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Solid-Phase Synthesis of a Biotin Derivative and its Application to the Development of Anti-Biotin Antibodies

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Abstract A biotin derivative, namely biotin–aminocaproic acid–lysine (BAL), was synthesized with solid-phase chemistry, conjugated to a carrier-protein, and used for rabbit immunization. The aminocaproic acid–lysine "long-arm" was used in order to project the biotin-hapten above the carrier-protein surface. Lysine was selected due to its N^{ε}-amino group, through which BAL was conjugated to the carrier-protein. BAL was synthesized on a commercially available resin with the Fmoc-solid-phase strategy; this has simplified the experimental procedure, overcome the need for intermediate purification steps, and led to a final product of high purity, with high yield. The anti-BAL antibodies recognized free biotin, as shown with an in-house-developed ELISA, in which biotin conjugated to a synthetic "lysine–dendrimer" was used to coat the ELISA microwells. In immunocytology and Western-blot experiments, the anti-BAL antibodies led to similar results with those obtained with streptavidin. Synthetic derivatives of hapten molecules that can be easily prepared with solid-phase chemistry, such as BAL, may be used for the development of specific antibodies for the corresponding hapten.

Keywords Solid-phase synthesis · Anti-biotin antibodies · Streptavidin · ELISA · "Lysine–dendrimer" · Immunocytology · Western blot · Anti-hapten antibodies

Lists of abbreviation

2-CTC	2-Chloro-trityl-chloride
ABTS	2,2′-Azino-bis-(3-ethylbenzthiazoline-6 sulfonic acid) diammonium salt
Aca	Aminocaproic acid
BCA	Bicinchoninic acid

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Boc	Tert-butoxycarbonyl
Cy3	Cyanide dye 3
DAB	3,3′-Diaminobenzidine
DCM	Dichloromethane
DIC	*N*,*N*′-Diisopropylcarbodiimide
DMEM	Dulbecco's modified eagle medium
DMF	*N*,*N*′-Dimethylformamide
DMSO	Dimethyl-sulfoxide
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethyl-amino-propyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetra acetate
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Fmoc	9-Fluorenylmethoxycarbonyl
GA	Glutaraldehyde
HOBt	Hydroxybenzotriazole
HRP	Horseradish peroxidase
MW	Molecular weight
NHS-LC-Biotin	*N*-Hydroxy-succinimidyl-long chain-biotin
NP-40	Nonyl phenoxylpolyethoxyl-ethanol
OD	Optical density
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PPD	*p*-Phenylenediamine
r.t.	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid

Introduction

Biotin is a low molecular weight molecule (hapten) which serves important biological functions in the living cell. The classical role of biotin is that of a vitamin belonging to the B-complex, which is involved in intermediate metabolism as a covalently bound coenzyme for cellular carboxylases [1]. Autosomal recessive disorders of biotin metabolism have been described in humans, which mainly result from deficient activity of the enzymes responsible for the in vivo recycling of biotin. Affected patients, who usually demonstrate a combination of all carboxylase deficiencies known as multiple carboxylase deficiency syndrome, respond successfully to pharmacological doses of biotin [2]. According to recent literature, in addition to its classical function as a vitamin, biotin may be associated with other important biological processes as well, such as development, cellular homeostasis, and regulation, and may also play a yet not completely elucidated role as a ligand attached to histones in the cell nucleus [3, 4].

Anti-biotin antibodies are useful bioanalytical tools, since they may be used for conducting research on biotin and biotin-containing macromolecules; moreover, they may serve as alternative reagents in various "universal" multicomponent biotechnology systems (e.g., detection or purification systems, etc.) that employ biotin or biotinylated probes along with specific biotin-binding proteins. The most widely used among the latter are avidin and

streptavidin, two naturally occurring proteins which bind biotin with high affinity and specificity [5–7]. Nevertheless, anti-biotin antibodies are still considered useful, alternative to (strept)avidin, biotin-binding tools, while some of them have proven to be, in certain experimental systems, even advantageous to avidin or streptavidin [8]. Due to their numerous potential applications, various anti-biotin antibodies have been developed so far and are still being developed and/or commercially distributed and used [9–11]. From a different point of view, anti-biotin antibodies may be considered as an efficient model of anti-hapten antibodies, since their binding characteristics for the biotin-hapten can be easily evaluated by using avidin or streptavidin as control biotin-binding proteins. For all the above-mentioned reasons, development of anti-biotin antibodies is considered an interesting task.

