

Role of N- or C-Terminal Biotinylation in Autoantibody Recognition of Citrullin Containing Filaggrin Epitope Peptides in Rheumatoid Arthritis

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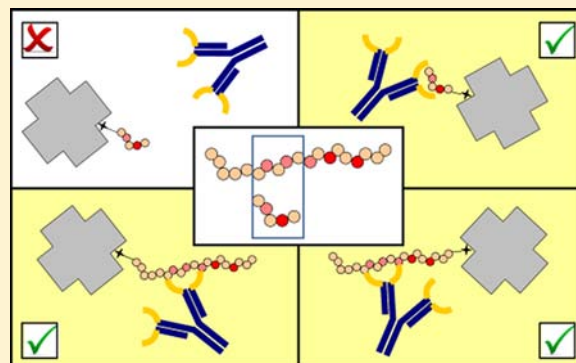
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Supporting Information

ABSTRACT: Here, we report on the synthesis, conformational analysis, and autoantibody binding properties of new sets of rheumatoid arthritis (RA) specific biotin–peptide conjugates derived from filaggrin epitope peptides. The biotin with or without a linker was attached to the Cit or Arg containing epitope core (³¹¹TXGRS³¹⁵) or epitope region (³⁰⁶SHQESTXGXSGRSGRSGS³²⁴) peptide (where X = Cit), through an amide bond at the N- or C-terminal of the epitopes. Antibody binding was detected by indirect enzyme-linked immunosorbent assay (ELISA) using sera from RA, Systemic lupus erythematosus (SLE) patients, as well as healthy individuals, and the secondary structure of conjugates was investigated by electronic circular dichroism (ECD). We found that autoantibodies from RA patients recognize specifically both filaggrin epitope region (³⁰⁶SHQESTXGXSGRSGRSGS³²⁴) and short epitope core (³¹¹TXGRS³¹⁵) peptides. Our data also indicate that the positioning of the biotin label within a peptide sequence can markedly influence the antibody binding, but the length of the linker incorporated has essentially no effect on the recognition. ECD experiments demonstrate that the Arg/Cit change does not influence the solution conformation of the peptide conjugates. However, the presence and position of the biotin moiety has a pronounced effect on the conformation of the 5-mer epitope core peptides, while it does not alter the secondary structure of the 19-mer epitope region peptides.



INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease affecting 0.5–1.0% of the population worldwide and is characterized by chronic inflammation of the synovial joints. The current therapeutic strategy uses increasingly aggressive regimens early in the course of the disease. Thus, diagnosis of RA at a very early stage is crucial.

Although the 1987 American College of Rheumatology (ACR) classification criteria for RA¹ are used in clinical practice as diagnostic tool for RA, they are not very well suited for the diagnosis of early RA, since at this time the clinical parameters are often not yet manifest. Therefore a specific and sensitive (serological) marker, which is presented very early in the disease, is still needed.

Various autoantibodies have been detected in sera from RA patients,² and were found to recognize citrullinated proteins or peptides: the so-called anticitrullinated protein/peptide antibodies (ACPA).³ In this report we are dealing with the filaggrin (filament-aggregating protein), which is one of the possible

targets of these antibodies. The possibility that citrulline (Cit) residues might be present in the epitopes was investigated by Schellekens.⁴ Several peptides were synthesized and an epitope region consisting of nineteen amino acids, ³⁰⁶SHQESTRGRSRGRSGRSGS³²⁴, was found, which contained five arginine residues that might be target of citrullination. Numerous analogues were synthesized substituting arginine residues into citrulline or glutamine, glutamic acid, alanine, and ornitin. The results suggested that not the absence of arginine, but the presence of citrulline specifically rendered the peptide autoantigenic.

The best binding was obtained by the peptide once-substituted analogue at the 312 position: ³⁰⁶SHQESTXGRSRGRSGRSGS³²⁴ (X = citrulline).⁴ The cyclic derivative of this peptide — SHQ(CESTXGRSRGRC)_{cyclo}SGRSGS — was introduced as

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target antigen first to detect antibodies by ELISA in the clinical practice.⁵

By systematic change of the arginine residues to citrulline an applying multipin ELISA approach with the RA patients' sera,⁶ we have identified an analogue, in which three Arg residues at positions 312, 314, 316 are replaced by Cit: ³⁰⁶SHQESTXGXSGRSGRSGS³²⁴. Furthermore, by N- and C-terminal shortening of the peptide sequence the minimum epitope peptide ³¹¹TXGRS³¹⁵ was determined.⁷

To detect the epitope binding we have designed a novel group of peptide conjugates with biotin. The biotin–avidin interaction, applied in ELISA to anchor the peptides to the plates, is one of the strongest noncovalent interactions known and it is stable toward a variety of harsh conditions.^{8,9} Biotin-labeled peptides are widely used for example in affinity purification,¹⁰ FRET-based flow cytometry,¹¹ solid-phase immunoassays,¹² and receptor localization.¹³

The biotin, as label, is most frequently attached directly to the N-terminal amino group,^{14–16} and in only a few cases to the C-terminal¹⁷ of the B-cell epitope peptides. According to our knowledge no systematic studies were reported on the effect of the position of biotin on the antibody recognition of a peptide epitope. The distance between the epitope binding site and the biotin could also influence the interaction between the partners. To avoid this several linker types were developed and were utilized in the biotinylation methods: aminohexanoic acid (biotin LC, aminocaproic acid),^{18–20} tetrapeptide–SGSG,²¹ or β -alanine²² was incorporated. Interestingly, essentially no data are available on the analysis of the effect of spacer moiety between biotin and B-cell epitope on antibody binding properties.

The aim of the present work was to answer interesting and very important questions: how can the position of the biotin influence the antibody recognition and how far should the biotin have been located from the minimum flaggrin epitope. We also examined if the secondary structure is influenced by the biotin's position. Based on our preliminary results⁷ we have synthesized a new set of N- and C-terminal biotin conjugates of peptides with Arg or Cit using various linkers between the epitope core and the biotin (Figure 1) to test the availability of the epitope as well as the specificity of antibody binding when the peptide conjugates are coupled to neutravidin coated plates. We were also interested in

clarifying the role of the secondary structure in antibody binding by ECD.

