Analytica Chimica Acta 1192 (2022) 339334

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Selection and characterization of DNA aptamers for highly selective recognition of the major allergen of olive pollen Ole e 1

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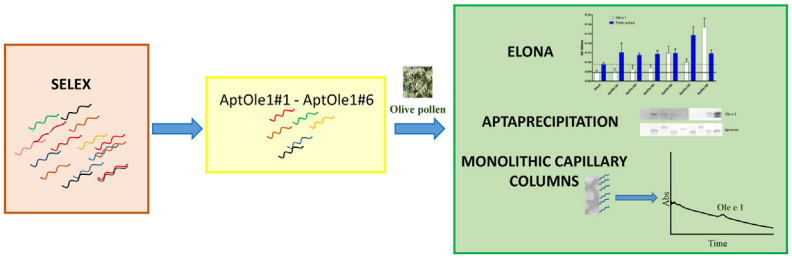
HIGHLIGHTS

- Aptamers able to specific recognize Ole e 1 were firstly selected by SELEX.
- Binding of aptamers to Ole e 1 was evaluated via ELONA and aptaprecipitation studies.
- Aptamer-based monoliths were prepared for Ole e 1 binding.
- The best affinity monolith was used to recognize Ole e 1 from allergy skin tests.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 July 2021
Received in revised form
23 November 2021
Accepted 25 November 2021
Available online 2 December 2021

Keywords:
Allergen
Olive pollen
Aptamer
Enzyme-linked oligonucleotide assay
Affinity-based monolith
Ole e 1

ABSTRACT

In this study, single-stranded DNA aptamers with binding affinity to Ole e 1, the major allergen of olive pollen, were selected using systematic evolution of ligands by exponential enrichment (SELEX) method. Binding of the aptamers was firstly established by enzyme-linked oligonucleotide assay (ELONA) and aptaprecipitation assays. Additionally, aptamer-modified monolithic capillary chromatography was used in order to evaluate the recognition of this allergenic protein against other non-target proteins. The results indicated that AptOle1#6 was the aptamer that provided the highest affinity for Ole e 1. The selected aptamer showed good selective recognition of this protein, being not able to retain other non-target proteins (HSA, cyt *c*, and other pollen protein such as Ole e 9). The feasibility of the affinity monolithic column was demonstrated by selective recognition of Ole e 1 in an allergy skin test. The stability and reproducibility of this monolithic column was suitable, with relative standard deviations (RSDs) in retention times and peak area values of 7.8 and 9.3%, respectively (column-to-column reproducibility). This is the first study that describes the design of an efficient DNA aptamer for this relevant allergen.

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1. Introduction

Olive (*Olea europaea* L.) pollen is one of the main health concerns in Mediterranean countries and other regions where this

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https://doi.org/10.1016/j.aca.2021.339334
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agronomic species is intensively cultivated [1]. The allergenicity of olive pollen is dependent on a number of allergenic proteins, 12 of which (Ole e 1 to Ole e 12) have been identified and characterized up to date [2,3]. Most of these olive pollen allergens are polymorphic and exhibit several IgE-reactive forms [3]. In particular, Ole e 1 consists of a single polypeptide chain of 145 amino acid residues with a molecular weight of approximately 20 kDa [2]). Indeed, it has been described as the major olive pollen allergen, representing up to 5–20% of the total olive pollen proteins [3,4]. Only this protein causes more than 80% of allergies related to olive pollen [2]. Consequently, the detection and quantification of this protein in allergen extracts for clinical purposes and/or in atmospheric air samples is of utmost interest to assure efficiency and safety in the diagnosis and specific immunotherapy procedures [5].

In this sense, enzyme-linked immunosorbent assay (ELISA) and immunoelectroforesis assays have been particularly useful for detection/quantification of Ole e 1 in pollen samples [6–9]. However, these methodologies rely on the use of antibodies as affinity probes, which are often challenged by high production costs, short shelf-life and requirement of live hosts for antibody production.

To overcome these drawbacks, a new generation of affinity ligands, called aptamers, have been described. Concretely, aptamers are short single-stranded oligonucleotides that can be chemically synthesized with specific target recognition and binding properties similar or higher than those of antibodies [10]. They can be synthesized through an iterative process known as systematic evolution of ligands by exponential enrichment (SELEX) [11,12], giving oligonucleotide sequences with high-affinity target. Besides, they exhibit other benefits such as low production costs, easy modification, thermal and chemical stability and prolonged shelf-life/reusability. Indeed, these target-specific ligands have been widely applied in analytical, bioanalytical, imaging, diagnostic and therapeutic fields [13–21].

Regarding the analytical applications, several aptamer-based methodologies have been developed, including enzyme-linked oligonucleotide assays (ELONA, a variant of ELISA with aptamers instead of antibodies) [22], biosensors ("aptasensors") [23,24], the use of aptamers as solid-phase extraction (SPE) sorbents [15,16] and as ligands in affinity chromatography [25].

Within aptamer modified columns in affinity chromatography, monolithic supports have been considered as suitable supports due to their merits such as high permeability, fast mass transfer rate, easy preparation and large loading capacity. Thus, studies on aptamer-based affinity monoliths for selective recognition of target analytes have attracted much interest and have been recently reviewed [26]. To date, aptamers have been immobilized on organic polymer monolithic supports [27–29] or siloxane-based hybrid monoliths [30–33] to produce aptamer-modified monolithic capillary columns. However, most of these works have been commonly focused on the recognition of thrombin and ochratoxin binding as model targets. To the best of our knowledge, there is no report on applications of aptamer-based affinity monoliths to the recognition of allergenic proteins, such as Ole e 1.

In this work, the preparation and characterization of several aptamers against Ole e 1 is described. The recognition ability of aptamers was first investigated through ELONA and aptaprecipitation assays. Subsequently, an aptamer-based affinity chromatography method for this allergenic protein using the synthesized aptamers is developed. For this purpose, a glycidyl methacrylate (GMA)-based monolithic capillary column was synthesized and functionalized with streptavidin. Then, biotinylated aptamers of Ole e 1 were bound to streptavidin-modified monolithic columns. These columns were tested to separate Ole e 1 and other non-target proteins (e.g., human serum albumin and cytochrome *c*). The aptamer-modified monolith that gave the largest affinity to Ole e 1 was used for selective capture of this protein in commercial pollen protein extracts for allergy diagnosis.