In the present work, we have produced polyclonal anti-biotin antibodies, which were employed in the development of an in-house ELISA and evaluated in immunocytology and Western blot experiments in parallel with streptavidin. The anti-biotin antibodies were developed against a new synthetic biotin derivative, namely biotin–aminocaproic acid–lysine (BAL), which was prepared with solid-phase chemistry. As it will be discussed, the employment of solid-phase technology in the synthesis of BAL has led to specific advantages and may be thus considered as a general approach for preparing derivatives of hapten molecules.

Experimental

Materials

D-Biotin was a product of Sigma. The 2-CTC polystyrene resin used in the synthesis of BAL, the Fmoc-protected amino acids, and HOBt were purchased from Chemical and Biopharmaceutical Laboratories (Patras, Greece). The Fmoc-protected aminocaproic acid (Fmoc-Aca-OH) was prepared in-house. The 4-CN-tritylamidomethyl polystyrene resin used in the synthesis of the "lysine dendrimer" was prepared in-house, as previously described [12]. DMF, DCM, THF, TFA, HPLC solvents (analytical grade), and other common laboratory chemicals were obtained from Merck or Sigma. DMSO was from Fluka. Sulfo-NHS-LC-Biotin was purchased from Pierce. Bovine thyroglobulin, glutaraldehyde, EDC, Tween-20, goat anti-rabbit IgG/HRP, sheep anti-rabbit IgG/Cy3, streptavidin-FITC, DAB tablets, and ABTS were products of Sigma. Freund's Adjuvant was from Difco. The ELISA microplates were obtained from Corning. The cell lines were obtained from the American Type Culture Collection (ATCC). DMEM and FCS were purchased from PAA Laboratories. Trypsin/EDTA [0.05%/0.02% (*w*/*v*) in PBS (free of Ca^{2+}, Mg^{2+})] was obtained from Biochrom AG. Prestained SDS-PAGE Standards, Broad Range, were from Fermentas.

Synthesis of BAL

BAL was synthesized on a commercially available 2-CTC polystyrene resin. The first amino acid, Fmoc-Lys(Boc)-OH, was anchored on the resin as previously described [13]. Couplings of Fmoc-Aca-OH and biotin were performed by dissolving an excess (4 mol equiv.) of the molecule to be coupled and of HOBt and DIC in DMF (biotin was first dissolved in DMSO); the corresponding solution was preactivated for 10 min at r.t. and then added to the resin and allowed to react for 3 h. Anchoring and coupling efficiency was monitored using the Kaiser ninhydrin test [14]. In the case of an intense, positive result, the coupling step was repeated. The crude product was cleaved from the resin using a mixture of TFA/H_2O/ethanedithiol/triisopropylsilane (94.5/2.5/2.5/1, *v*/*v*/*v*/*v*) and subsequently

purified with semi-preparative RP-HPLC (Waters, pump 600E, detector UV 484). Pure BAL was characterized by analytical RP-HPLC (Waters, pump 616, detector 996 PDA) and ESI-MS (Finningan AQA Thermoquest).

Synthesis of aminocaproic acid–lysine (AL) and biotin–aminocaproic acid (BA) was performed following the above described protocol. The final products were purified and characterized as described above.

Preparation of the Immunogen (BAL Conjugated to Bovine Thyroglobulin) and Immunization of Rabbits

This was performed following a protocol based on the well-established glutaraldehyde method [15]. Briefly, in a reaction vial containing 200 μL of a BAL solution (0.5 mg/mL in 0.01 M PBS, pH 7.4), 200 μL of a bovine thyroglobulin solution (10 mg/mL in 0.01 M PBS, pH 7.4), 360 μL 0.01 M PBS, pH 7.4, and 40 μL of an aqueous solution of glutaraldehyde (25%) were added, and the mixture was allowed to react for 3 h at 25 °C and then overnight at 4 °C. Then, the mixture was dialyzed against distilled water (MW cutoff: 6,000–8,000 Da) for 48 h and finally diluted with 0.9% NaCl to a final protein concentration of 200 μg/mL. The diluted mixture ("working solution") was subsequently used for immunization as follows:

New Zealand white rabbits (2-month old) were intradermally or subcutaneously injected [16] with 500 μL of the working solution of the immunogen (i.e., 100 μg of the protein-conjugate) that had been emulsified with an equal volume of Complete Freund's Adjuvant. The animals were boosted initially 6 weeks after first exposure and subsequently every 4 weeks. Blood was collected 2 weeks after each booster injection. Antisera were obtained with low speed centrifugation of whole blood and stored at −35 °C.