Here we report on our findings that autoantibodies derived from RA patients recognize specifically both flaggrin epitope region (³⁰⁶SHQESTXGXSGRSGRSGS³²⁴) and short epitope core (³¹¹TXGRS³¹⁵) peptides (where X = Cit). Our data indicate that the positioning of the biotin label within a peptide sequence can markedly influence the antibody binding, but the length of the linker has essentially no effect on the recognition. Based on ECD experiments we demonstrate that the Arg/Cit change does not influence the solution conformation of the peptide analogues. However, the presence and position of the biotin moiety has a pronounced effect on the conformation of the 5-mer epitope core peptides, while it does not alter the secondary structure of the 19-mer epitope region peptides.

EXPERIMENTAL PROCEDURES

Materials. All amino acid derivatives and resins (MBHA, Rink Amide MBHA) and coupling agents (*N,N'*-diisopropylcarbodiimide (DIPCI), *N*-diisopropyl-ethylamine (DIEA)), and scavengers (triisopropylsilan (TIS)) were purchased from IRIS Biotech GmbH (Marktredwitz, Germany) or Reanal (Budapest, Hungary), (benzotriazole-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from NovaBiochem (Läufelfingen, Switzerland). Scavenger, coupling agents, and cleavage reagents (*p*-cresol, 1-hydroxybenzotriazole (HOBt), piperidine, trifluoroacetic acid (TFA), and hydrogen fluoride (HF)) were Fluka products (Buchs, Switzerland). Solvents for synthesis (dichloromethane (DCM), *N,N'*-dimethylformamide (DMF), and diethylether) were purchased from Molar Chemicals (Budapest, Hungary). Dimethylsulfoxide (DMSO) was from Sigma-Aldrich Kft (Budapest, Hungary) and HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were from Merck Kft (Budapest, Hungary). Biotin and biotinyl-6-aminohexanoic acid were Fluka products (Buchs, Switzerland) and the 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid (Ttds) was synthesized by us as described before.^{23,24} Rabbit anti human IgG HRPO conjugate was purchased from Dako Agilent Technologies Company (Glostrup, Denmark).

Synthesis of Free Peptides and N-Terminal Biotinylated Peptide Conjugates. The nonbiotinylated and the N-terminal biotinylated 5-mer and 19-mer derivatives listed in Table 1 were built up on Rink Amide-4-methylbenzhydrylamine (Rink Amide-MBHA) resin (0.56 mmol/g coupling capacity) using Fmoc/^tBu chemistry. Amino acids were coupled as Fmoc derivatives by the DIC/HOBt coupling method using a 3 molar excess over the resin capacity in DMF. The side chain of Glu residue was blocked with a *tert*-butyl ester protecting group and of His and Gln residues were blocked with trityl group. *tert*-Butyl ether group was applied for the protection of the OH function of Ser and Thr side chains, while the *tert*-butyloxycarbonyl group was used for blocking the ϵ -amino group of Lys residue and pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) group for the guanidino function of Arg. The N-terminal Fmoc group was removed by treatment of the resin-bound peptide with piperidine/DMF (30:70, v/v) mixture for 3 + 17 min at room temperature. The success of the coupling was controlled by ninhydrin reaction.²⁵ After removal of the last Fmoc group, the N-terminus was acetylated by acetic anhydride and DIEA (5:5 equiv) or biotinylated (peptide:biotin derivative:HOBt:PyBOP:DIEA = 1:3:3:3:10) in DMF/DMSO (25:75, v/v). After washing, the peptide-resin was dried in a desiccator. The peptides were cleaved from the resin with a mixture of TFA-

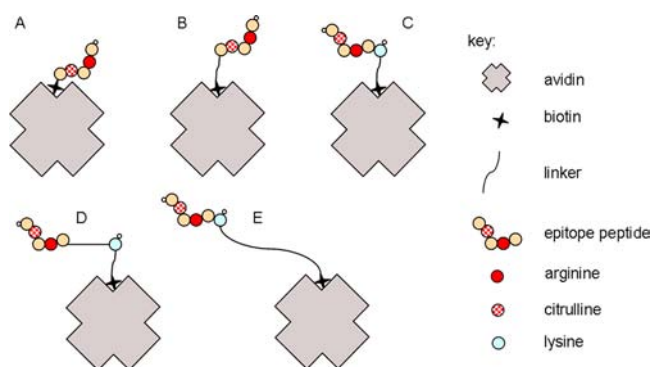


Figure 1. Schematic presentation of the N- and C-terminal biotinylated forms. (A) N-terminal biotin conjugated peptide, (B) N-terminal biotinyl-6-aminohexanoic acid conjugated peptide, (C) C-terminal biotinyl-6-aminohexanoic acid conjugated peptide, (D) C-terminal biotinyl-6-aminohexanoic acid conjugated peptide using 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid (Ttds) linker, (E) C-terminal biotinyl-Ttds conjugated peptide.

Table 1. Characteristics of the Conjugates and Free Peptides^a

code	sequence ^d	RP-HPLC ^b	ESI-MS ^c	
		R _t (min)	M _{av} calc.	M _{av} exp.
p1	Ac-TRGRS-NH ₂	13.1	616.7	616.5
p2	Ac-TXGRS-NH ₂	13.1	617.7	617.5
p3	Biotinyl-TRGRS-NH ₂	19.1	802.9	802.8
p4	Biotinyl-TXGRS-NH ₂	18.3	803.9	803.7
p5	BiotLC-TRGRS-NH ₂	16.2	914.1	914.0
p6	BiotLC-TXGRS-NH ₂	16.5	915.1	915.0
p7	Ac-TRGRSK(BiotLC)-NH ₂	17.1	1085.3	1085.6
p8	Ac-TXGRSK(BiotLC)-NH ₂	17.1	1086.3	1086.6
p9	Ac-TRGRSK(Biotinyl-Ttds)-NH ₂	18.0	1274.1	1274.2
p10	Ac-TXGRSK(Biotinyl-Ttds)-NH ₂	17.6	1275.1	1275.2
p11	Ac-TRGRS-Ttds-K(BiotLC)-NH ₂	18.4	1387.3	1387.4
p12	Ac-TXGRS-Ttds-K(BiotLC)-NH ₂	18.5	1388.3	1388.4
p13	Ac-SHQESTRGRSRGRSGSGS-NH ₂	15.6	2086.2	2086.2
p14	Ac-SHQESTXGXSGRSGRSGS-NH ₂	15.6	2089.2	2089.3
p15	BiotLC-SHQESTRGRSRGRSGSGS-NH ₂	13.4	2383.6	2383.8
p16	BiotLC-SHQESTXGXSGRSGRSGS-NH ₂	13.1	2386.6	2386.7
p17	Ac-SHQESTRGRSRGRSGSGSK(BiotLC)-NH ₂	17.8	2554.8	2554.8
p18	Ac-SHQESTXGXSGRSGRSGSK(BiotLC)-NH ₂	17.9	2557.8	2557.9
p19	Ac-SHQESTRGRSRGRSGSGSK(BiotLC-Ttds)-NH ₂	18.8	2857.4	2857.5
p20	Ac-SHQESTXGXSGRSGRSGSK(BiotLC-Ttds)-NH ₂	19.1	2860.4	2860.5
p21	Ac-SHQESTRGRSRGRSGSGS-Ttds-K(BiotLC)-NH ₂	20.0	2857.4	2857.5
p22	Ac-SHQESTXGXSGRSGRSGS-Ttds-K(BiotLC)-NH ₂	20.0	2860.4	2860.5

