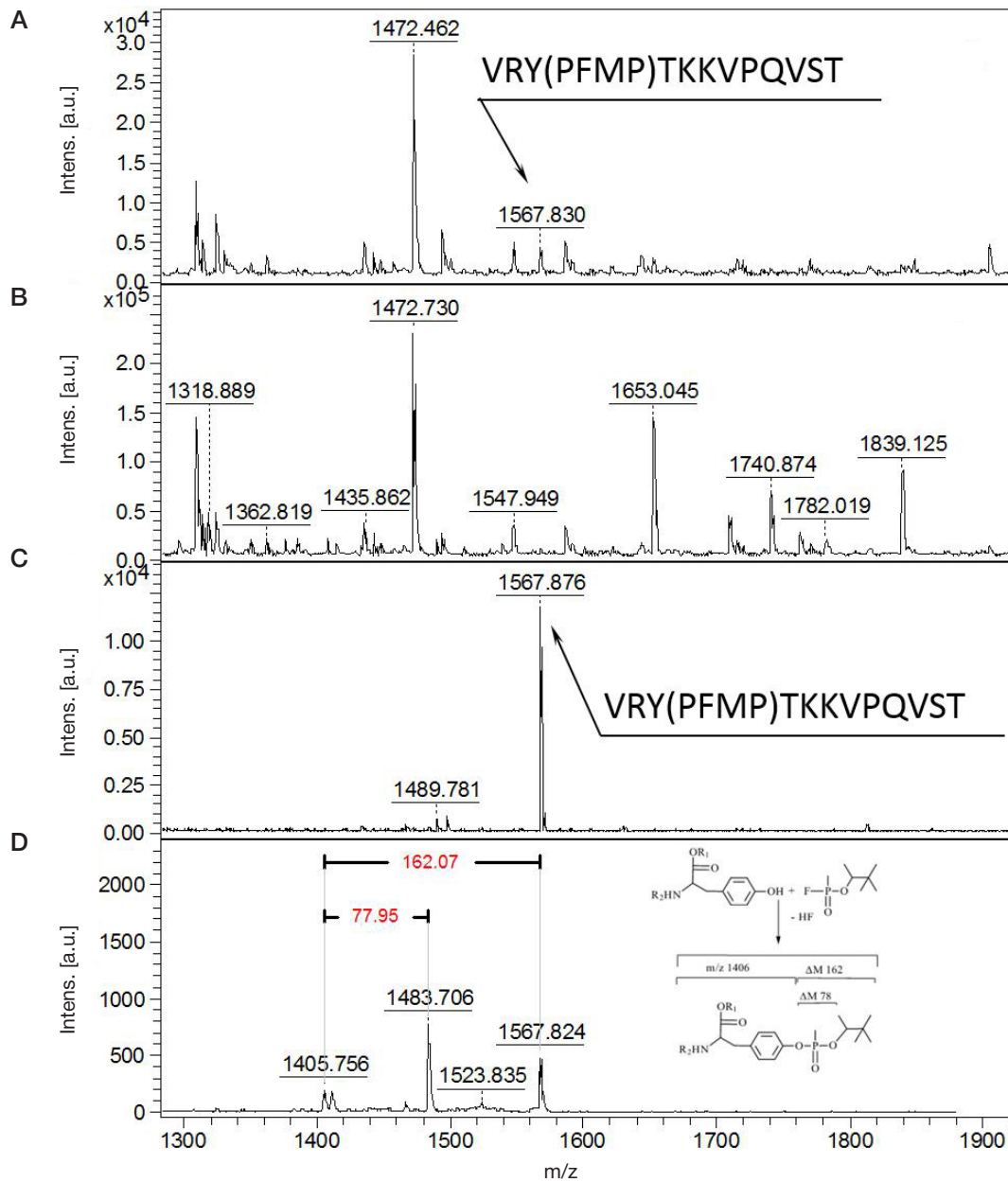


Fig. 2. Mass spectrum of human SA peptic hydrolyzate modified with PFMP at the modified-to-unmodified ratio of 1 : 10. **A.** Mass spectrum of the original sample. **B.** Mass spectrum of the unbound fraction. **C.** Mass spectrum of the adsorbent spot after IMAC. **D.** PFMP modification identification by neutral loss