Care of animals was in accordance to the corresponding European legislation.

Immunochemical Evaluation of the Anti-BAL Antibodies with ELISA

Biotin Immobilization on the ELISA Microwells: Synthesis of the "Lysine Dendrimer" The dendrimer was synthesized manually on 4-CN-tritylamidomethyl polystyrene resin, following the Fmoc strategy. Synthesis was performed through first the anchoring and then six consecutive coupling steps, in each of which an excess of the following Fmoc-protected amino acids was used: Anchoring: Fmoc-Aca-OH (2 mol equiv.); Couplings: (1) Fmoc-Lys(N^{ε}-Fmoc)-OH (4 mol equiv.), (2) Fmoc-Lys(N^{ε}-Fmoc)-OH (8 mol equiv.), (3) Fmoc-Lys(N^{ε}-Fmoc)-OH and Fmoc-Lys(N^{ε}-Boc)-OH (16 mol equiv., molar ratio 2/1), (4) Fmoc-Lys(N^{ε}-Fmoc)-OH and Fmoc-Lys(N^{ε}-Boc)-OH (32 mol equiv., molar ratio 2/1), (5) Fmoc-Lys(N^{ε}-Boc)-OH (64 mol equiv.), (6) Fmoc-Aca-OH (64 mol equiv.). In each of the above steps, HOBt (mol equiv. equal to those of the corresponding Fmoc-protected amino acid) in DMF was also added. The solution of each amino acid (or mixture of amino acids) was cooled on ice and then DIC (mol equiv. equal to those of the Fmoc-protected amino acid) was added. The reaction mixture was left on ice for 10 min and then at r.t. for another 10 min; afterwards, the reaction mixture was added to the resin and allowed to react at r.t. (for 3 h or overnight). Coupling efficiency was monitored using the Kaizer ninhydrin test. In case of an intense, positive result, the coupling step was repeated, once or more. Removal of the N-terminal Fmoc group (deprotection) was performed using a 20% (*v*/*v*) solution of piperidine in DMF. Deprotection was carefully monitored by UV spectroscopy at 301 nm and when necessary, suitably elongated until a baseline optical absorbance was obtained. After completing the synthetic procedure, the resin was extensively washed

(DMF, DCM and petroleum ether) and then dried under vacuum. The final product was cleaved from the resin using a mixture of TFA/triisopropylsilane/H_2O (95/2.5/2.5, *v*/*v*/*v*) for 2 h. After lyophilization, the above product was characterized by analytical RP-HPLC (Waters, pump 616, detector 996 PDA) and ESI-MS (Finningan AQA Thermoquest; MW calculated 4,578.18 Da).

Biotin Immobilization on the ELISA Microwells: Conjugation of Biotin to the "Lysine Dendrimer" ("Biotin/Dendrimer") To 100 μL of an aqueous solution of the purified synthetic dendrimer (10 μg/mL), 100 μL of a solution of sulfo-NHS-LC-Biotin (4.81 mg/mL in DMSO/H_2O, 2/1, *v*/*v*) were added. Then, 300 μL of distilled water were added, the pH was adjusted to 6.0, and the mixture was left to react for 5 h at r.t. and then overnight at 4 °C under gentle stirring. Finally, the mixture was dialyzed against distilled water (MW cutoff 1,000 Da) for 48 h.

ELISA Titer Curves ELISA microtiter plates were coated with "biotin/dendrimer" (1 μg/mL in 0.01 M PBS, pH 7.4, 100 μL/microwell, overnight incubation, 4 °C). The following day, the solution was discarded, and the microwells were washed twice with 0.01 M PBS, pH 7.4. Blocking was performed with a 2% BSA solution, in PBS-T, i.e., 0.01 M PBS, pH 7.4 containing 0.05%, *v*/*v*, Tween-20 (200 μL/microwell, 1 h, r.t.). After blocking, the solution was discarded, the microwells were washed three times with PBS-T and then incubated with serial dilutions of anti-BAL/thyroglobulin (short: anti-BAL) antisera in diluting buffer, i.e., PBS-T containing 0.2% BSA (100 μL/microwell, 2 h, 37 °C). After incubation, the solutions were discarded, the microwells were washed three times with PBS-T and then incubated with goat anti-rabbit IgG/HRP, diluted 1:3,000 in diluting buffer (100 μL/microwell, 2 h, 37 °C). Afterwards, the solution was discarded and the microwells were washed three times with PBS-T and incubated with an ABTS (1 mg/mL)/H_2O_2 (0.003%) solution (100 μL/microwell, 30 min, r.t.). Finally, the OD of the microwells was measured (405 nm), and the corresponding titer curves were plotted.