^aRP-HPLC chromatograms and mass spectra are shown in the Supporting Information (S1–S24). ^bColumn: Phenomenex Jupiter C18 (250 mm × 4.6 mm) with 5 μm silica (300 Å pore size); gradient: 0 min 0% B; 5 min 0% B; 50 min 90% B; eluents 0.1% TFA in water (A) and 0.1% TFA in acetonitrile–water (80:20, v/v) (B); flow rate: 1 mL/min; detection: λ = 220 nm. ^cBruker Daltonics Esquire 3000+ ion trap mass spectrometer. ^dX = citrulline, standard one-letter code is used for the other amino acid residues; Ac = acetyl group; BiotLC = Biotinyl-6-aminohexanoic acid, Ttds = 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid.

water-TIS (95:2.5:2.5, v/v/v) at room temperature for 2 h. The crude products were purified by semipreparative RP-HPLC and the purified compounds were characterized by analytical RP-HPLC and ESI-ion trap mass spectrometry. The yields were 50% to the resin capacity.

Synthesis of C-Terminal Biotinylated Peptide Conjugates. The C-terminal biotinylated 5-mer and 19-mer conjugates listed in Table 1 were built up on 4-methylbenzhydrylamine (MBHA) resin (0.7 mmol/g coupling capacity) using Fmoc/^tBu chemistry. Amino acids were coupled as described above. An additional Lys (using Fmoc-Lys(Boc)-OH) was built into the C-terminus of the peptide to conjugate easily the biotin derivatives to its ε-amino group. After removal of the Boc protecting group from the side chain the ε-NH₂ group of Lys was biotinylated (peptide:biotin derivative:HOBt:PyBOP:DIEA = 1:3:3:3:10) in DMF/DMSO (25:75, v/v). After washing, the remaining part of the sequence was built up by Fmoc strategy. The Fmoc group was removed by treatment of the resin-bound peptide with piperidine/DMF (30:70, v/v) mixture for 3 + 17 min. The success of the coupling was controlled by ninhydrin reaction.²⁵ After removal of the last Fmoc group, the N-terminus was acetylated by acetic anhydride and DIEA (5:5 equiv) and after washing with DMF, methanol, and diethyl ether, the peptide-resin was dried in a desiccator. The peptides were cleaved from the resin with anhydrous HF in the presence of *p*-cresol (HF-*p*-cresol 5 mL: 0.2 g) at room temperature. The crude products were purified by semipreparative RP-HPLC and the purified compounds were characterized by analytical RP-HPLC and mass spectrometry (Table 1). The yields were 40% to the resin capacity.

Synthesis of C-Terminal Ttds-Containing and Biotinylated Peptide Conjugates. The C-terminal biotinylated and Ttds-containing 5-mer and 19-mer conjugates listed in Table 1 were synthesized as described above, but with an additional Lys residue (using Boc-Lys(Fmoc)-OH) on the C-terminus of the peptide. After removal of the Fmoc protecting group from the ε-NH₂ function of Lys, Ttds linker was coupled as Fmoc-Ttds form by the DIC/HOBt coupling method using a 4 molar excess over the resin capacity in DMF. After washing, the Fmoc protecting group was cleaved from the Ttds residue and biotin derivatives were coupled (peptide:biotin derivative:HOBt:PyBOP:DIEA = 1:3:3:3:10) in DMF/DMSO (25:75, v/v). After washing and removing of the Boc protecting group by TFA, the remaining part of the sequence was built up by Fmoc strategy. The Fmoc group was removed by treatment of the resin-bound peptide with piperidine/DMF (30:70, v/v) mixture for 3 + 17 min. The success of the coupling was controlled by ninhydrin reaction.²⁵ After removal of the last Fmoc group, the N-terminus was acetylated by acetic anhydride and DIEA (5:5 equiv), and after washing, the peptide-resin was dried in a desiccator. The peptides were cleaved from the resin with anhydrous HF in the presence of *p*-cresol (HF-*p*-cresol 5 mL: 0.2 g). The crude products were purified by semipreparative RP-HPLC and the purified compounds were characterized by analytical RP-HPLC and mass spectrometry. The yields were 35% to the resin capacity.

High-Performance Liquid Chromatography (HPLC). Analytical RP-HPLC was performed on a Knauer (H. Knauer, Bad Homburg, Germany) system using a Phenomenex Jupiter C18 column (250 mm × 4.6 mm) with 5 μm silica (300 Å pore size) (Torrance, CA) as a stationary phase. Linear gradient

elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20, v/v)) was used at a flow rate of 1 mL/min. Peaks were detected at $\lambda = 220$ nm. The crude products were purified on a semipreparative Vydac 218 TP C18 column (250 mm \times 10 mm) with 10 μ m silica (300 Å pore size). The flow rate was 4 mL/min. The same eluents with a linear gradient of 20–60% B in 40 min were applied.

Electrospray Ionization Mass Spectrometry (ESI-MS).

Electrospray ionization mass spectrometry was performed with a Bruker Daltonics Esquire 3000+ mass spectrometer (Bremen, Germany), operating in continuous sample injection mode at 4 μ L/min flow rate. Samples were dissolved either in a mixture of acetonitrile–water (1:1, v/v) or 50% MeOH, 1% AcOH in water. Mass spectra were recorded in positive ion mode in the m/z 50–3000 range.

Electronic Circular Dichroism (ECD). ECD spectra were recorded using Jasco 810 polarimeter (Jasco Corporation, Japan) in quartz cell with an optical length of 0.02 cm at room temperature, under constant nitrogen flush. The polarimeter was calibrated with ammonium α -10-camphor-sulfonate. ECD spectra were measured in the region $\lambda = 180$ –300 nm. The samples were dissolved at concentrations of 0.5 mg/mL in distilled water or in trifluoroethanol (TFE) (Aldrich, NMR grade). ECD band intensities are expressed in molar ellipticity $([\theta]_{MR})$ in deg cm²/dmol where MR = mean residue).