in such a way that the ratios of VRY(PFMP)TKKVPQVST/VRYTKKVPQVST were 1 : 10, 1 : 100, 1 : 1000, and 1 : 10,000. We applied a sample with initial SA concentration of 1 mg/ml to the formed and prepared FLa spots in accordance with the data presented in Table 1. The results of the IMAC-MALDI-MS analysis (Table 1; Fig. 2) indicate the high efficiency of the suggested approach to metal-affinity extraction of SA adducts with PFMP in the "lab-on-a-plate"

format. We have not detected the adduct signal only in case of application of 1 µl of the mixture at the VRY(PFMP)TKKVPQVST/VRYTKKVPQVST ratio of 1 : 10,000. However, application of even 5 µl of this solution yields detection of the m/z 1567.87 signal with the signal-to-noise ratio (S/N) of 2–3 (various replicates). In case of 1 µl of a 1 : 1000 mixture, the S/N ratio was 5–6. At higher concentrations, the adduct is reproducibly and reliably detected.

Table 1. Results of investigation of selectivity of the developed technique with dilution of PFMP-modified SA with human blood plasma

Molar ratio VRY(PFMP)TKKVPQVST/ VRYTKKVPQVST	Volume of sample applied to the adsorbent, µl	Signal-to-noise ratio
1 : 10	1	51
1 : 100	1	35
1 : 1000	5	13
	1	5
1 : 10,000	5	3
	1	–