ELISA Displacement Curves ELISA microwells were coated, washed, blocked, and washed again as described above. Then, the microwells were incubated (2 h, 37 °C) with 50 μL of a solution of the anti-BAL antiserum, diluted 1:25,000 in diluting buffer, and 50 μL of a series of standard solutions of free biotin, BAL, BA, AL, aminocaproic acid, or lysine (in diluting buffer, 0.1 ng/mL to 10 mg/mL), which had been preincubated (18 h, 4 °C). After incubation, microtiter plates were washed, and the procedure described in the above paragraph was followed. Finally, after measuring the OD (405 nm), the displacement curves were plotted.

In an additional series of experiments, 50 μL of serial dilutions of a commercially available biotin solution for pharmaceutical use ("Biotine Roche 0,5 pour cent") and 50 μL of a solution of the anti-BAL antiserum, diluted 1:25,000 in diluting buffer, were preincubated (18 h, 4 °C) and tested in the ELISA displacement system, in parallel with biotin standard solutions.

Biotin ELISA-Type Assay Based on the "Biotin/Dendrimer" and Streptavidin

ELISA-Type Titer Curves ELISA microwells were coated with the "biotin/dendrimer," washed, blocked, and washed again as described in "ELISA titer curves." Then, they were incubated (1 h, r.t.) with 100 μL of serial dilutions of a streptavidin/HRP solution (50–500 ng/mL in diluting buffer). After incubation, the solution was discarded, and the

microwells were washed and incubated with ABTS/H_2O_2 as described in "ELISA titer curves." Finally, the OD was measured (405 nm) and the corresponding titer curve was plotted.

ELISA-Type Displacement Curves ELISA microwells were coated, washed, blocked, and washed again as described above. Then, the microwells were incubated (1 h, r.t.) with 50 μL of a streptavidin/HRP solution (120 ng/mL in diluting buffer) and 50 μL of a series of standard solutions of biotin (100 – 800 pg/mL) or serial dilutions of the commercially available pharmaceutical biotin solution, which had been preincubated (18 h, 4 °C). Then, the procedure described in the above paragraph was followed and the displacement curves were plotted.

In all ELISA or ELISA-type experiments, various blank microwells were included in each run, in which (1) the "biotin/dendrimer" coating solution was replaced with plain PBS, (2) the primary antiserum or the streptavidin/HRP solution was replaced with pre-immune rabbit serum or the diluting solution, respectively.

Evaluation of the Anti-BAL Antibodies in Immunocytology Experiments

Protocol A: Fluorescence-Staining of HeLa Cells HeLa cells were grown on special glass coverslips and fixed with 4% paraformaldehyde for 30 min at r.t. The coverslips were washed twice with PBS (for 2 min each time), incubated with Triton X-100 (0.5% in PBS) for 10 min at r.t., and washed three times with PBS (for 5 min each time). Blocking was performed by incubating with blocking buffer (1% BSA, 20% FCS in PBS) for 1 h at r.t. The slides were washed three times with PBS (for 5 min each time) and incubated with rabbit anti-BAL antiserum, diluted 1:5,000 in diluting buffer (0.2% BSA, 2% FCS in PBS), or streptavidin/FITC (1.5 μg/mL in diluting buffer), for 1 h at r.t. The slides were washed three times with PBS (for 5 min each time), and those incubated with the anti-BAL antiserum were then incubated with sheep anti-rabbit-IgG/Cy3, diluted 1:500 with diluting buffer, for 1 h at r.t., and finally washed three times with PBS (for 5 min each time). A PPD solution was added on the slides which were then observed with a fluorescence microscope (Nicon E400 equipped with a complete fluorescence unit, Nikon C-SHG1).

Protocol B: Color-Staining of HeLa Cells HeLa cells were grown on special glass coverslips; the coverslips were then washed and fixed with MeOH, rehydrated by passing through a graded series of alcohol and water mixtures, washed with PBS, and then treated with Triton X-100, and washed as described above. Then, they were incubated with H_2O_2, 0.3% (30 min, r.t.), washed twice with PBS (for 2 min each time), blocked with blocking solution (2 h, r.t.), and washed three times with PBS (for 5 min each time). Afterwards, the coverslips were incubated with the anti-BAL antiserum (diluted 1:500 in diluting buffer) or streptavidin/HRP (8 μg/mL in diluting buffer) for 1 h at r.t. The coverslips were washed three times with PBS (for 5 min each time), and those incubated with the anti-BAL antiserum were incubated with anti-rabbit-IgG/HRP, diluted 1:500 in diluting buffer for 1 h at r.t., washed three times with PBS (for 5 min each time), and finally incubated with a DAB solution; the coverslips incubated with streptavidin/HRP were directly incubated with DAB. Immediately after color development, the coverslips were counterstained with Harris hematoxylin, dehydrated by passing through a suitably graded series of alcohol and water, fixed with Entellan, and observed with an optical microscope (Nicon E400).