Indirect Enzyme-Linked Immunosorbent Assay (ELISA). For binding of biotinylated peptides, NeutrAvidin (5 μ g/mL in phosphate buffered saline (PBS)) coated plates were used. After adding biotinylated peptides (1 μ g/mL in PBS), wells were blocked by 40 mM Tris-HCl, 150 mM NaCl, 0.1% Tween, 0.5% BSA in deionized water. After washing 3 times with 0.5% Tween/PBS, sera samples were added (1:100 dilution) for an hour at 37 °C. After washing and incubation with rabbit antihuman IgG (1:2000 dilution), conjugated to horse radish peroxidase, for an hour at 37 °C, signal was developed by tetramethyl benzidine (10 mg/mL, diluted in 0.1 M acetate buffer pH 5.5 adding 30% H₂O₂) for 5 min at room temperature. The reaction was stopped by 4 M H₂SO₄. Absorbance was measured at 450 nm (correction wavelength set at $\lambda = 620$ nm) using a Labsystems Multiskan MS spectrophotometer. Optical density (OD) value was defined as the absorbance of each individual well, after subtracting the background. Then OD ratios were calculated from the means of two parallel OD values as follows: the OD value of the citrulline containing peptide divided by the OD value of the arginine containing analogue. Cutoff values for two peptide pairs were calculated from OD ratios of healthy samples (mean \pm 2 SD (standard deviation)). Specificity was calculated as the percentages of healthy samples having an OD ratio below the cutoff value as compared to total number of samples; while sensitivity was calculated as the percentage of RA patient samples giving an OD ratio higher than the cutoff value, as compared to the total number of patient samples. For analyzing the data one-way ANOVA test (in case of normal distribution) and Kruskal–Wallis test (in other cases) was used for comparison.

Patients. Sera samples from 16 age-matched (63 \pm 18 years) RA, 16 CCP (citrulline containing protein/peptide) negative, non-RA patient with other autoimmune disease (Systemic lupus erythematosus, SLE), and 16 age-matched (57 \pm 14 years) healthy individuals as controls were collected with ethical permission. The diagnosis of the disease was established on the basis of the revised diagnostic criteria for classification of

rheumatoid arthritis, suggested in 1987 by the American Association for Rheumatism. All sera were previously tested for anti CCP positivity by Immunoscan CCPlus kit (Eurodiagnostica)² according to the manufacturer's instructions. Samples with results <25 U/mL are defined as negative; samples \geq 25 U/mL are defined as positive.

RESULTS

In order to understand the effect of biotinylation of a B-cell epitope on the binding of polyclonal autoantibodies from RA patients' sera, first we have prepared two sets of N- and C-terminal biotinylated peptides. In these conjugates biotin was linked at the N- or at the C-terminal part of either a pentapeptide epitope core (³¹¹TXGRS³¹⁵) or a 19-mer epitope region peptide (³⁰⁶SHQESTXGXSGRSGRSGS³²⁴) without or with two types of spacer moieties (Figure 1). After appropriate characterization by RP-HPLC and MS analysis we have performed ECD studies to obtain information on the solution conformation of the above peptide conjugates in comparison with those of Arg containing 5-mer and 19-mer counterparts in aqueous solution, as well as in ordered structure promoting TFE. The antibody binding properties of these groups of biotin peptide conjugates were studied by using sera from three groups of individuals. Namely, serum samples from healthy individuals, from RA patients, and also from SLE patients were comparatively analyzed.

Synthesis of the Conjugates. In both groups of biotinylated conjugates the N- or C-terminal of the peptides were coupled with biotin using two types of spacers between the partners (Figure 2). We have utilized the long chain biotin in

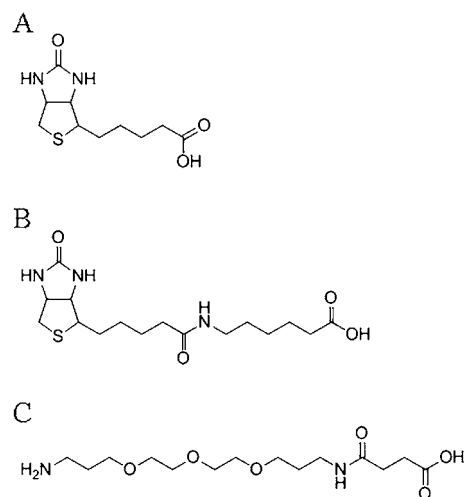


Figure 2. Biotin derivatives and linker: (A) N-(+)-biotin, (B) N-(+)-biotinyl-6-aminohexanoic acid = BiotLC, (C) 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid = Ttds.

which biotin is extended by aminohexanoic acid (biotinyl-6-aminohexanoic acid) (Figure 1B and C). In conjugates with C-terminal biotin a Lys residue was introduced to the 5-mer or 19-mer peptide sequence and its ϵ -amino group was acylated with biotin or biotin derivative (Figure 1C–E). In addition, in conjugates with C-terminal biotin we have incorporated an oligoethyleneglycol derivative spacer developed by us^{23,24} based on 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid (Ttds). This moiety was positioned either between the epitope and the biotinylated Lys residue (Figure 1D) or between the Lys residue and the biotin (Figure 1E). Arginine containing analogues of

both groups of peptides ($^{306}\text{SHQESTRGRS}\text{GRSGS}^{324}$ and $^{311}\text{TRGRS}^{315}$) were also synthesized and used as controls for the conformation as well as antibody binding studies.

All peptides were synthesized by solid phase methodology using Fmoc-chemistry. The *N*-terminal biotinylation of the peptides were carried out via amide bond formation. In case of the C-terminal biotinylated conjugates a Lys was attached to the peptide and the biotin derivatives was coupled to the ϵNH_2 group. In that case the *N*-terminals of the analogues were acetylated. It was a surprise that the biotinylation step took at least 1 day, and in the case of the C-terminal biotinylated derivatives repetition of the conjugation was needed. There can be a sterical reason for this difficulty because the ϵNH_2 group of the lysine is not far enough away from the resin.

All bioconjugates were purified by semipreparative RP-HPLC and the purified compounds were characterized by analytical RP-HPLC and ESI mass spectrometry (Table 1 and Supporting Information, Figures S1–S22). The purity of the compounds was over 95% and all compounds were obtained with 30–50% yield.