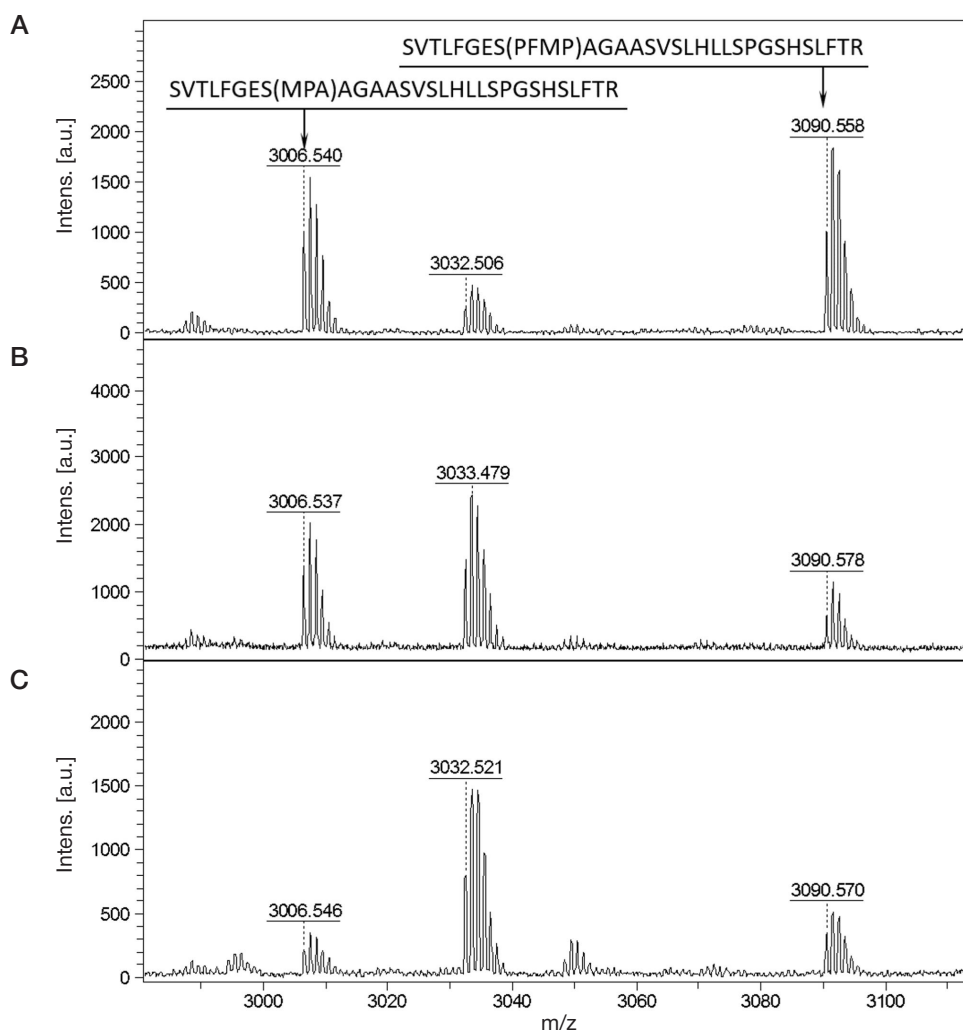


Fig. 3. Results of IMAC(FLa)-MALDI-MS analysis of the tryptic hydrolyzate of BChE (1 mg/ml) modified with PFMP, modified-to-unmodified ratios of 100 : 0 (A); 1 : 10 (B); 1 : 100 (C)

At the next stage of the work, we studied the possibility of extraction of BChE adducts with PFMP by IMAC on FLA, followed by MALDI-MS. The BChE tryptic peptide modified with OPs is a more convenient analyte for MALDI-MS than BChE active site peptic nonapeptide, which is widely used for HPLC-MS/MS analysis. The tryptic peptide containing active site serine-198 consists of 29 amino acid residues: SVTLFGES*AGAASVSLHLLSPGSHSLFTR (m/z 2928.521). However, unlike SA adducts with OPs, BChE adducts are subject to dealkylation (aging) [22, 23], and MS most often detects BChE adduct with a methylphosphonic acid (MPA) residue. We experimented with BChE isolated from human blood plasma that was incubated with PFMP until complete inhibition of the enzyme. One μ l of 1 mg/ml tryptic hydrolyzate of the modified BChE protein was applied to FLA, the application followed by IMAC-MALDI-MS, which allows drawing a conclusion (Fig. 3) that FLA formed on the target exhibit specificity towards BChE adducts with both PFMP and MPA. The mass spectrum contains signals at m/z 3006.51, which corresponds to SVTLFGES(MPA)AGAASVSLHLLSPGSHSLFTR adduct, and at m/z 3090.61 which represents the SVTLFGES(PFMP)AGAASVSLHLLSPGSHSLFTR adduct. When the hydrolyzate of modified BChE is diluted with hydrolyzate of unmodified BChE, specificity and selectivity of IMAC-MALDI-MS is noticeably lower compared to the analysis of adducts of human serum albumin with PFMP. However, at the 1 : 100

ratio of the modified form to the unmodified form, signals of BChE adducts with PFMP and MPA are reliably detected at $S/N \geq 6$.

Detection of adducts in human blood plasma samples incubated with PFMP

We used human blood plasma incubated with PFMP at concentrations of 1 and 10 ng/ml as samples. The degree of BChE inhibition was < 5 and 40%, respectively. The adduct of SA with PFMP was searched for in the peptic hydrolyzate of the total blood plasma protein; after protein precipitation and reconstitution in distilled water, the concentration of SA was 1 mg/ml. In case of BChE, it was important to isolate its purest form possible, therefore, we resorted to a highly specific method of immunoprecipitation and used 250 μ l of blood plasma for the purpose. For IMAC-MALDI-MS, a solution with a BChE concentration of 1 mg/ml was used; Figure 4 and Table 2 show the results thereof. Applying the developed approach to analyze the peptic hydrolyzate of the total blood plasma protein, we encountered the VRY(PFMP)TKKVPQVST adduct signal in the mass spectra of both samples. However, with only 1 μ l of the first sample, which was incubated with PFMP at a concentration of 10 ng/ml, applied to the adsorbent, we received S/N of 5 for the signal of VRY(PFMP)TKKVPQVST, whereas the second sample, with the final concentration of PFMP at 1 ng/ml, required enrichment, and we had to apply

