Microchemical Journal 92 (2009) 123–128

Contents lists available at ScienceDirect

Microchemical Journal

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

Selective recognition of ovalbumin using a molecularly imprinted polymer

Wei-Xiang Su [a], John Rick [a,b], Tse-Chuan Chou [a,b,*]

[a] Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan
[b] Department of Chemical Engineering, Tatung University, Taipei 104, Taiwan

ARTICLE INFO

Article history:
Received 4 December 2008
Accepted 14 December 2008
Available online 31 December 2008

Keywords:
Molecular imprinting
Ovalbumin
Protein
Synthetic molecular recognition
Microcontact

ABSTRACT

Micro-contact imprinting has been used to form thin-film molecular imprints of ovalbumin (OVA) in polymers supported on glass slides. Thermocalorimetric data was used to optimise the choice of functional monomer and cross-linker to maximise selectivity and minimise non-specific recognition.

A polymer comprising polyethyleneglycol 400 dimethacrylate (95 vol.%) and methacrylic acid (5 vol.%) showed both maximum recognition for OVA when made as a molecularly imprinted polymer (MIP), and minimal recognition when made as a non-imprinted, i.e. control polymer. OVA rebinding to the molecularly imprinted polymer, from a buffered 2 μM OVA solution, was 1.55×10^{-11} mol cm^{-2}, while the control polymer showed 10-fold less re-binding, i.e. 0.154×10^{-11} mol cm^{-2}.

Experiments in which human serum albumin (HSA), conalbumin, ovomucoid or lysozyme, were re-bound to the polymers, either as single proteins or in competition with OVA, showed them to have low affinity for the polymer formulation used. Of the competing proteins examined, in non-competitive binding experiments, HSA showed the greatest affinity 0.45×10^{-11} mol cm^{-2} for the OVA imprinted polymer. In two protein competition experiments, i.e. with OVA and a competing protein present at equal concentrations (2 μM), OVA binding to the OVA imprinted polymer was in all cases significantly greater than that of the competitor.

1. Introduction

Ovalbumin is a glycosylated 385 amino acid 45 kDa protein, which comprises approximately 55–65% of the total protein content of egg white [1]. Smaller contributions to the egg white's total protein composition are made by: conalbumin [(13%), mol. wt. 80,000 Da], ovomucoid [(~11%), mol. wt. 28,000 Da], and lysozyme [(~3.5%), mol. wt. 14,600 Da], together with smaller amounts of the globulins (G2, G3) and ovomucin [2].

Allergies against one, or more, of the proteinacious components of eggs are quite common affecting approximately 1.6% of children. Vaccines, against such diseases as influenza, are frequently cultured in chickens' eggs. Ideally, after purification the sought-after immunogenic materials should be free of contaminants, especially proteins potentially able to induce an adverse immune response. However, while an individual who is aware of their sensitivity to a given food component can attempt to limit their exposure to the allergen through abstinence, they may be unaware of the risk that vaccination, as a consequence of incomplete allergen removal, could potentially expose them to. Thus, a method with high specific recognition for detecting unwanted proteinacious allergens would be potentially useful.

Molecular imprinting has developed rapidly during the past three decades as a branch of synthetic recognition chemistry. The non-covalent route to molecular imprinting results in polymers with recognition characteristics that are formed under the direction of a template resulting in polymeric features that are ideally complimentary in both their dimensions and also their functionality to the template [3]. Molecular imprinting methodology employs functional monomers to mediate specific chemical recognition together with crosslinking agents that serve to hold the functional monomers in place while forming shape and size selective cavities, or surface impressions. The resulting artificial recognition structures are formed either, imbedded within three-dimensional matrices, or on the surface of thin-film polymers [4–9].

Until recently most imprinting targets tended to be relatively small well-functionalised molecules; however, recently several groups have made significant in-roads into the imprinting of bio-macromolecules, especially proteins [3]. Several excellent reviews have recently been published relating to the state-of-the-art with respect to protein imprinting [10–12].