Recognition of the *N*- and C-Terminally Biotinylated Peptides by RA Sera. The *N*-terminally biotinylated core epitope (p4) showed no binding to the patients' sera and the insertion of aminohexanoic acid linker between the biotin and the peptide (p6) could not increase the antibody recognition. In contrast, all the C-terminal biotinylated core peptides (p8, p10, p12) were recognized by RA sera that were also positive in the most widely used diagnostic test, detecting anticitrullinated peptide antibodies (anti-CCP2). Interestingly even with the increase of the length of the linker between the peptide and biotin, the strength of the binding decreases but the difference is not significant (by one-way ANOVA). The control healthy sera showed no reactivity to any of the peptides (Figure 3 and Table 2).

In case of the epitope region peptide ($^{306}\text{SHQESTXGXSGRSGS}^{324}$) of filaggrin the *N*-terminal biotinylated

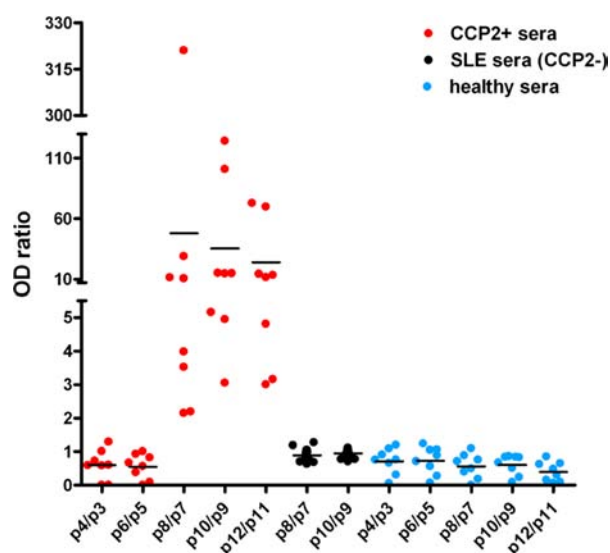


Figure 3. Binding of antibodies from RA sera to peptides containing the minimum epitope ($^{311}\text{TXGRS}^{315}$) of filaggrin as detected by indirect ELISA. The peptides were biotinylated either at the *N* terminal (p3–p6) or the C terminal (p7–p12) position. The red drops represent the OD ratio of the peptides tested with CCP2 positive sera. Sera from healthy subjects were used as control (blue drops) and SLE sera were used as disease control (black drops).

Table 2. Interpretation of the OD Ratio Values of the Conjugates

peptide pairs	mean OD ratio			<i>p</i> -value
	RA sera (CCP2+)	SLE sera (CCP2-)	healthy sera	
p4/p3	0.59	n.d.	0.72	-
p6/p5	0.56	n.d.	0.73	-
p8/p7	47.91	0.89	0.56	$p < 0.001^a$
p10/p9	35.21	0.95	0.61	$p < 0.001^a$
p12/p11	23.98	n.d.	0.40	$p < 0.001^a$
p16/p15	3.55	0.52	0.92	$p < 0.05^b$
p18/p17	2.26	0.41	0.64	$p < 0.05^b$
p20/p19	2.08	0.43	0.59	$p < 0.05^b$
p22/p21	2.37	0.42	0.72	$p < 0.05^b$

^aComparison of the C-terminal biotinylated 5-mer peptides and the *N*-terminal biotinylated conjugates using one-way ANOVA test (in case of normal distribution) and Kruskal–Wallis test (in other cases). ^bThe data from the RA patients were compared with the data from the healthy controls analyzing by one-way ANOVA test (in case of normal distribution) and by Kruskal–Wallis test (in other cases).

compound (p16) and the C-terminal biotinylated molecule (p18) exhibited the same binding profile with the CCP2 positive sera. The distance between the peptide and the biotin (p18, p20, p22) has no marked influence (by one-way ANOVA) on the recognition (Figure 4 and Table 2). The control sera (healthy

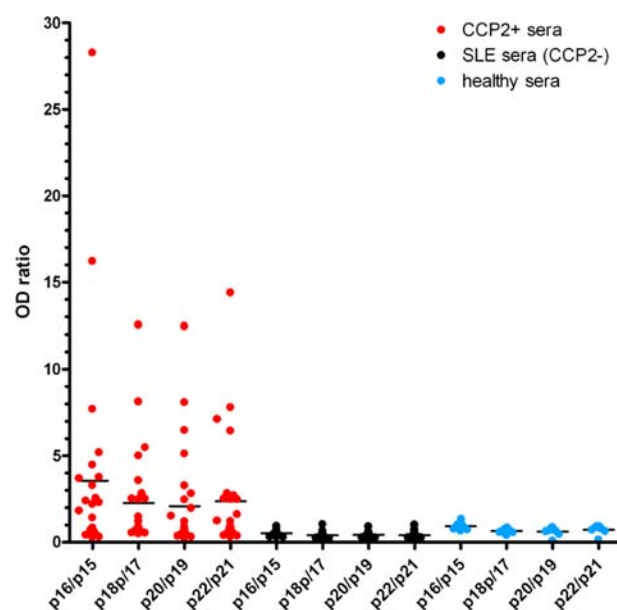


Figure 4. Binding of anticitrullinated peptide antibodies (ACPA) to peptides containing the epitope region ($^{306}\text{SHQESTXGXSGRSGS}^{324}$) of the filaggrin. The red drops represent the OD ratio of the peptides tested with the CCP2 positive sera. Sera from healthy subjects were used as control (blue drops) and SLE sera were used as disease control (black drops).

subjects and SLE patients) showed no reactivity to any of the peptides, the differences between the RA and control sera are significant ($p < 0.05$ by one-way ANOVA).