2. Experimental

2.1. Reagents and materials

3-(Trimethoxysilyl)propyl methacrylate, glycidyl methacrylate (GMA), trimethylolpropane trimethacrylate (TRIM) cyclohexanol, dodecanol, azobisisobutyronitrile (AIBN), sodium chloride, potassium chloride, magnesium chloride ($MgCl_2$), streptavidin, ammonium persulfate, acrylamide, bisacrylamide, bovine and human serum albumin (BSA and HSA, respectively), cytochrome *c* (cyt *c*, from horse heart), 2-mercaptoethanol, sodium dodecyl sulfate (SDS), tetramethylethylenediamine, and tris(hydroxymethyl)-aminomethane (Tris) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Aptamers targeting Ole e1 (denoted as AptOle1#1 to AptOle1#6, see Table 1), primers and deoxynucleotide triphosphates (dNTPs) were purchased by IBA GmbH (Göttingen, Germany). A biotin label was attached to the 5' end of each aptamer, and the biotinylated aptamers were used for ELONA, aptaprecipitation assays and affinity-based monolithic columns as described below. A molecular-weight-size protein standard (6.5–200 kDa) was also provided by Sigma-Aldrich. Disodium hydrogen phosphate, ammonium bicarbonate, HPLC-grade methanol (MeOH) and hydrochloric acid were purchased from Scharlau (Barcelona, Spain). Deionized water was obtained using a Crystal B30 EDI Adrona water purification system (Riga, Latvia). Fused-silica capillaries with UV transparent external coating (100 μm i.d. × 375 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Isolation, purification and characterization of Ole e 1 and Ole e 9

Olea europaea L. pollen samples from Picual were provided by Iberpolen (Alcala la Real, Jaén, Spain). Ole e 1 and e 9 were purified from olive pollen as previously described [34–37]. Briefly, the pollen (3 g) was extracted in 60 mL of 50 mM ammonium bicarbonate (pH 8.0) at room temperature for 1 h. The pellet was reextracted twice under the same conditions. After centrifugation at 10,000 *g* for 20 min at 4 °C, the resulting supernatant was chromatographed on a Sephadex G-75 column (2.0 cm × 67.0 cm; flow rate, 0.8 mL min^{-1}; Sigma), equilibrated with the same buffer. The different fractions were collected and subjected to SDS-PAGE analysis to select the fractions containing Ole e 1 and Ole e 9 as major components. The fractions corresponding to a molecular mass of about 20 and 19 kDa (Ole e 1) and 46 kDa (Ole e 9) were pooled and lyophilized. The SDS-PAGE analysis of the selected fractions (see Fig. S1) showed the characteristic pattern of Ole e 1 and Ole e 9 proteins previously reported [34–37]. The Ole e 1 and Ole e 9 concentration found in the above fractions was also determined by absorbance measurements as previously described [34–37].

2.3. Aptamer selection process (SELEX)

Purified Ole e 1 was biotinylated by using EZ-Link Micro-PEO4-Biotinylation kit (Thermo Fisher Scientific, Madrid, Spain) and biotin-labeled Ole e 1 quantified using the Pierce Biotin Quantitation kit (Thermo Fisher Scientific) following the manufacturer instructions. Next, 100 μg of biotinylated Ole e 1 protein diluted in 500 μL of phosphate buffer solution (PBS) were incubated in the presence of 300 μL of Streptavidin Magnetic Beads (SoluLINK, Vector Laboratories, Burlingame, USA), previously equilibrated in 1 mL of PBS during 16 h at 4 °C. Next, the biotinylated Ole e 1:resin

Table 1
List of DNA aptamers with their sequences and Gibbs free energy values for Ole e 1 recognition used in this study.

Aptamer	Sequence	*ΔG*
AptOle1#1	5′-GCGGATGAAGACTGGTGTCTCGGCACCCCGTCACTAATGTGGCTGTTCCCTGTTCAGAGCCCTAAATACGAGCAAC-3′	−8.78
AptOle1#2	5′-AGAGAGAGAGGCGGATGAAGACTGGTGTCTCGGCACCCCGTCACTAATGTGGCTGTTCCCTGTTCAGAGCCCTAAATACGAGCAAC-3′	−8.78
AptOle1#3	5′-GCGGATGAAGACTGGTGTCACATTCGAAAACCTCGTCGCACTGTCAGCTCCGTCTATTGCCCTAAATACGAGCAAC-3′	−8.53
AptOle1#4	5′-AGAGAGAGAGGCGGATGAAGACTGGTGTCACATTCGAAAACCTCGTCGCACTGTCAGCTCCGTCTATTGCCCTAAATACGAGCAAC-3′	−8.95
AptOle1#5	5′-GCGGATGAAGACTGGTGTTCGGTTCTGGTTGACTGGTCGCACAGTCGCTTCCCTTTTGGCCCTAAATACGAGCAAC-3′	−5.40
AptOle1#6	5′-GCGGATGAAGACTGGTGTAGAGGCATCGTTGCCTGGTTTTATTTCCTGCCCCATTGCTGCCCTAAATACGAGCAAC-3′	−8.34

complexes were washed 3 times with 1 mL of PBS and resuspended in 500 μL of selection buffer (20 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 150 mM NaCl, 5 mM KCl).

A SELEX process was used to selected DNA aptamers with binding affinity to Ole e 1. In brief, as the initial population we used an ssDNA library (named RND40), containing a central randomized region of 40 nucleotides flanked by two conserved 18-nucleotides regions in each end (5′-GCGGATGAAGACTGGTCT-40N-GTTGCTCGTATTTAGGGC-3′). For the initial SELEX round, 1 nmol of ssDNA was denatured at 90 °C for 10 min, cooled on ice for 10 min, and mixed with 100 pmol of biotin-Ole e 1:resin complexes in 200 μL of selection buffer with 0.2% BSA and incubated at 25 °C for 1 h. After washing three times with 1 mL of selection buffer, the ssDNA-protein complexes were resuspended in 20 μL of deionized water and amplified by PCR using the primers named F3 (5′ GCGGATGAAGACTGGTGT 3′) and R3 (5′ GTTGCTCGTATTTAGGGC 3′) under the conditions of 1 μM/primer, 250 μM dNTPs, in a final volume of 200 μL at 56 °C at different cycles (typically 5.10, 15 and 20 cycles) in order to optimize the number of cycles in each round. The cycles used has been 15 cycles (round 1), 10 cycles (round 2) and 5 cycles (round 3–6). PCR product was ethanol-precipitated (Fig. S2). After round 3 and 6, the pools, named Rd3 and Rd6, were amplified and labeled by PCR using 1U Taq DNA polymerase (Biotools, Madrid, Spain) in 50 μL of reaction also containing 1xPCR buffer (75 mM Tris HCl pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 125 mM dNTPs, 1 μM F3 primer (5′-GCGGATGAAGACTGGTGT-3′) and 1 μM R3 primer (5′-GTTGCTCGTATTTAGGGC-3′). The dsDNA product with 'A'-overhangs was cloned into pGEM-T Easy-cloning vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Individual clones were sequenced using T7 (5′-TAATACGACTCACTATAGGG-3′) and Sp6 (5′- ATTTAGGTGACACTATAGAA-3′) primers.

Selected ssDNA molecules were subjected to secondary structure prediction using the mFold software (http://www.unafold.org/mfold/applications/dna-folding-form.php) [38] at 25 °C in 150 mM Na (I) and 1 mM Mg (II) tertiary structure prediction using RNAComposer server (http://rnacomposer.cs.put.poznan.pl/) with CentroidFold secondary structure prediction method [39], and G-quadruplexes in nucleotide sequence prediction using QGRS Mapper (http://bioinformatics.ramapo.edu/QGRS/analyze.php) [40].

2.4. Aptamer binding capacity

2.4.1. Enzyme-linked OligoNucleotide assay (ELONA)

ELONA was used to analyze the binding of the selected aptamers for the target as in García-Recio et al. [41]. Briefly, 200 μL of biotin-labeled Ole e 1 protein at 4 μg mL^{-1} or pollen extract at 4.5 μg mL^{-1} were plated in a 96-well microtiter plate (NUNC, Roskilde, Denmark) in selection buffer with 0.2% BSA and then incubated overnight at 4 °C. Next, the wells were washed three times with selection buffer and blocked with BSA 5% in PBS. Afterwards, biotin-labeled aptamers (IBA GmbH) were diluted in selection buffer at 1 μg mL^{-1}, denatured for 10 min at 95 °C and cooled for 10 min on ice to fold in the proper structure. Then, 200 μL of the solution were added to each well, and the plate was incubated at 25 °C for several incubation times after which individual wells were washed four times with selection buffer to remove unbound ssDNA. Next, 200 μL of a 1/1000 dilution of streptavidin antibody conjugated with horse-radish peroxidase (POD) (Roche, Basel, Switzerland) were added to the individual wells. Following 30 min incubation at room temperature on a shaking platform, the plates were washed three times and developed using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution (Boehringer Mannheim, Ingelheim am Rhein, Germany) according to the manufacturer's instruction. Optical density (OD) values at 405 nm were determined using a microplate reader from TECAN (Mannedorf, Switzerland).