The non-covalent route to molecular imprinting allows the production of materials that are: easy to fabricate, robust, and cost effective to produce. Having become established as viable materials in separation techniques such as HPLC and solid phase chromatography [13–18], MIPs are now employed as functional materials useful for bio-macromolecular recognition and separation [19–22].

Given that undesirable allergenic materials may remain, as described above, in vaccines; MIPs potentially offer a simple and cost-effective route for their detection.

* Corresponding author. Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan. Tel.: +886 6 2757575x62639; fax: +886 6 2366836.
E-mail address: tcchou@mail.ncku.edu.tw (T.-C. Chou).

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doi:10.1016/j.microc.2008.12.010

2. Materials and methods

2.1. Materials

Ovalbumin (OVA); 2-hydroxyethyl methacrylate (HEMA); methacrylic acid (MAA); Poly(ethylene glycol) dimethacrylate [mol. wt. 550, (PEG400DMA)]; Poly(ethylene glycol) dimethacrylate [mol. wt. 875, (PEG600DMA)]; Tween 20 (polyoxyethenesorbitanmonolaurate); trypsin; bovine serum albumin (BSA); ovomucoid; lysozyme; conalbumin; human serum albumin (HSA) as an HSA-FITC conjugate; anti-ovalbumin (mouse derived); anti-mouse IgG (H & L chain specific) were all obtained from Sigma. N-vinyl-pyrrolidone (NVP) was obtained from ACROS. Styrene (SM) was purchased from Lancaster. Methyl methacrylate (MMA) was supplied by the Kanto Chemical Co. Inc., Tokyo, Japan. Ethylene glycol dimethacrylate (EGDMA) and tetraethylene glycol dimethacrylate (TEGDMA) were supplied by Fluka. Sodium dodecyl sulphate was purchased from J.T. Baker. Potassium dihydrogen phosphate (KH_2PO_4); Potassium c4e dibasic dehydrate ($Na_2HPO_4 \cdot 2H_2O$); sodium hydroxide, and sodium chloride were purchased from Riedel-de-Haën. 2,2′-dimethoxy-2-phenyl-acetophenone (DMPAP) was from TCL. Methacrylic acid 3-triethoxysilylpropyl ester was from the San Fu Chemical company. Enhanced Chemiluminescence (ECL) reagents were supplied by Amersham Biosciences. All other chemicals/solvents were obtained as either Analar or HPLC reagent grade materials from normal commercial sources.

Microcalorimetry measurements were made using a Thermometric 2277 thermal activity monitor supplied by Thermometric AB Sweden operating under dedicated software from the same company. Atomic force microscopy images were made using a scanning probe microscope from Digital Instruments Inc., Santa Barbara, USA. Ultraviolet/visible spectra/absorbances were recorded using a Shimadzu UV-160A spectrophotometer. Film thickness determinations were made using an Alpha-step 500 supplied by Tencor. Photochemical reactors manufactured by the Panchum Scientific Corp., Taiwan were used to form the polymeric thin-films.

3. Methods

3.1. Preparation of materials

A micro-contact approach was used to form imprinted sites, for OVA, on the surface of thin-film polymers supported on substrate glasses. A cover glass, modified by silanisation to increase protein adhesion, was used to introduce the OVA into the polymer mixture, on the substrate glass, prior to polymerisation. Before use, the supporting slide and the cover glass were prepared in a manner broadly similar to our previous studies.

In brief, the supporting substrate glasses (1.3 cm^2) were sequentially washed (10 min each stage in a sonicator bath) in: sodium hydroxide solution (1 M); deionised water; hydrochloric acid (1 M) and finally again deionised water. After drying, the support glasses were modified to increase protein adhesion, by immersion in a homogenous solution of glacial acetic acid (69 μl), additionally containing 0.4% methacrylic acid 3-triethoxysilylpropyl ester (769 μl), at 80 °C for 4 h. After drying in a nitrogen stream the glasses were stored under inert gas prior to use.