To evaluate the specificity of the antibody recognition toward RA individuals, sera from patients with systemic lupus erythematosus (SLE), a different autoimmune disease, and serum samples of healthy subjects were also comparatively examined. These sera from SLE patients did not show any

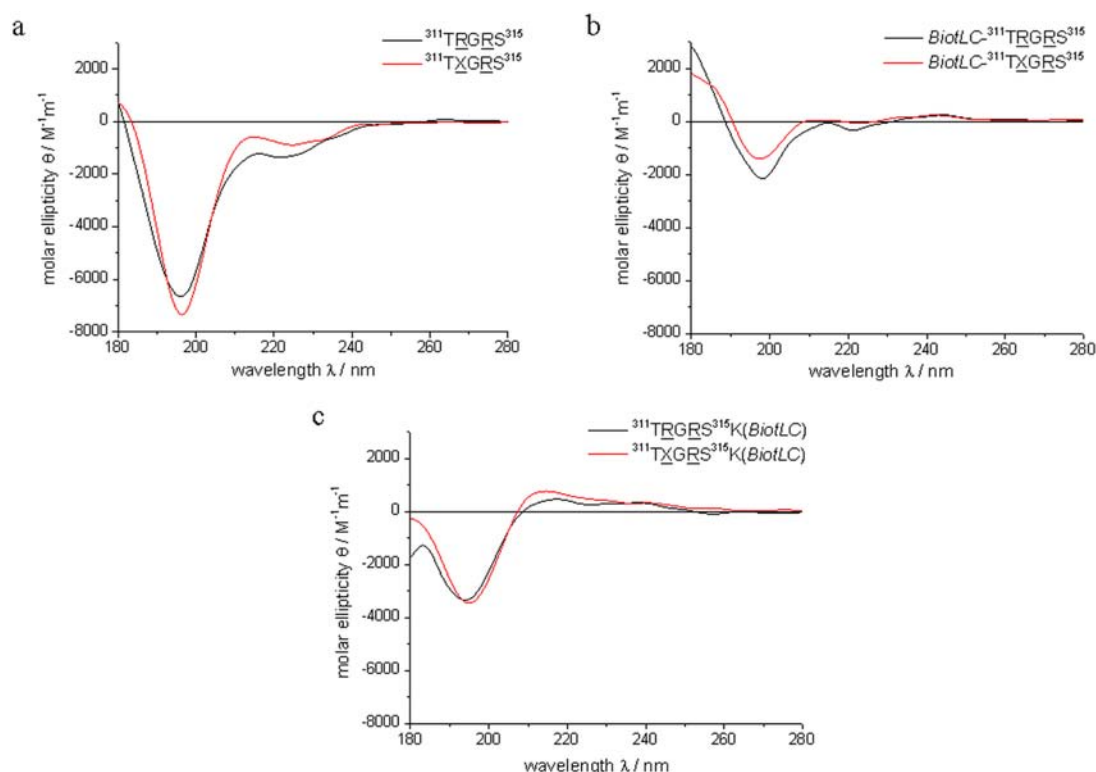


Figure 5. ECD spectra of the $^{311}TXGRS^{315}$ epitope core peptide and its biotin conjugates in aqueous solution. (a) ECD spectra of the biotin free peptides (1, 2), (b) ECD spectra of the N-terminal biotinylated conjugates (5, 6), (c) ECD spectra of the C-terminal biotinylated conjugates (7, 8).

binding to the target antigens, indicating that the recognition of both the C-terminally biotinylated short epitope peptide and the N- or C-terminally biotinylated long epitope region peptide is specific for RA.

In order to investigate the specificity and sensitivity and correlation of our results with data obtained by the standard (clinically) used methods, we have determined the relevant values in case of two peptide-conjugate (p10, p18). The results are the following: For the core epitope peptide (p10) cutoff value was 1.352, the specificity was 98%, and the sensitivity was 51%. In case of the epitope region peptide (p18) the cutoff value was 1.253, the specificity was 98%, and the sensitivity was 51%.

To test that our results are correlated to the standard or clinically used methods,²⁶ we have calculated Spearman correlation between the strength of binding (OD ratio) and the anti-CCP2 titers. The recognition of peptides and the anti-CCP2 titer has shown a weak, but significant correlation. For the core epitope peptide (p10) the following values were obtained: $r = 0.3379$ and $p < 0.0001$. For the epitope region peptide (p18) we found $r = 0.2339$ and $p = 0.0025$ values. Stronger and significant correlations were detected ($p < 0.0001$ in both cases with the MCV (mutated citrullinated vimentin) titers, for the core epitope peptide (p10) $r = 0.4562$, and for the epitope region peptide (p18) $r = 0.3531$.

Collection of data covering peptide conjugates obtained with >100 serum samples are in progress and the results will be published fully as a separate contribution in an assay development oriented journal.

Electronic Circular Dichroism (ECD). ECD spectroscopy is a useful tool for monitoring the secondary structures of peptides and proteins. The ECD spectrum of periodic ordered (e.g., different helices, β -sheets), aperiodic ordered (different type of turns) structures, as well as the unordered structures, are well

documented. ECD curve of an α -helix has a weak but broad negative band at $\lambda = 222$ nm assigned with the $n\pi^*$ transition and an exciton couplet ($\pi\pi^*$) localized at $\lambda = 208$ nm (negative band) and at $\lambda = 192$ nm (very intense positive band).^{27,28} ECD spectrum of β -sheet consists of a negative band near $\lambda = 215$ nm ($n\pi^*$ transition) and a positive band near $\lambda = 198$ nm ($\pi\pi^*$ transition).

Different turn-types have characteristic chiral contributions and their detection and discrimination is also possible by ECD spectroscopy. Among the three subgroups of turns, the contributions of β -turns to the circular dichroism has been studied in detail.²⁹ The spectrum of a type I or III β -turn resemble the ECD spectrum of α -helix where the band intensities are weaker, the $\pi\pi^*$ bands are blue-shifted, and the $n\pi^*$ bands are shifted to the longer wavelength region (C-like spectrum).³⁰

Type II β -turns have similarity to the β -sheet spectrum but the bands are red-shifted by 5–10 nm. The γ -turn also represents a main type of folded secondary structures, an inverse γ -turn shows a negative $n\pi^*$ band near $\lambda = 230$ nm, and a $\pi\pi^*$ a positive band near $\lambda = 190$ nm.³¹ It should be noted that ECD spectroscopy itself cannot distinguish between an inverse γ -turn and a type II β -turn.