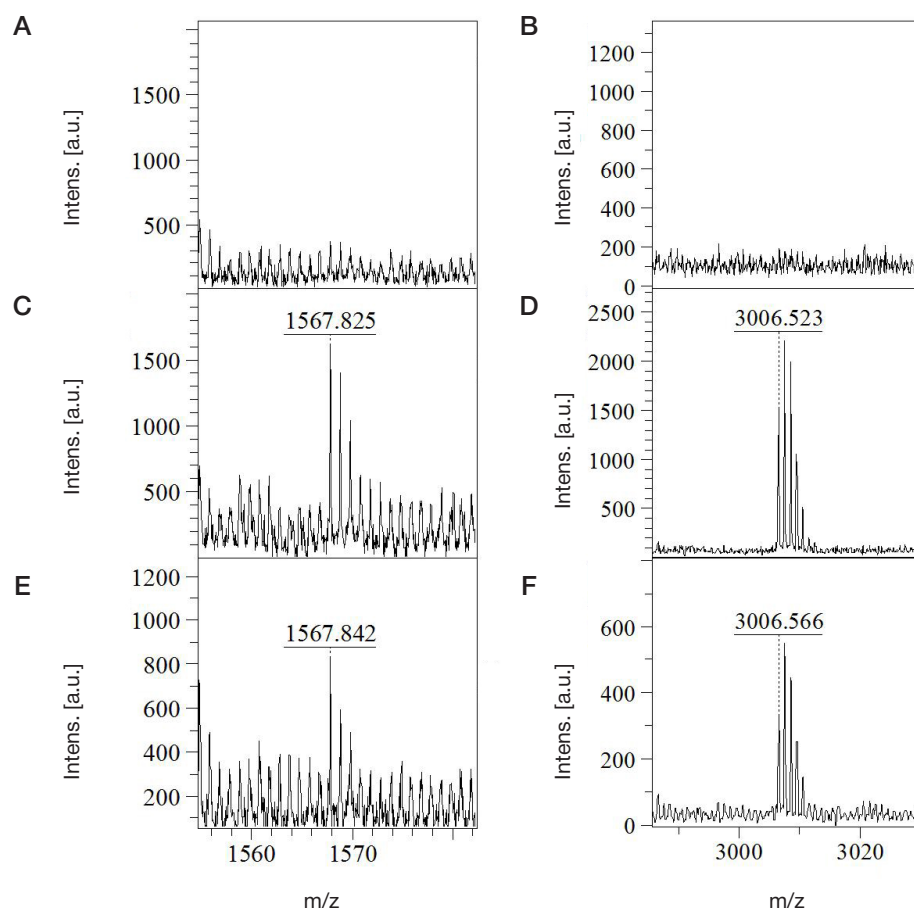


Fig. 4. Results of IMAC(FLa)-MALDI-MS analysis of blood plasma samples incubated with PFMP. The figure shows mass spectra of control samples (**A, B**); adducts VRY(PFMP)TKKVPQVST and SVTLFGES(MPA)AGAASVSLHLLSPGSHSLFTR, PFMP concentration 10 ng/ml (**C, D**); VRY(PFMP)TKKVPQVST and SVTLFGES(MPA)AGAASVSLHLLSPGSHSLFTR adducts, PFMP concentration 1 ng/ml (**E, F**)

5 μ l thereof to the adsorbent, after which we registered the signal with the S/N ratio of 4. Moreover, in both blood plasma samples we identified SVTLFGES(MPA)AGAASVSLHLLSPGSHSLFTR, the BChE adduct isolated through immunoprecipitation with dealkylated residue of PFMP. It exhibited good S/N value and required no additional method optimization.

Detection of adducts of OPs with BChE in human blood plasma samples

We applied the suggested approach to detect OPs adducts with BChE when doing the Seventh Official OPCW Biomedical

Proficiency Test. Preliminarily, BChE was isolated from 250 μ l of blood plasma supplemented with OPs. After immunoprecipitation, we eluted BChE from the adsorbent with 0.6% formic acid, transferred the eluate into a bicarbonate buffer and hydrolyzed it with trypsin. Subsequent IMAC-MALDI-MS revealed a native peptide and modified peptides at m/z 3034.5397 (VX) and m/z 3062.5650 (GE) in the m/z range from 2880 to 3100 (Fig. 5). Other OPs, like VR and CVX, have the molecular weight similar to that of the GE residue. In such a case, a feasible approach to the task of agent identification involves its reactivation from protein adducts and gas chromatography — mass spectrometry as means of detection.