The cover glasses were cleaned (30 min each stage in a sonicator bath) in Sodosil®RM02 (10 mL in 30 mL deionised water) at 55 °C, prior to similar sonication treatment in: deionised water, isopropanol, water and ethanol.

3.1.1. Imprint formation and template protein extraction

The cover glasses, treated as above, were immersed in a solution of OVA [2 μM in phosphate buffer (adjusted to pH 7.4)] at 25 °C for 16 h prior to drying in a nitrogen stream. The crosslinkers and functional monomers examined in this study were mixed and added to the substrate glass together with the initiator 2,2 dimethoxy-2-phenylacetophenone [DMPAP (2 wt.%)]. Imprinted films were formed by bringing together, i.e. contacting, the support and cover glasses prior to UV irradiation for 1 h. Non-imprinted polymers, made as controls, were formed in the same way except that no protein was adhered to the cover glass prior to contacting.

Protein was removed from the films in a two stage process. Initially the films were treated in trypsin/phosphate buffer at 37 °C for 3 h prior to being washed (4 washes each of 10 min) in phosphate buffer. The films were subsequently washed in 2% SDS, containing 0.4 wt.% NaOH, at 60 °C for 30 min and then washed again (4 washes each of 10 min) in phosphate buffer.

3.1.2. Protein detection

OVA adhering to the surfaces of the thin-films, was quantified by ELISA. Briefly, the films were immersed in a blocking solution comprising reconstituted dried milk powder (5 wt.% in PB), additionally containing BSA (2 wt.%), and then left overnight in a refrigerator, after which they were washed (4 times, 10 mL/10 min each wash) in phosphate buffered saline (PBS) also containing Tween20 (0.1 wt.%), adjusted to pH 7.4. The detection of OVA is complicated by the need to use the anti-OVA antibodies in the ELISA protocol at a reduced temperature (4 °C). Therefore, in order to establish satisfactory working conditions for the detection system two antibody incubation experiments were undertaken using an arbitrary polymer formulation (5 vol.% MAA in PEG400DMA) to establish the optimal incubation time for the antibodies at 4 °C.

A primary anti-ovalbumin antibody solution (1 mL), made as 1 μl/mL in PBS, pH 7.4, was added to each film. The films were then incubated at 4 °C for varying times from 2 to 16 h (Fig. 1). A secondary antibody (2 μL/mL, anti-mouse IgG), carrying the peroxidase conjugate to the anti-OVA antibody was introduced and incubation allowed to proceed for 8 h. A similar procedure was adopted to establish the optimal conditions for the second antibody except that two concentrations (1 μL/mL, or 2 μL/mL) were examined. Again, as with the first antibody, samples were taken to allow the examination of the OVA re-binding profile up to a maximum time of 16 h. OVA binding profiles with respect to time for both the primary and secondary antibodies, as shown in Fig. 2, were obtained by adding ECL reagents to the incubation wells in accordance with the manufacturer's instructions and allowing incubation to proceed for 3 min prior to plate reading.

The results from both figures indicate that saturation is achieved after approximately 7 h incubation time and also that a clearer saturation profile was found when using 2 μL/mL of the secondary antibody. These conditions for incubation in ELISA assays were used for the remainder of this study.

4. Results and discussion

4.1. Selection of functional monomer

Isothermal titration calorimetry results were used as a rational basis for the selection of the functional monomer and crosslinker used to form the thin-film imprinted polymers and the controls. Ideally, the imprinted films should have maximum selectivity for OVA with respect to a panel of competing proteins, while for the non-imprinted control (NIP) OVA-polymer recognition should be minimal.

Initially, five functional monomers, namely: MMA; HEMA; NVP and MAA, were used in individual experiments in which each functional monomer (5 μL) was titrated into the reaction cell of the calorimeter containing the target protein (OVA) adhered to glass slides (prepared as previously described) in phosphate buffer (pH 7.4). This injection procedure was repeated at hourly intervals, for 10 h, to generate an accumulated heat response profile, for the monomers, as is shown in Fig. 2.