Protocol C: Fluorescence-Staining of Nuclei of HeLa Cells HeLa cells were cultured for 48 h in DMEM, supplemented with 10% FCS at 37 °C in a 5% CO_2 incubator. Then, the culture medium was discarded, and the cells were incubated with a hypotonic solution (0.075 M KCl) for 3 min at r.t. and centrifuged (1,500×*g* for 10 min). The nuclei-pellet was suspended in MeOH/acetic acid, 3/1, *v*/*v* and centrifuged (1,500×*g* for 10 min); that step was repeated twice, and finally, small aliquots of the nuclei suspension were put on special object slides and air-dried. The slides were rehydrated as described above (Protocol B), then blocked and treated as described in Protocol A, and finally observed with a fluorescence microscope (Nikon E400 equipped with a complete fluorescence unit, Nikon C-SHG1).

Pre-immune rabbit serum or streptavidin preincubated with a BAL solution (100 μg/mL) for 15 min at r.t. were used as negative controls in the above protocols. A rabbit polyclonal antibody that recognizes the nuclear polypeptide prothymosin alpha [17] was used as a positive control for nuclear staining.

Evaluation of the Anti-BAL Antibodies in Western Blot Experiments

Cell Culture—Preparation of HeLa Cell Lysates HeLa and MDA-MB-231 cells were cultured in DMEM supplemented with 10% FCS at 37 °C in a 5% CO_2 incubator. The culture medium was discarded, and the cell monolayers were washed with 0.9% NaCl. Trypsin/EDTA was added, and the cells were incubated for 10 min at 37 °C. The cells were resuspended in NaCl 0.9% and centrifuged (1,500×*g* for 10 min). The supernatant was discarded, the cells were resuspended in single-detergent cell-lysis buffer (0.05 M Tris–HCl pH 8.0, 0.9% NaCl, 0.02% NaN_3, 100 μg/mL PMSF, 1 μg/mL aprotinin, 1% NP-40), incubated on ice for 10 min, and centrifuged (1,500×*g* for 10 min). The supernatant was transferred to a microtube and stored at 4 °C. Total protein concentration of the lysates was determined with the BCA method [18] slightly modified.

Western Blot Analysis of Cell Lysates HeLa and MDA-MB-231 cell lysates (aliquots corresponding to 30 μg total protein) were treated for 3 min at 100 °C in SDS-loading buffer (0.05 M Tris–HCl pH 6.8, 0.1 M DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and were further subjected to SDS-PAGE on 10% polyacrylamide gel slabs (Blue Vertical 101 Mini Slab Gel System, Serva). The gels were either stained with Coomassie Brilliant Blue R-250 as control SDS-PAGE, or the isolated protein bands were electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell, 0.20 or 0.45 μm). Electrophoretic transfer was performed on a Mini Trans-Blot Cell (BIO-RAD, USA) using a Tris–glycine continuous buffer system (0.025 M Tris, 0.192 M glycine, 20% methanol, pH 8.3). The gels were electroblotted for 1 h at 100 V. The membranes were rinsed twice with 0.01 PBS, pH 7.4, containing 0.05%, *v*/*v*, Tween-20 (PBS-T), blocked with blocking buffer (3% BSA in PBS-T) for 1 h at r.t., and then rinsed twice with PBS-T. The membranes were incubated for 1 h at r.t. with a solution of anti-BAL antiserum, diluted 1:500 in diluting buffer (0.3% BSA in PBS-T) or streptavidin/HRP (5 μg/mL in diluting buffer), and then rinsed three times with PBS-T. The membranes treated with the antiserum were further incubated for 30 min with a solution of goat anti-rabbit IgG/HRP, diluted 1:500 in diluting buffer, and then rinsed three times with PBS-T. All membranes were finally stained with a DAB solution.

Pre-immune rabbit serum or streptavidin preincubated with a BAL solution (100 μg/mL) for 15 min at r.t. were used as negative controls.