2.4.2. Aptaprecipitation assays

To test the ability of individual aptamers to specifically bind to purified Ole e 1, binding experiments using streptavidin magnetic microparticles were carried out. Nanolink Streptavidin magnetic beads (SoluLINK) (7.5 μL) were incubated with 5 μg of aptamer for 1 h at 25 °C with stirring in selection buffer. Then, 2 pmol of Ole e 1 were added to the microparticles:aptamer complexes and incubated for 1 h at 25 °C. Finally, samples were placed on the magnet until pellet forming and the supernatant was discarded. Then, pellet was washed three times with selection buffer, resuspended in 3x loading buffer, resolved by 15% SDS-PAGE and Ole e 1 was detected by western blot analysis using an anti-Ole e 1 antibody and silver staining to detect the aptamers.

2.5. Preparation of affinity monolithic capillary columns

UV transparent fused-silica capillaries (100 μm i.d. × 375 μm o.d.) were treated with 3-(trimethoxysilyl)propyl methacrylate to ensure covalent attachment of the monolith to its inner wall [42]. A polymerization mixture composed of GMA (150 μL), TRIM (50 μL), cyclohexanol (510 μL), dodecanol (90 μL), and AIBN (2 mg) as initiator was used for the synthesis of monolithic supports [27]. The mixture was sonicated for 10 min and purged with nitrogen for 10 min. The silanized capillary (33.5 cm) was filled with the polymerization mixture using a syringe pump (at a flow rate of 0.5 μL min^{-1}) up to a total length of 8.5 cm with a syringe, whereas the other 25 cm was kept empty. To initiate the photo-polymerization, the capillaries were placed into a UV irradiation chamber equipped with five UV lamps (5 × 8 W, 254 nm) (model CL1000, UVP, Upland, CA, USA) at 0.9 J/cm^2 for 10 min at room temperature. After polymerization, the resulting monolithic columns (of 8.5 cm length) were flushed with MeOH using an HPLC pump for 30 min to remove the pore-forming solvents and possible unreacted monomers.

To immobilize aptamers on bare monolithic columns, streptavidin was firstly attached onto pore surface of monolith via ring opening reaction of epoxide groups of monolith, followed by the immobilization of the biotinylated aptamers. This protocol, adapted from Zhao et al. [27,28], is summarized in Fig. 1.

Briefly, the monolithic column was rinsed in sequence with water and 10 mM PBS (pH 9.0) using a syringe pump at a flow rate

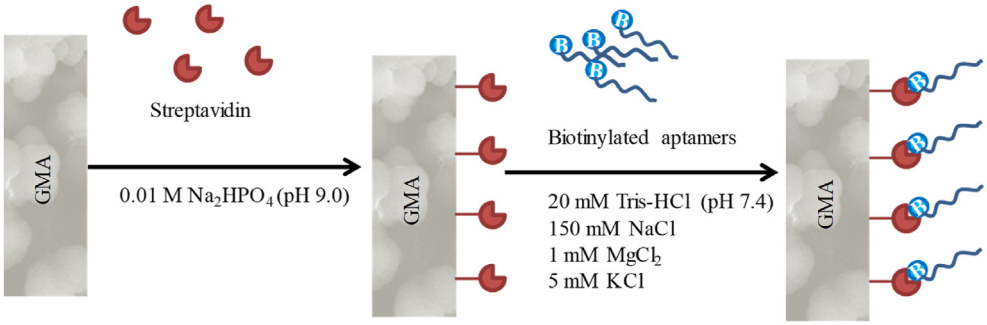

Fig. 1. Schematic illustration of the immobilization of aptamer on the polymeric monolithic columns.

of 80 μL h^{-1} for 30 min each one. Afterwards, 200 μL of streptavidin solution (1 mg mL^{-1} streptavidin dissolved in PBS solution) was pumped through the monolithic column for 24 h at room temperature. Streptavidin concentration was determined both in the initial solution as well as in the percolated fraction by measuring absorbance at 280 nm with a NanoDrop™ spectrophotometer (DeNovix DS-11, Wilmington, USA) to evaluate the amount of streptavidin attached. The column was then rinsed with PBS to remove the unreacted streptavidin and flushed with 40 μL of selection buffer. Finally, 50 μL of 50 μM biotinylated aptamer solution (prepared in selection buffer) was pumped through the streptavidin-modified monolithic column for 2.5 h. The obtained affinity monolithic column was rinsed with the selection buffer to remove the unbound aptamer. To evaluate the aptamer coverage density in the columns, the aptamer concentrations in the initial solution introduced into the monolith column, and in the percolated and washing fractions, were determined with a NanoDrop™ spectrophotometer. The amount of attached aptamer was calculated by subtraction of the aptamer amount in the percolated and washing fractions from that present in the initial solution. The aptamer coverage density was calculated by dividing the amount of immobilized aptamer by the volume of capillary affinity monolith (considering that the 8.5-cm long monolith inside the capillary of 100 μm i.d. has a geometric volume of 0.67 μL).

The morphology of the affinity monolithic columns was examined by scanning electron microscopy (SEM) (S-4800, Hitachi, Ibaraki, Japan). Prior to SEM analysis, these capillaries were sputter-coated with Au/Pd for 2 min to avoid charging problems. This microscope was also used to perform energy dispersive X-ray spectrometry (EDS) experiments of these materials.

Infrared spectrum measurements were done with an ATR-FTIR instrument (Agilent Cary 630 FTIR spectrometer) with Microlab PC software. Spectra were collected from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.6. Chromatographic conditions

The monolithic columns were placed into an $HP^{3D}CE$ instrument (Agilent, Waldbronn, Germany) equipped with a diode array UV detector, and pressurized with an external nitrogen supply. Data acquisition was performed with the ChemStation Software (Rev.A.10.01, Agilent). Samples were injected in the capillary (containing the monolithic bed) through short-end injection mode by applying a pressure of 8 bar for 5 s at the outlet (defined in the instrument). Pressure-driven chromatographic separations of proteins were done in the $HP^{3D}CE$ system by applying an external pressure of 12 bar at the outlet using the binding or selection buffer (see composition in Section 2.3) as mobile phase. Separations were done at 20 °C and detection absorbance was set at 214 nm.

2.7. Recognition of Ole e 1 in diagnosis skin prick-test extracts

A skin prick-test positive to olive pollen protein containing Ole e 1 and Ole e 9 was obtained from ALK (Madrid, Spain). A certain volume of this extract was properly diluted with the selection buffer solution, and it was subjected to the chromatographic conditions indicated above.