Table 2. Sensitivity of IMAC-MALDI-MS for peptic SA peptides and BChE tryptic peptides modified with PFMP

No	Peptide (m/z)	Ratio PFMP / plasma, ng/ml	Volume of sample applied to the adsorbent, μ l	Signal-to-noise ratio
CA				
1	VRY(PFMP)TKKVPQVST (1567,89)	10 : 1	1	5
2	VRY(PFMP)TKKVPQVST (1567,89)	1 : 1	5	4
БХЭ				
3	SVTLFGES(MPA)AGAASVSLHLLSPGSHSLFTR (3006,54)	10 : 1	1	34
4	SVTLFGES(PFMP)AGAASVSLHLLSPGSHSLFTR (3090,56)	10 : 1	1	–
5	SVTLFGES(MPA)AGAASVSLHLLSPGSHSLFTR (3006,54)	1 : 1	1	14
6	SVTLFGES(PFMP)AGAASVSLHLLSPGSHSLFTR (3090,56)	1 : 1	1	–

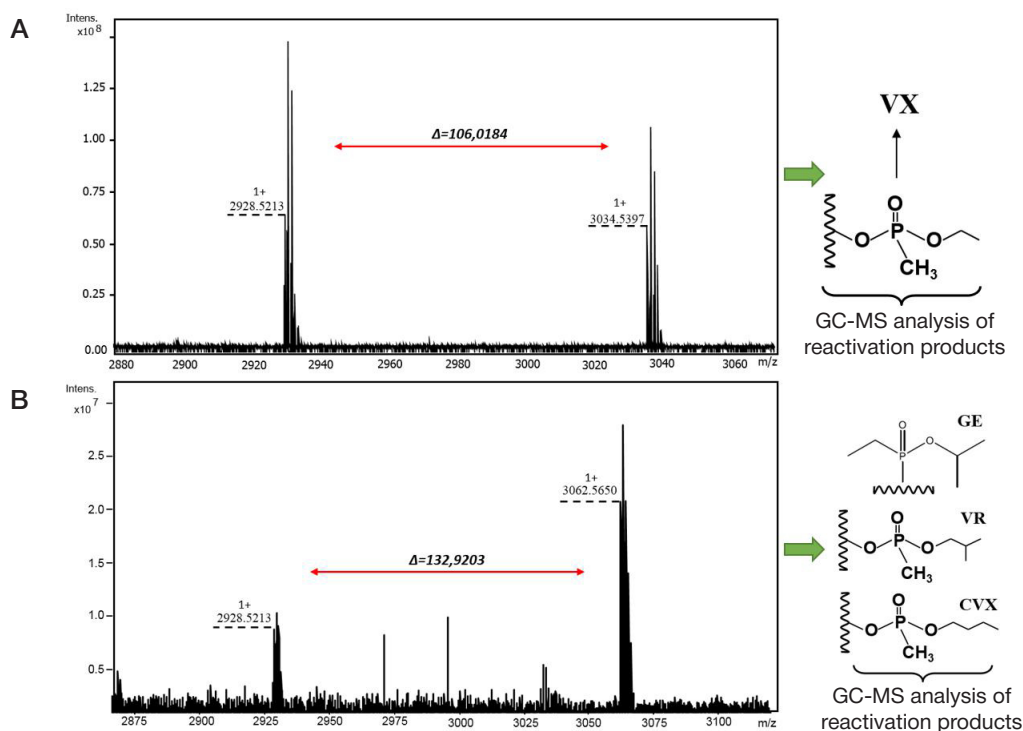


Fig. 5. Results of IMAC(FLa)-MALDI-MS analysis of samples from the Seventh Official OPCW Biomedical Proficiency Test. **A.** MALDI mass spectrum of tryptic hydrolyzate of BChE modified with VX, signal at m/z 3034.5397, SVTLFGES(Vx)AGAASVSLHLLSPGSHSLFTR. **B.** MALDI mass spectrum of tryptic hydrolyzate of BChE modified with GE, signal at m/z 3062.5650 - SVTLFGES(GE)AGAASVSLHLLSPGSHSLFTR

Fourier transform ion-cyclotron resonance mass spectrometry allowed determining the exact mass of the attached residue, which translated into a significantly narrower list of candidate OPs.