Results

Synthesis of BAL

Crude BAL was obtained in almost quantitative yield. Highly pure BAL (>95%) was obtained using semi-preparative RP-HPLC and characterized with analytical RP-HPLC and ESI-MS. According to the ESI-MS analysis data (not shown here), the expected and observed molecular mass was 485.6 and 485.4, respectively; the main ion (m/z=486.4) was the +1 charged state. Synthetic BA and AL were also characterized with analytical RP-HPLC and ESI-MS.

Immunochemical Evaluation of the Anti-BAL Antibodies: ELISA Titer and Displacement Curves

Conjugation of BAL to bovine thyroglobulin was performed following a well-established protocol. Formation of the conjugate was indirectly verified by coating ELISA microwells with various dilutions of the working solution of the immunogen ("Experimental"), then incubating the wells with streptavidin/HRP (at three different concentrations, i.e., 500, 250, and 100 ng/mL) and with ABTS/H_2O_2, and finally measuring the OD values (405 nm).

A high titer value, approximately 1:50,000, was obtained with the anti-BAL antiserum (pool of four consecutive bleedings). In all cases, very low OD values were obtained in the various blanks.

A full displacement curve was obtained with the anti-BAL antiserum in the presence of standard solutions containing 10 ng/mL–10 μg/mL of free biotin, which were prepared by serially diluting a stock solution of biotin (1 mg/mL in DMSO/H_2O, 1/1, *v*/*v*). This finding indicates good affinity for the non-derivatized biotin-hapten. Shifted-to-the-left displacement curves (data not shown) were obtained in the presence of BAL (1 ng/mL up to 1 μg/mL) and BA (1 ng/mL up to 100 ng/mL). No displacement was observed in the presence of lysine, aminocaproic acid, or AL.

The anti-BAL antiserum led to parallel displacement curves by using either biotin standard solutions or serial dilutions of a commercially available biotin solution for pharmaceutical use (Fig. 1). Parallel displacement curves were also obtained by using streptavidin instead of the anti-BAL antiserum (Fig. 2).

Evaluation of the Anti-BAL Antibodies in Immunocytology Experiments

HeLa cells were stained and observed with either optical or fluorescence microscopy using anti-BAL antiserum and anti-rabbit IgG/HRP parallel with streptavidin/HRP, or anti-BAL antiserum, and anti-rabbit IgG/Cy3 parallel with streptavidin/FITC, respectively. According to the results obtained (Fig. 3) in all cases, the cell cytoplasm was clearly stained, while no staining was observed in the cell nucleus. The above results were confirmed by suitably treating HeLa cells, so as to remove their cytoplasm, and then staining the cell nuclei. Contrary to that obtained with the anti-BAL antiserum and with streptavidin, clear nuclear staining (data not shown here) was observed with the positive control, i.e., a well-characterized rabbit antiserum recognizing the nuclear polypeptide prothymosin alpha [17].

Further in-depth experimental work, along with the employment of the most sensitive analytical techniques available, is necessary in order to detect the presence of any biotinylated proteins, e.g., biotinylated histones, in the cell nucleus of various cells and investigate whether and how this may be associated with cell state and functioning.

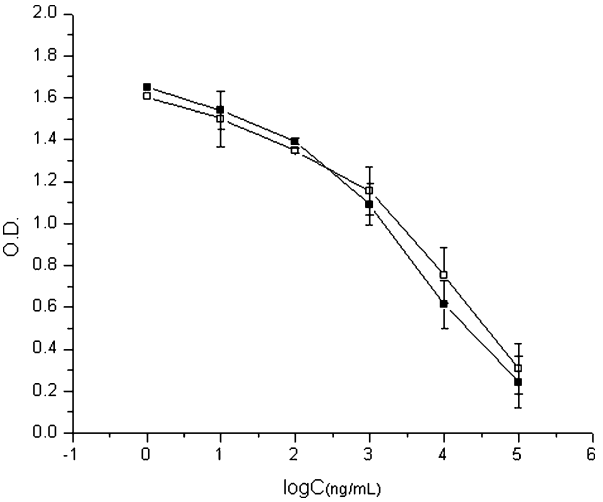

Fig. 1 ELISA displacement curves obtained with the anti-BAL antiserum in the presence of standard solutions of free biotin (*filled squares*) or serial dilutions of a commercially available pharmaceutical solution containing biotin (*empty squares*). An in-house synthesized, biotinylated "lysine dendrimer" ("biotin/dendrimer") was used to immobilize biotin on the ELISA microwells

Evaluation of the Anti-BAL Antibodies in Western Blot Experiments

Using anti-BAL antiserum and anti-rabbit IgG/HRP parallel with streptavidin/HRP in the analysis of HeLa and MDA-MB-231 cell lysates, similar Western blot patterns were obtained. Various slightly stained protein bands with MW values ranging from 34 to 130 kDa were observed, while a band corresponding to ~26 kDa predominated in both cell lysates, independently of whether anti-BAL antiserum or streptavidin was used for visualization (Fig. 4). The less intensive staining of the bands with MW higher than 26 kDa might be due to non-quantitative transfer. The biotin-containing bands probably correspond to biotinylated carboxylases, other reported biotinylated proteins, or degradation products thereof [19–21].