3. Results and discussion

3.1. Selection and characterization of high-affinity aptamers against Ole e 1

Aptamers were selected from libraries of oligonucleotides by iterative cycles of selection (SELEX methodology). We performed a selection of specific DNA aptamers against Ole e 1 as indicated in the Experimental section. We carried out six rounds of selection using streptavidin magnetic beads that binds the biotin-labeled Ole e 1. Afterwards, we isolate and characterize 6 individual aptamers from rounds 3 (AptOle1#1 and AptOle1#2) and 6 (AptOle1#3, AptOle1#4, AptOle1#5 and AptOle1#6), as described in Section 2.3 (Table 1). A preliminary ELONA assay showed that the aptamers AptOle1#4 and AptOle1#6 had a high binding capacity to the purified protein, and AptOle1#6 also to the protein present in the pollen extract (Fig. S3). However, due to the low values obtained using ELONA assays, we have not been able to calculate a K_d for these aptamers. Alternatively, aptaprecipitation assays were performed as described in Section 2.4.2. Fig. 2 shows that a high signal is detected after a short time of exposition when Ole e 1 is aptaprecipitated with the aptamers 5 and 6. However, it is necessary a high exposition to detect Ole e1 band when aptamers 1 to 4 are used for the aptaprecipitation. From these results, it could be concluded that the binding degree is higher for the aptamers 5 and 6 than for the aptamers 1, 2 and 3, meanwhile aptamer 4 was not able to aptaprecipitate Ole e 1.

3.2. Bioinformatics analysis of the secondary structures

To predict the most stable secondary structures of Ole e 1 aptamers, we performed a bioinformatic analysis of their sequences using the mFold, software. In Fig. S4 we showed the most probable secondary structures of these aptamers, taking into account the lower free energy (ΔG). Because many of the aptamers described in the literature contain G-quadruplex structures [22–24], which strongly stabilize the tertiary structure, the capacity of the aptamers to form G-quadruplex structures was studied using the QGRS Mapper program. The analysis of the results indicated that the aptamers AptOle1#2 and AptOle1#5 could form G-quadruplex structures (Fig. S4). Although the Gibbs free energy (ΔG) is similar

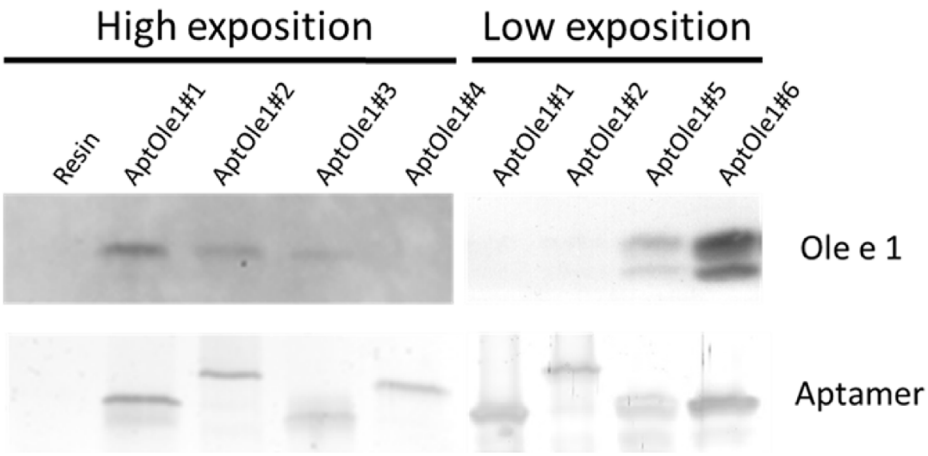

Fig. 2. Aptaprecipitation assays to analyze the binding of the selected aptamers for Ole e 1. Western blot (top) and silver stain (bottom).

in almost all the aptamers (except AptOle1#5, that is higher) (Table 1), aptamers AptOle1#2 and AptOle1#5 contain G-quadruplex motifs in its structure, which provide greater stability for these molecules; therefore, the AptOle1#2 aptamer, with lower ΔG and containing G-quadruplex motifs could be most stable aptamer. In addition, a prediction of the tertiary structure of the aptamers using the RNA Composer software is shown (Fig. S4).

3.3. Affinity studies using aptamer modified monolithic columns

The conditions to prepare photo-polymerized GMA-based monoliths as host supports for posterior aptamer immobilization were adapted from a previous work, where the columns were thermally polymerized [27,28]. Unlike this initiating mode, UV initiation is significantly fast (performed in few minutes) with low energy requirements and cost. Besides, the resulting photoinitiated columns showed higher permeabilities (with macropores around 1–2 μm) [43,44] than the thermally-initiated ones, which extremely facilitated the surface modification protocols. Additionally, the large pores present in the bare monolith (Fig. S5A) can favor the transfer of the target analyte and provide enough space for the attached aptamers forming appropriate 3D structures for the target protein binding. The immobilization of the aptamer on the surface of this monolith gave similar morphological structure than that found in the bare monolith (Fig. S5B). In order to confirm the covalent immobilization of aptamer on the bare monolith, EDS analysis was performed. As shown in Fig. S6B, the presence of P (with a content of *ca.* 2.7 wt%) was found in the aptamer-modified monolithic column, whereas the bare monolith (Fig. S6A) did not indicate the presence of this element. It demonstrates that the surface of monolithic column was successfully functionalized with the aptamer.

Additionally, FT-IR spectra of monolithic materials (prepared in bulk format) were measured. Fig. S7A shows a strong peak at 1720 cm^{-1} attributed to the carbonyl group, characteristic of this bare polymer. After aptamer immobilization onto this monolith, a new peak at 1650 cm^{-1} is emerged, which corresponded to heterocyclic structure of aptamer [45,46], Also, this spectrum showed the 3,400 cm^{-1} imidazole band corresponding to the biotinylated aptamer. These results corroborated that the aptamer was successfully attached to the surface of polymer monolith.

Next, the coverage density of aptamers on the monolithic columns was evaluated for the 6 different investigated aptamers. In all cases, the aptamer coverage density was similar 1350 and 1400 pmol μL^{-1}, which is higher than those obtained for other studies using affinity monoliths for target proteins such as thrombin [27,28].

In order to investigate the specific capture of Ole e 1 on monolithic columns modified with different affinity aptamers (AptOle1#1 - AptOle1#6), the previously isolated Ole e 1 protein was injected and separated on these affinity monoliths. Apart from Ole e 1 protein, other nonspecific proteins (such as HSA and cyt *c*), and another relevant olive pollen allergen (Ole e 9) were also tested. The retention time of these three proteins (HSA, cyt *c* and Ole e 9) was similar (*ca.* 1.5 min), eluting at the void time; however, different retention times were observed for the different aptamer-modified columns using Ole e 1 fractions as can be evidenced in the representative chromatograms depicted in Fig. S8. According to this figure, monolithic columns modified with AptOle1#4 did not retain the target protein, whereas the monoliths functionalized with AptOle1#3 and AptOle1#1 showed a very weak retention (*ca.* 1.7 and 2.0 min, respectively). On the other hand, the monolithic columns immobilized with AptOle1#2, AptOle1#5 and AptOle1#6 showed an enhanced retention of Ole e 1 (2.4, 4.5 and 16 min, respectively). From these results, it can be derived that AptOle1#6 gave the highest affinity against Ole e 1, which was consistent with the previous ELONA and aptaprecipitation studies. At sight of these findings, the affinity monolith modified with AptOle1#6 was chosen for further studies. Fig. 3 shows the chromatograms of the target protein (Ole e 1, trace A) and non-target proteins (HSA, cyt *c* and Ole e 9, traces B, C and D, respectively) on this aptamer monolithic column. Additionally, a bare monolithic column (without the immobilized aptamer) was also used to separate Ole e 1 and the non-target proteins for comparison (data not shown). In this case, the column did not show strong retention for Ole e 1, which coeluted with the non-target proteins. The results showed that the retention of HSA, cyt *c* and Ole e 9 was very weak, which suggested that the columns modified with AptOle1#6 capture preferentially Ole e 1 due to its specific interaction.