DISCUSSION

The suggested approach enables screening for OPs-modified SA and BChE peptides in an array of real blood plasma samples when poisoning by this class of compounds is suspected. In such cases, the concentration of these highly toxic OPs in blood plasma can reach 100 ng/ml. Identification of the mass of attached residue with the help of high resolution MS allows reasonable assumptions about the parent compound.

A number of studies [24–27] take SA adducts with OPs as markers of intoxication that allow not only establishing the fact of poisoning but also enable identification of the OPs by the attached residue. However, the majority of researchers consider the adduct of BChE, which attaches the OPs residue by serine-198, to be the main protein marker [28]. The reason behind this fact is the great difference in concentrations of BChE and SA in human blood plasma: 5 and 40000 $\mu\text{g}/\text{ml}$, respectively. Even when the BChE is inhibited completely, the degree of SA modification will be extremely low. At the same time, in contrast to BChE, SA adducts with OPs do not undergo dealkylation, which in most cases allows either identifying the OPs by ΔM between modified and native peptides or selecting an empirical formula, which greatly facilitates further identification by other methods. Accordingly, the stage of sample enrichment with SA adducts with OPs becomes a compulsory one. Serum albumin is the main component of blood plasma, therefore, enzymatic hydrolyzates of blood plasma can be used to identify SA adducts without protein isolation. Indeed, IMAC on the surface of a MALDI target functionalized with FLA allows specific and selective extraction of adducts of SA with PFMP even from a peptic hydrolyzate of the total blood plasma protein, with SA concentration at 40% and the remaining protein components enhancing suppression of the target signals. It should be noted

that for incubation of plasma with OPs we selected PFMP concentrations that do not imply a lethal outcome if OPs in such a dose entered human body. With PFMP concentration of 1 ng/ml, BChE inhibition was below 5%, which often does not even cause clinical manifestations. The suggested "lab-on-a-plate" format revealed presence of a dealkylated adduct, which signals of BChE inhibition, and, furthermore, provided information enabling identification of PFMP by $\Delta M = 162$. Compared to extraction in spin columns, the approach translates into significant reduction of the reagent costs and time expenditures while noticeably increasing sensitivity of the analysis, which is essential when working with extremely limited amounts of biosamples.

Sequential isolation of the BChE enzyme from blood plasma and subsequent IMAC on FLA of the enzyme's tryptic hydrolyzate allow detecting potential covalent adducts of OPs with BChE active site serine by MALDI-MS in the m/z range of 3000–3100. Information about the mass of the attached inhibitor significantly shortens the list of potential substances that caused the poisoning. The probable structural isomers that have similar molecular weight can be identified through targeted chemical analysis by gas chromatography in combination with MS after reactivation from protein adducts, or by analysis of tyrosine adducts of blood plasma proteins that do not dealkylate spontaneously.

The Research Institute of Hygiene, Occupational Pathology and Human Ecology is an accredited laboratory of the OPCW; the developed approach allows rapid screening of potential organophosphorus toxicants in blood plasma when investigating poisoning with this class of compounds.

CONCLUSIONS

We developed a technique of specific and selective extraction of blood protein adducts with OPs on the surface of a MALDI target functionalized with multimolecular structures based on lanthanum stearate using MAC in the "lab-on-a-plate" format. The study has shown that the adsorbent is capable of retaining

both full-length and dealkylated adducts of blood proteins with 2-(fluoromethylphosphoryl)oxy-3,3-dimethylbutane (PFMP). The technique allowed extraction and identification of peptides of SA and BChE modified with PFMP and MPA (after incubation of human blood plasma with OPs in concentrations from 1 to

100 ng/mL), which makes this approach applicable for the purposes of OPs identification in the context of investigation of real cases of intoxication. We applied the suggested approach in the context of doing the Seventh Official OPCW Biomedical Proficiency Test.

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