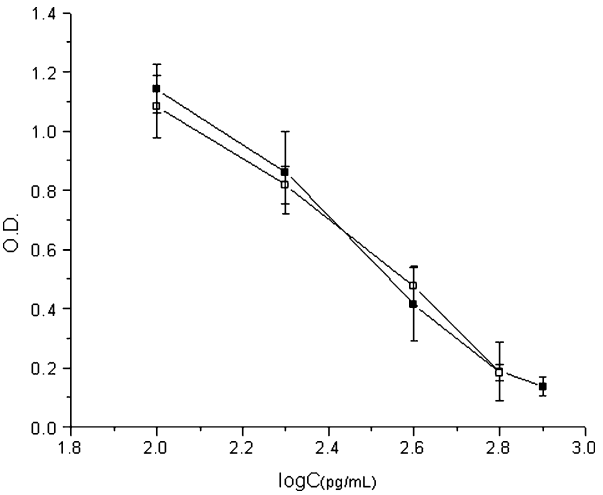

Fig. 2 ELISA-type displacement curves obtained with streptavidin in the presence of standard solutions of free biotin (*filled squares*) or serial dilutions of a commercially available pharmaceutical solution containing biotin (*empty squares*). An in-house synthesized, biotinylated "lysine dendrimer" ("biotin/dendrimer") was used to immobilize biotin on the ELISA microwells

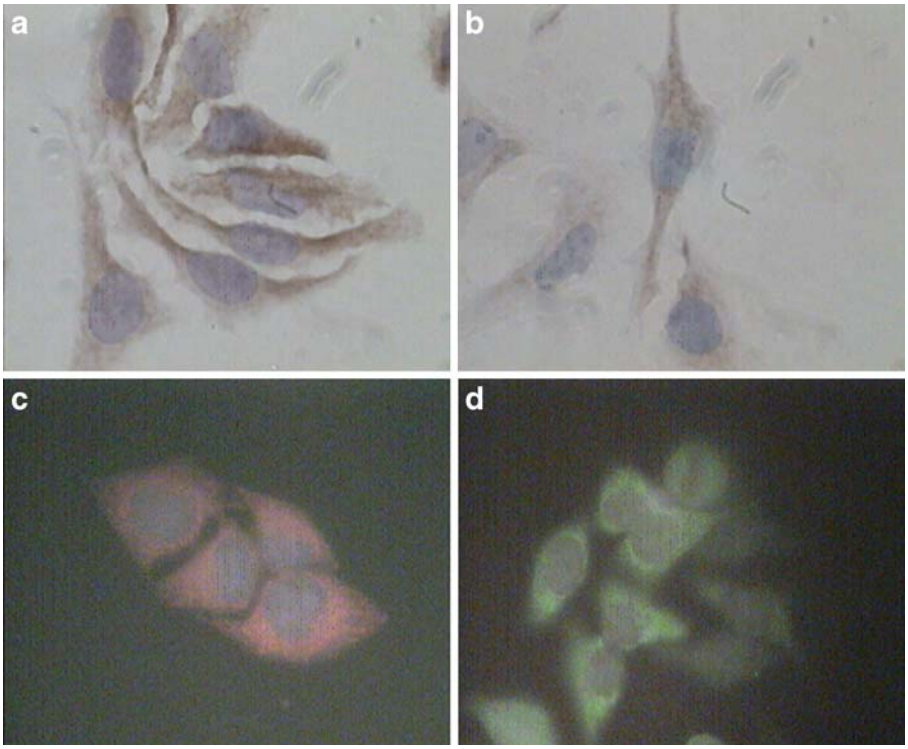

Fig. 3 Immunostaining of HeLa cells with: rabbit anti-BAL antiserum and anti-rabbit IgG/HRP (**a**); streptavidin/HRP (**b**); rabbit anti-BAL antiserum and anti-rabbit IgG/Cy3 (**c**); streptavidin/FITC (**d**). In all cases, cell cytoplasm was clearly stained