Using the aptamer-modified monolithic column, the present procedure was evaluated in terms of linearity, sensitivity and precision. A good linear relationship between the peak area and the concentration of Ole e 1 was obtained in the range of 0.05–2.0 mg mL^{-1} ($r > 0.988$). Also, the limit of detection (LOD) and quantitation (LOQ) of Ole e 1 based on the peak signal to the baseline noise ratio of 3 and 10, respectively, were estimated, giving values of 10 and 33 μg mL^{-1}.

The reproducibility of preparation of aptamer monolithic columns was also investigated. For this purpose, precision (as relative standard deviation (RSD, %)) was evaluated under the optimized

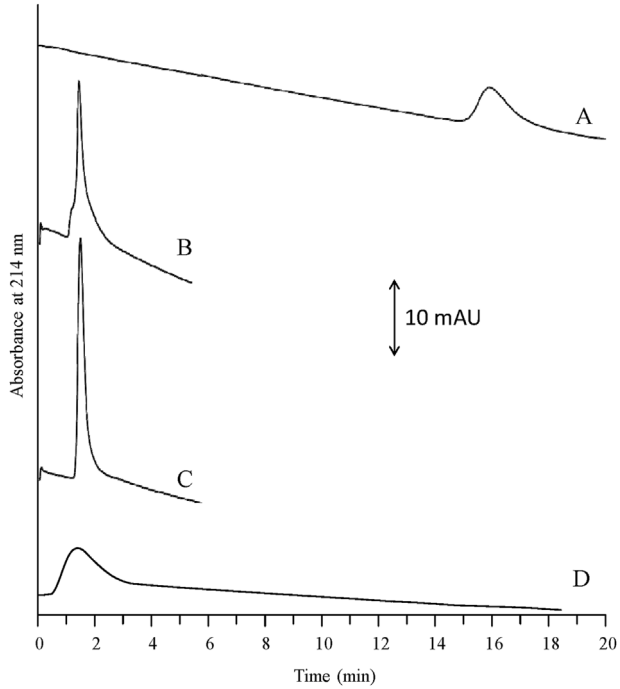

Fig. 3. Retention of Ole e 1 (0.25 mg mL^{-1}) (A), HSA (0.25 mg mL^{-1}) (B), cyt *c* (0.25 mg mL^{-1}) (C) and Ole e 9 (0.25 mg mL^{-1}) (D) on the aptamer affinity monolithic column prepared with AptOle1#6. Sample injection was done at pressure of 8 bar for 5 s; separation was carried out at 20 °C using as mobile phase the selection buffer solution.

conditions using the Ole e 1 solution. The run-to-run repeatability was evaluated from series of three injections of Ole e 1, whereas the column-to-column reproducibility was estimated with three columns prepared from the same polymerization mixture in different days. The RSD values of the retention times and peak areas were below 3.2% and 4.2% in run-to-run repeatability, respectively; whereas the RSD values were less than 7.8% and 9.3% in the column-to-column reproducibility, respectively. These results indicated a satisfactory reproducibility in the preparation of the aptamer-modified monolithic columns developed in this work.

Another key feature of chromatographic supports is related to their long-term stability. The aptamer-based monolithic capillary column did not show loss of separation performance after a period of continuous use exceeding than 25 injections. Indeed, the RSDs values for retention time and for peak areas of Ole e 1 were determined as 3.1% and 6.7%, respectively. Besides, the aptamer affinity monolithic columns were stored at 4 °C in selection buffer up to 5 months without significant change in the retention behavior of Ole e 1, thus demonstrating the good stability of the polymer rod.

3.4. Recognition of Ole e 1 in olive pollen extracts for skin prick-tests

In order to demonstrate the practical application, the prepared aptamer affinity column was used to recognize Ole e 1 in commercial olive pollen extracts developed by pharmaceutical companies in order to perform skin prick tests. In particular, as stated in the label, the used extracts are characterized by a relatively important concentration of Ole e 1 allergen, although other proteins (such as Ole e 9) can also be found. As shown in Fig. 4, other analytes present in the extract did not exhibit retention on the affinity monolithic column, eluting at void time (*ca.* 1.5 min), while a

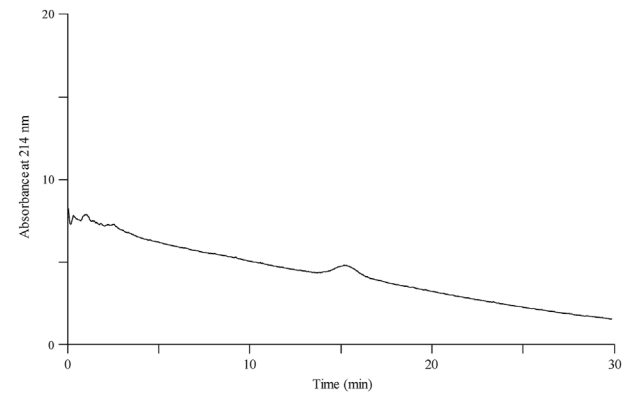

Fig. 4. Identification of Ole e 1 in commercial olive pollen extract for skin prick test (diluted 1/10 with the selection buffer) using the aptamer affinity monolithic column prepared with AptOle1#6. Experimental conditions as indicated in Fig. 3.

peak was observed at the same retention time of Ole e 1 (16 min). These results demonstrated that Ole e 1 can be separated and from protein mixtures by taking advantage of the specific recognition of the aptamer affinity monolithic column.

4. Conclusions

In this study, we report the selection and characterization of ssDNA aptamers having binding affinity to Ole e 1, the major allergen of olive pollen. Thus, SELEX technique was used to provide six sequences with high affinity to Ole e 1. Several binding assays (ELONA, aptaprecipitation and aptamer-based monolithic capillary chromatography) were carried out in order to evaluate aptamer specificity and affinity for this target protein. Our results indicate that aptamer AptOle1#6 provided the highest binding for this allergenic protein. Besides, the aptamer affinity monolith with the selected oligonucleotide sequence was successfully applied to recognize Ole e 1 in allergy skin test. All these results demonstrate that the aptamers and aptamer-based methodologies (*e.g.* affinity monolithic columns) can potentially serve as powerful analytical tools in separation, enrichment and diagnostic of allergenic proteins in therapeutic and clinical fields.

CRediT authorship contribution statement

María Vergara-Barberán: Investigation, Formal analysis, Writing – original draft. **María Jesús Lerma-García:** Conceptualization, Methodology, Validation, Writing – review & editing. **Ernesto F. Simó-Alfonso:** Funding acquisition, Supervision, Writing – review & editing. **Marta García-Hernández:** Investigation. **M. Elena Martín:** Conceptualization, Writing – review & editing, Supervision. **Ana García-Sacristán:** Project administration, Writing – review & editing. **Víctor M. González:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **José Manuel Herrero-Martínez:** Conceptualization, Methodology, Validation, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors acknowledge the financial support of project RTI2018-095536-BI00 (Ministry of Science, Innovation and Universities, Spain) funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe" by the European Union. M.V.B. thanks the Generalitat Valenciana for a VALi + D postdoctoral research contract (ref. APOSTD/2020/213). M.E.M. and V.M.G. are researchers from FIBio-HRC supported by Consejeria de Sanidad (CAM). We gratefully acknowledge to Dr. De La Hoz for kindly providing skin prick-test positive to olive pollen protein.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.339334.