ECD spectrum with an intense negative band around $\lambda = 200$ nm and with a weak negative shoulder $\lambda \sim 230$ nm can be interpreted as a random coil (class U-spectrum). The same spectral features but with a weak positive shoulder can be identified as polyproline type II helix (PPII) structure.^{32,33}

ECD of biotin-modified protein/peptide can be characterized by an extra positive band at $\lambda \sim 240$ nm if the biotin is interacting with the amino acid residue noncovalently.³⁴

In solution the conformation of the biotinyl-6-aminohexanoic containing derivatives and the nonbiotinylated peptides were studied by ECD spectroscopy. Spectra were recorded in water

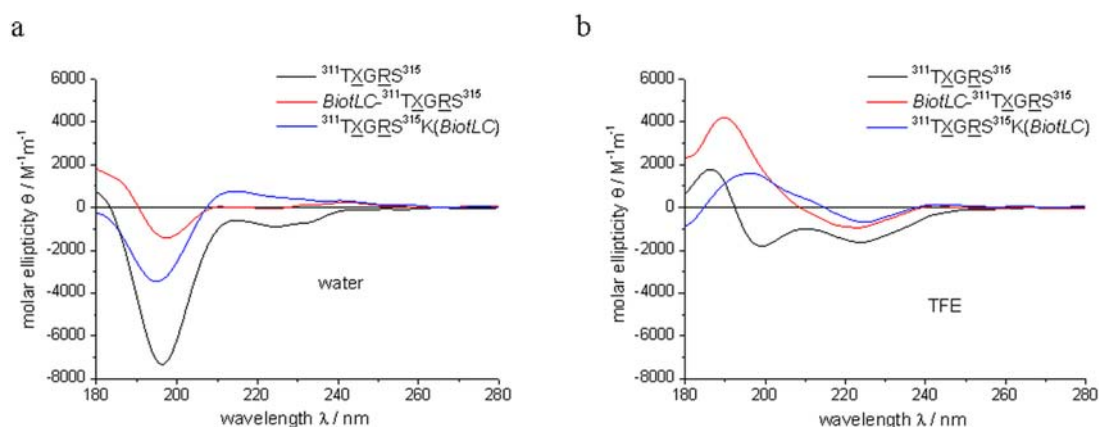


Figure 6. ECD spectra of the $^{311}\text{TXGRS}^{315}$ flaggrin epitope core and its biotin conjugates (a) in water and (b) in trifluoroethanol.

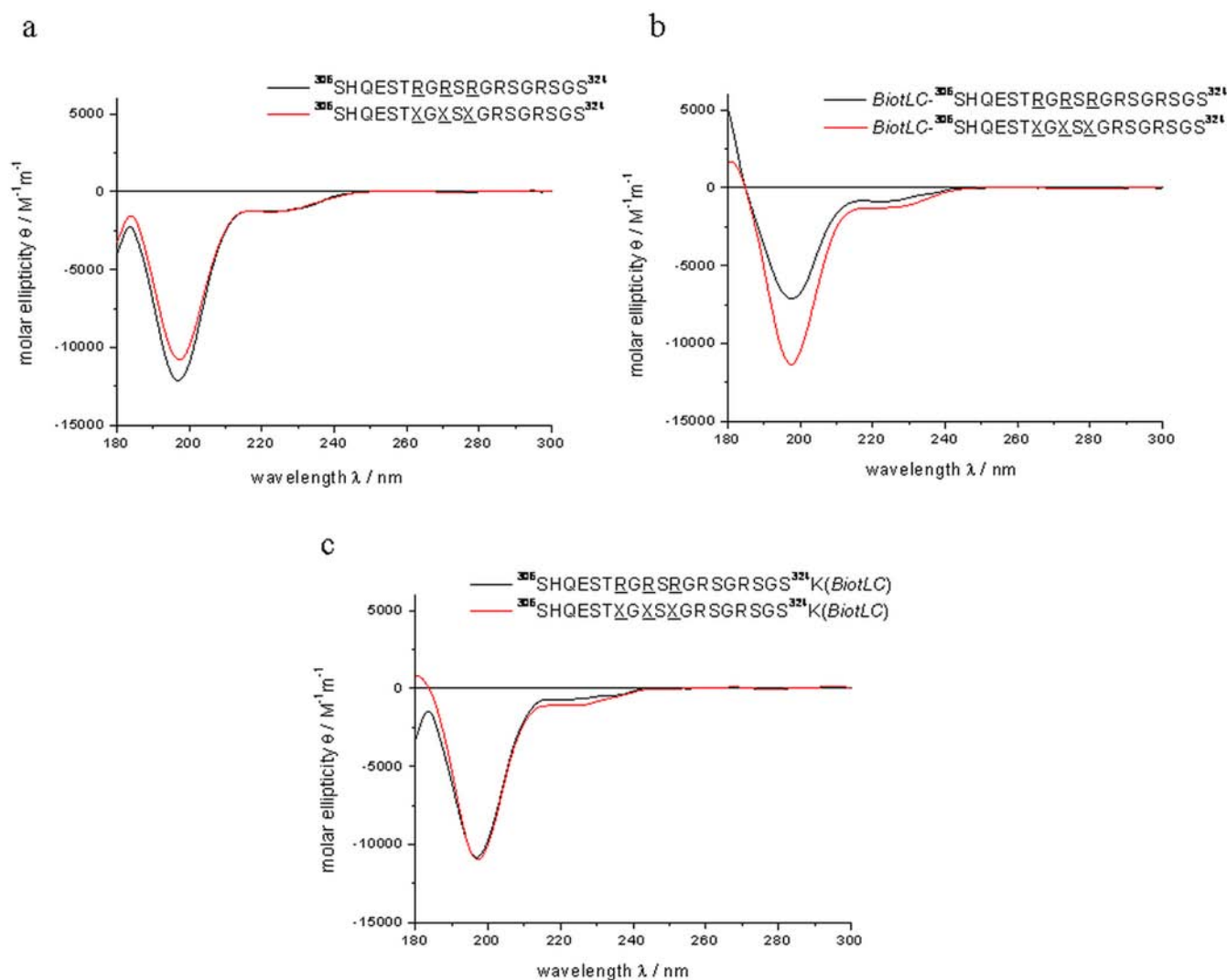


Figure 7. ECD spectra of the $^{306}\text{SHQESTXGXSGRSGRSGS}^{324}$ flaggrin epitope region peptide in water. (a) ECD spectra of the biotin free peptides (13, 14), (b) ECD spectra of the N-terminal biotinylated derivatives (15, 16), (c) ECD spectra of the C-terminal biotinylated derivatives (17, 18).

and in TFE, which is considered as ordered structure promoting solvent.³⁵

Considering that in ELISA experiments antibody binding occurs in aqueous medium, first we compared the solution conformations of the citrulline and arginine containing analogue pairs with or without biotin in water (Figures 5 and 7).