Discussion

In the present work, we have developed and immunochemically characterized rabbit polyclonal antibodies to biotin, as model anti-hapten antibodies. The synthetic derivative biotin–aminocaproic acid–lysine (BAL) was used for immunization. BAL was synthesized on a commercially available resin with the Fmoc-solid-phase strategy which is widely applied to peptide synthesis [22]; this has simplified the experimental procedure, ameliorated undesirable reactions (due to the presence of suitable protecting groups), overcome the need for intermediate purification steps, and led to a final product of high purity and yield. The

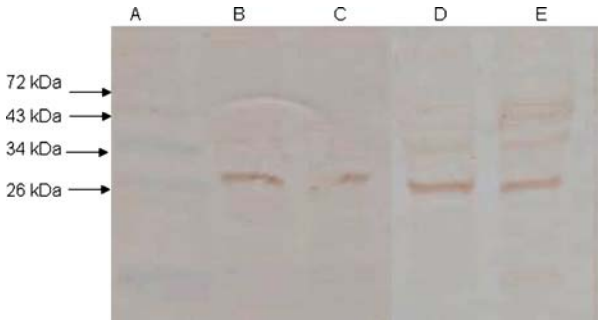

Fig. 4 Western blot analysis of HeLa (*lanes B*, *D*) and MDA-MB-231 (*lanes C*, *E*) cell lysates (30 μg total protein). Staining was performed with either rabbit anti-BAL antiserum and anti-rabbit IgG/HRP (*lanes D*, *E*) or streptavidin/HRP (*lanes B*, *C*); pre-stained molecular weight markers were also used (*lane A*)

aminocaproic acid–lysine "long arm" was used in order to project the hapten above the carrier-protein surface [23, 24]. The lysine moiety was selected due to its N^{ε}-amino group, through which BAL was conjugated—via glutaraldehyde—to the carrier protein for animal immunization. Other than lysine, amino acids may be used, e.g., cysteine; in this case, the synthetic biotin derivative obtained can be conjugated to the carrier protein via the –SH groups. In addition to BAL, a "lysine–dendrimer" was synthesized with the Fmoc-solid-phase strategy, to which biotin was conjugated; the "biotin–dendrimer" conjugate was used to immobilize biotin on ELISA microwells, as it will be discussed below.

The anti-BAL antibodies were immunochemically evaluated in an ELISA system through titer and displacement curves, and as revealed, they could recognize both BAL and free biotin. These antibodies were able to detect biotin levels in a commercially available pharmaceutical preparation (Fig. 1), although at a different range of concentrations than those measured by streptavidin in a similar, ELISA-type assay (Fig. 2).

In the ELISA system developed, a biotin–"lysine dendrimer" ("biotin/dendrimer") conjugate was used for the immobilization of the vitamin on the microwells. The efficiency of the "biotin–dendrimer" as a means for immobilizing biotin was indirectly evaluated, through the ELISA-type assay in which streptavidin was used. The "lysine–dendrimer" was synthesized following the Fmoc-solid phase strategy and using Fmoc-Lys(N^{ε}-Fmoc)-OH and Fmoc-Lys(N^{ε}-Boc)-OH as building blocks, as described in the "Experimental." This dendrimer may be used for the immobilization of other hapten molecules on solid surfaces as well. Moreover, similar biotinylated "lysine–dendrimer" derivatives may prove to be useful in other areas of biotechnology, e.g., in signal amplification systems, and relevant research is currently underway.

The anti-BAL antibodies were evaluated, parallel with streptavidin, in immunocytology experiments in which HeLa cells were used. In these experiments, both the anti-BAL antibodies and streptavidin led to a clear staining of the cell cytoplasm, while no staining was observed in the cell nucleus (Fig. 3). The anti-BAL antibodies were also evaluated in Western blot experiments, in which HeLa and MDA-MB-231 cell lysates were analyzed. In these experiments, the anti-BAL antibodies detected the same main protein band with that detected by streptavidin, as shown by the corresponding Western blot patterns (Fig. 4).

Concluding, efficient polyclonal anti-biotin antibodies were produced in-house against a new synthetic derivative of biotin, i.e., BAL. BAL was synthesized easily, in high purity and with high yield, by following a solid-phase approach, instead of the conventional chemistry that is usually used to derivatize hapten molecules. In general, synthetic derivatives of the type "hapten–aminocaproic acid–lysine (or, another proper amino acid, e.g., cysteine)" may be used for the development of specific antibodies for the corresponding hapten.

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