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Supplementary material

Selection and characterization of DNA aptamers for highly selective recognition of the major allergen of olive pollen Ole e 1

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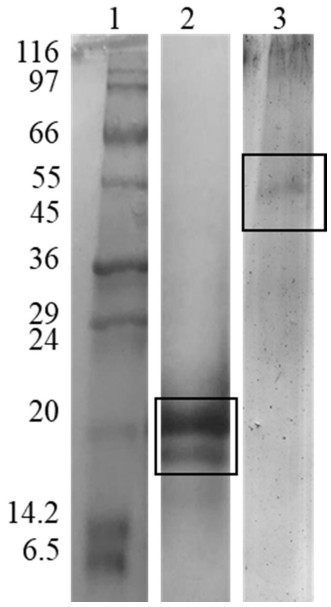


Figure S1. SDS-PAGE of pooled fractions from Sephadex G-75 chromatography. Identification: lane 1, molecular weight standards (Mr in kDa); lane 2 and 3, fractions obtained from Picual pollen corresponding to Ole e 1 and Ole e 9, respectively.

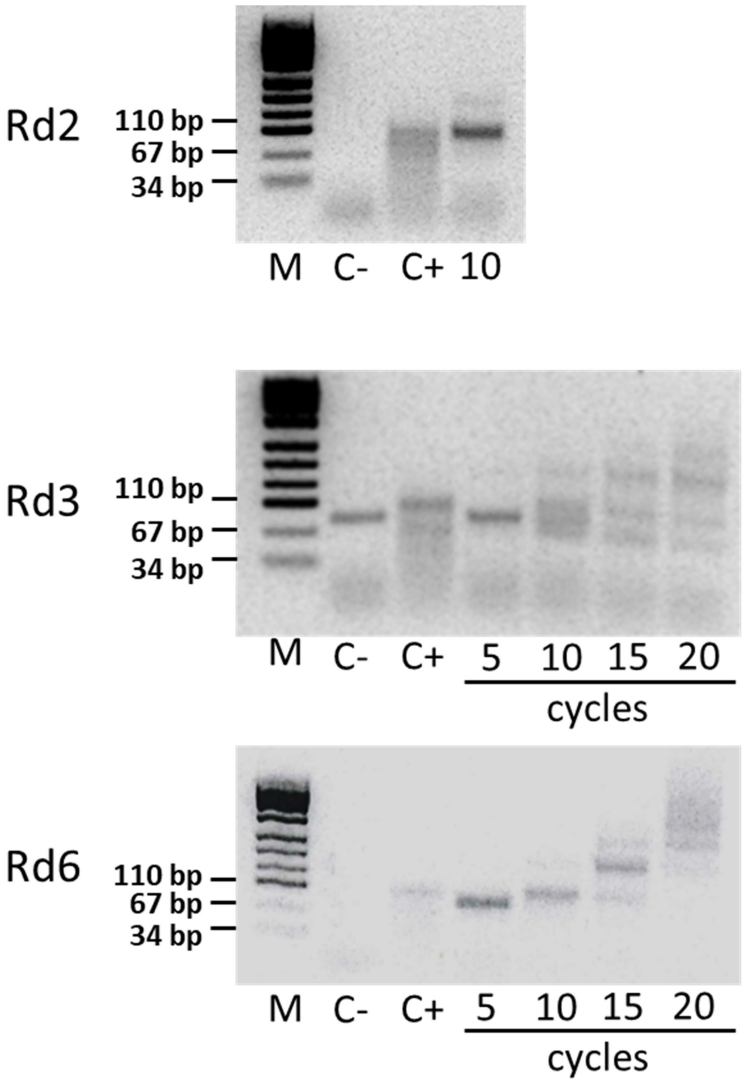
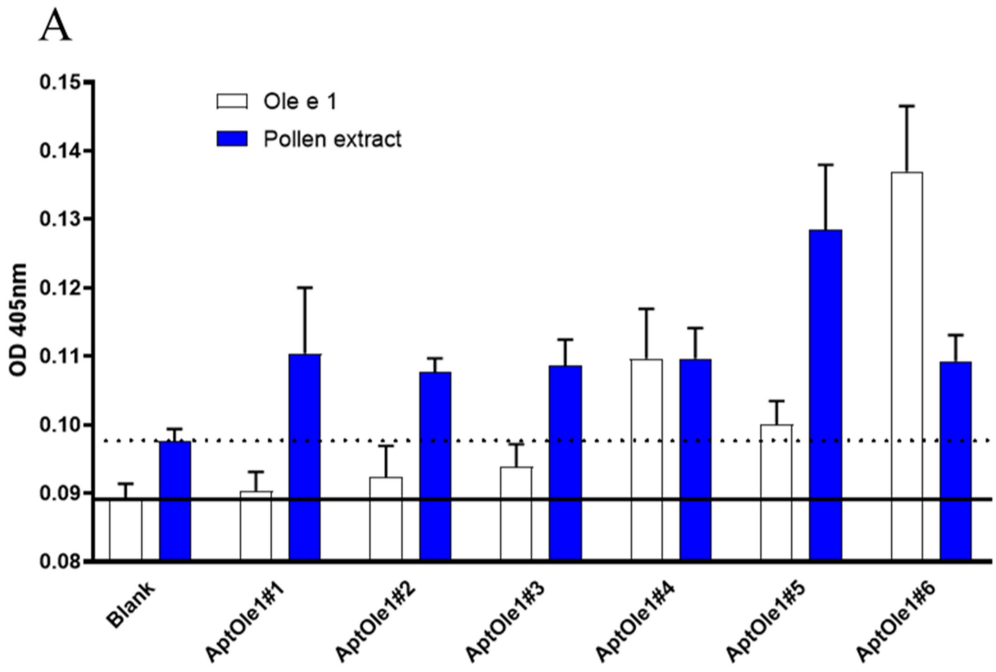


Figure S2. Agarose gels showing the PCR products obtained after several cycles of amplification in rounds 2, 3 and 6 of the SELEX process (M, pUC19 DNA/MspI (HpaII) Marker; C+, positive control; C-, negative control).



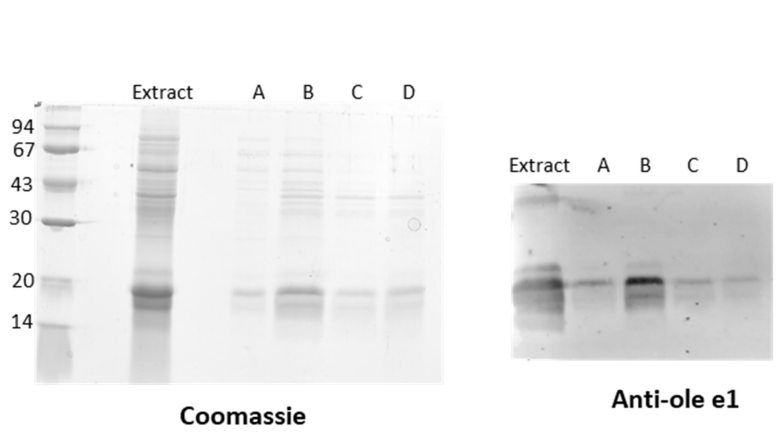


Figure S3. (A) ELONA assay to analyze the binding of the selected aptamers for Ole e 1. Blank is secondary antibody, without aptamer **(B)** PAGE gel stained with Coomassie and a western blot using anti-OleE1 antibody showing the pollen extract and different Ole e 1 purifications (A-D). Sample D was used in the ELONA assay.

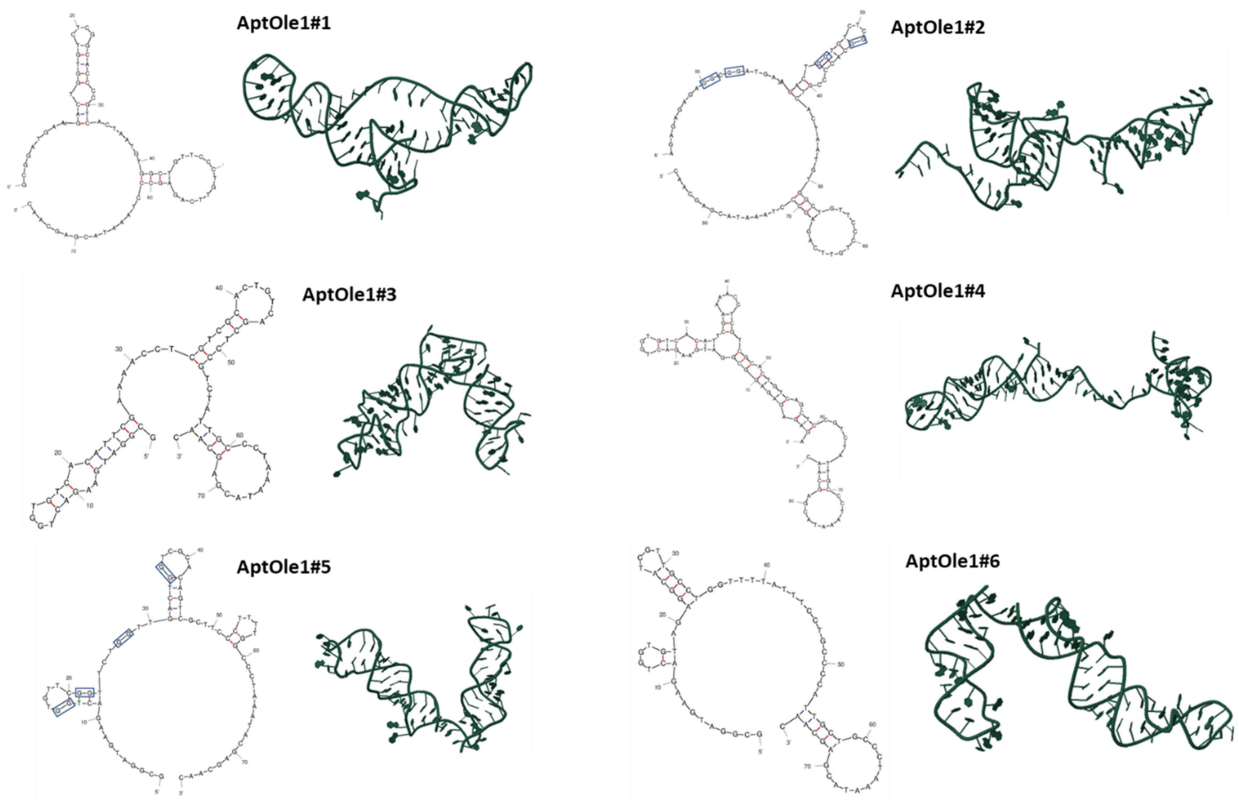


Figure S4. Predicted secondary and tertiary structures and potential G pairs implicated in the formation of G-quadruplex of the selected aptamers for Ole e 1.

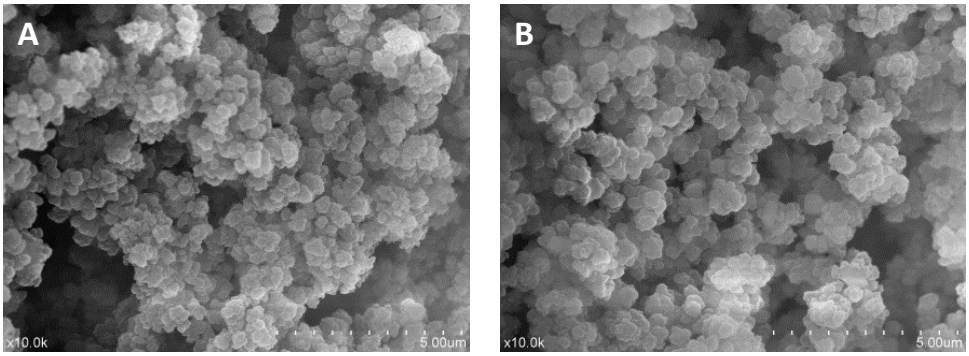
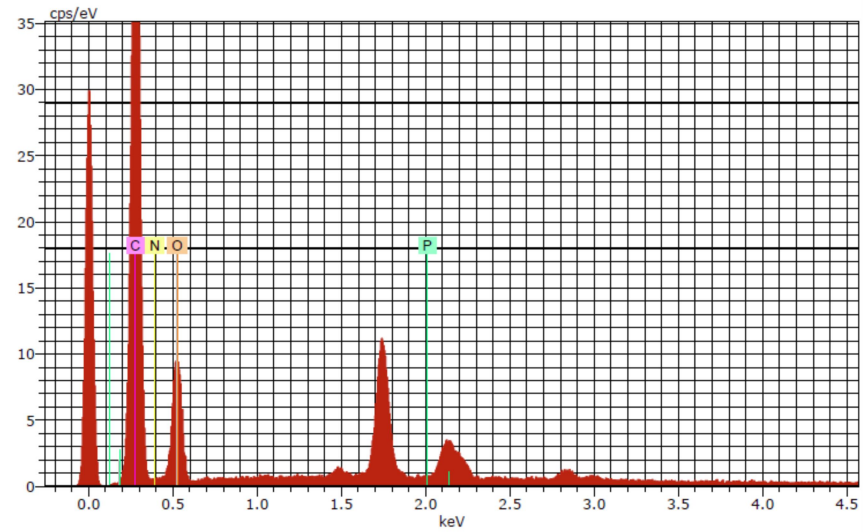


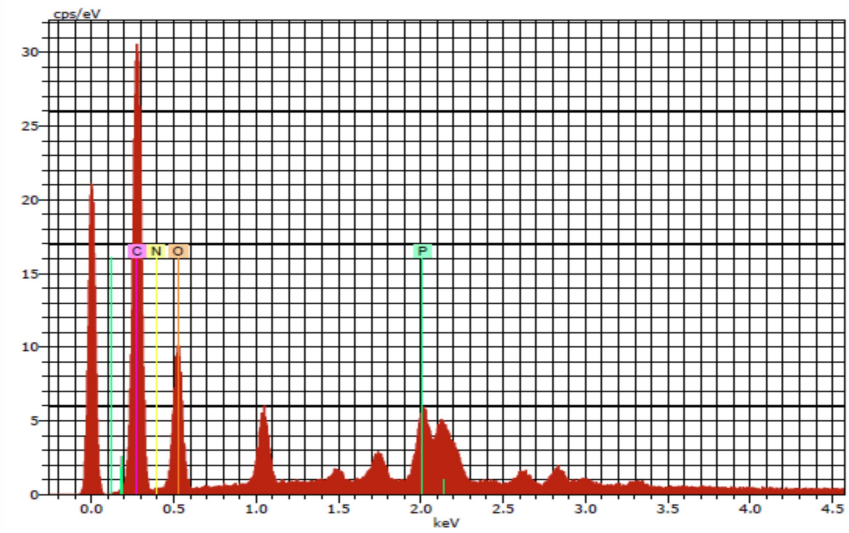
Figure S5. SEM micrographs of GMA-based monolith (A) and aptamer affinity monolithic column (B) prepared with AptOle1#6. Magnification at 10,000×.



A

Spectrum: 1

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error [wt.%]
Carbon	K-series	62.29	62.29	68.31	7.3
Nitrogen	K-series	5.95	5.95	5.60	1.4
Oxygen	K-series	31.61	31.61	26.03	4.3
Phosphorus	K-series	0.14	0.14	0.06	0.0
	Total:	100.00	100.00	100.00	



B

Spectrum: 1

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error [wt.%]
Carbon	K-series	58.90	58.90	66.12	7.2
Nitrogen	K-series	2.83	2.83	2.72	0.9
Oxygen	K-series	35.61	35.61	30.01	4.9
Phosphorus	K-series	2.66	2.66	1.16	0.1
	Total:	100.00	100.00	100.00	

Figure S6. EDS analysis of the bare monolith (A) and aptamer affinity monolithic column prepared with AptOle1#6 (B).

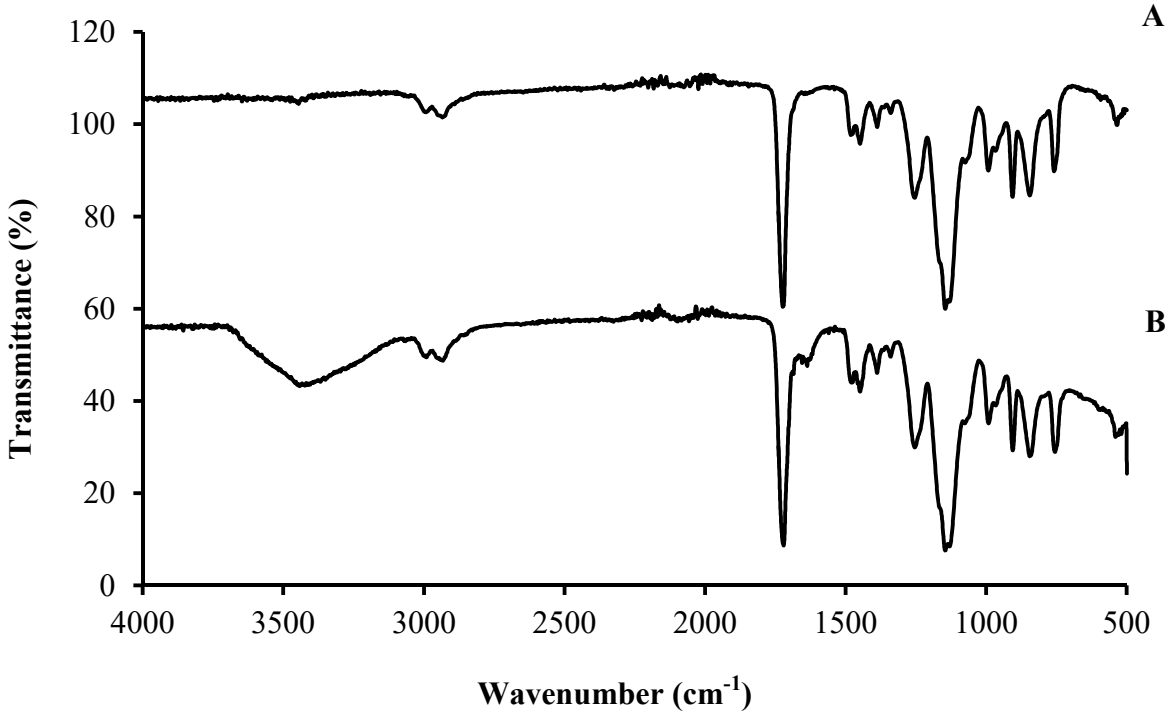


Figure S7. FTIR spectra of the bare monolith (A) and aptamer affinity monolithic column prepared with AptOle1#6 (B).

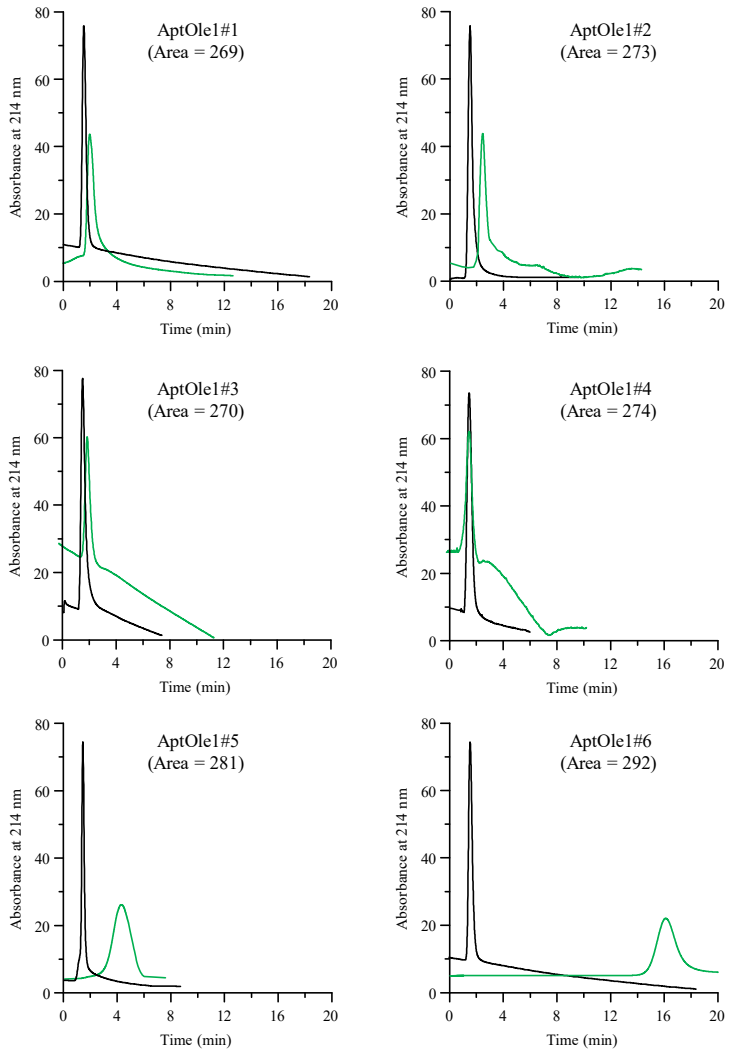


Figure S8. Retention of non-target protein (HSA, 1 mg mL^{-1}) and target protein (Ole e 1, 1 mg mL^{-1}) on several affinity monolithic columns prepared with different aptamers. Peak area values of Ole e 1 for each affinity monolithic column are included. Chromatographic conditions used indicated in Section 2.6.