The ECD spectra of pentapeptides representing the epitope core without biotin ($^{311}\text{TXGRS}^{315}$ vs $^{311}\text{TRGRS}^{315}$) indicate the presence of conformer-mixture composed of mainly unstructured conformers (Figure 5a) exhibiting at $\lambda \sim 222$ nm a weak and near $\lambda \sim 196$ nm a strong negative band. The spectra of the N-terminal biotin conjugates ($\text{BiotLC-}^{311}\text{TXGRS}^{315}$ vs Bi-

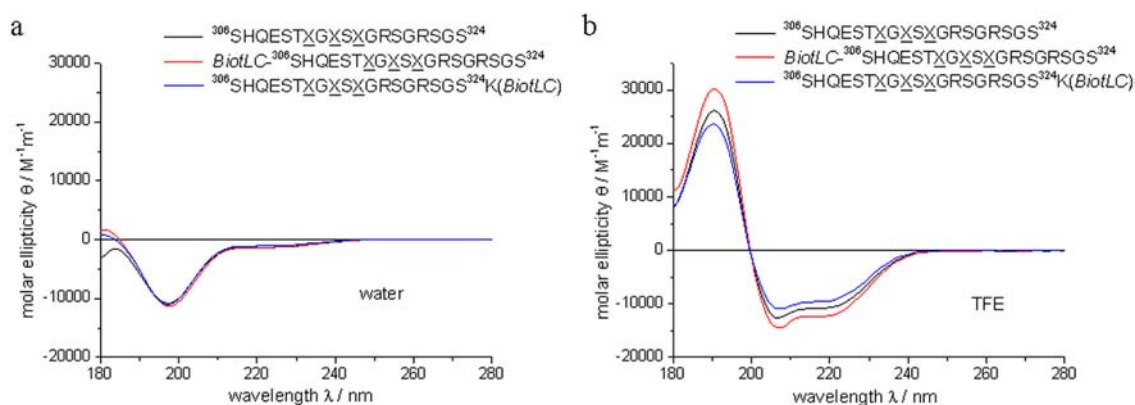


Figure 8. ECD spectra of the $^{306}\text{SHQESTXGXSGRSGRS}^{324}$ flaggrin epitope region and its biotin conjugates (a) in water and (b) in TFE.

$^{311}\text{TXGRS}^{315}$) are characterized by two negative bands, a weaker at $\lambda \sim 222$ nm and a stronger one near $\lambda \sim 198$ nm, as well as with a weak positive band at $\lambda \sim 243$ nm and intense positive ECD values at $\lambda \sim 180$ nm. These ECD curves represent higher ordered structures: in the conformational mixture a significant amount of β turns (mainly β I–III turn) nearby the extended conformers appeared (Figure 5b). The presence of a positive band at $\lambda \sim 243$ nm is indicative of the biotin moiety.³⁴ The spectra of the C-terminal biotinylated epitope peptides ($^{311}\text{TXGRS}^{315}\text{K}(\text{BiotLC})$ vs $^{311}\text{TRGRS}^{315}\text{K}(\text{BiotLC})$) are characteristic of PPII type helix and/or unstructured conformation (Figure 5c). There are two broad positive bands at $\lambda \sim 239$ and 215 nm and a strong negative band at $\lambda \sim 195$ nm, where the band $\lambda \sim 239$ nm as the biotin's band could also be recognized.

In comparison of spectral patterns of the three pentapeptide-pairs, the similar shapes indicate that the arginine/citrulline change in a given pair does not significantly influence their conformation in water. However, small but marked differences in the spectral characteristics (position and intensities of the positive bands) between the N- and C-terminally biotinylated peptides could be well documented.

Next we have compared the ECD spectra of the above compounds in TFE and observed essentially no differences in spectral features between the Arg- and Cit-containing analogues (data not shown). The spectra of citrulline containing free and biotin conjugated pentapeptides in TFE are presented in Figure 6. The ECD spectrum of biotin free epitope core peptide ($^{311}\text{TXGRS}^{315}$) showed predominantly β I–III turn conformation (Figure 6b, black color) with characteristic C-type spectrum:³⁶ a weak, blue-shifted helix spectrum with a positive band at $\lambda = 186$ nm and two negative bands at $\lambda = 224$ nm and at $\lambda = 199$ nm. The spectrum of the N-terminal biotinylated derivative ($\text{BiotLC-}^{311}\text{TXGRS}^{315}$) (Figure 6b, red color) may represent significant amount of β II turn conformers: there is a strong positive band at $\lambda = 190$ nm and a negative band near $\lambda = 223$ nm and appeared at $\lambda = 243$ nm the biotin's positive band. The spectrum of the C-terminal biotinylated pentapeptide ($^{311}\text{TXGRS}^{315}\text{K}(\text{BiotLC})$) represents a mixed set of conformers composed of β II turn (or γ turn) and unstructured conformation (Figure 6b, blue color) with two positive bands at $\lambda = 243$ nm (a broad, very weak) and at $\lambda = 196$ nm (stronger one) and a negative band at $\lambda = 225$ nm. The biotin's band at $\lambda = 243$ nm could be also recognized. As in the case of spectra recorded in water, we found marked differences in the spectral pattern (position and intensity of the bands) between the N- and C-terminally biotinylated peptides.

The ECD spectra of the free and biotin conjugated epitope core peptides ($^{311}\text{TXGRS}^{315}$) recorded in water and also in TFE (Figure 6a,b) suggest that conformations of the compounds are very dependent on the presence as well as position of the biotin-moiety in both solvents.

Next we have studied and compared the ECD spectra of the peptide-pairs covering the epitope region of flaggrin ($^{306}\text{SHQESTXGXSGRSGRS}^{324}$) in water and also in TFE. It is worth mentioning that the $^{311}\text{TXGRS}^{315}$ epitope core peptide studied above comprises this 19-mer peptide.

In water we found minor differences (stronger negative band near $\lambda \sim 197$ nm) between the 19-mer peptide with Arg residues as compared to the one containing Cit at positions 312, 314, and 316 (Figure 7a). Similarly the two peptides conjugated with biotin at their C-terminal displayed almost the same kind of spectra with some alteration (near $\lambda \sim 220$ nm) (Figure 7c). We detected differences only in the intensities in the case of the N-terminal biotinylated peptides (Figure 7b). This could indicate that the replacement of Arg by Cit induce no or only minor changes in the steric arrangement. Also in water the presence of the biotin in C-terminal position essentially does not influence the conformation (PPII and/or unstructured conformation) (Figure 7a vs c) and the presence of biotin at the N-terminus resulted in only limited changes in the population of conformers (appearance of some ordered structure nearby PPII and unordered conformation) (Figure 7a vs b). The biotin's band at $\lambda \sim 240$ nm could not be recognized in any ECD spectra, which means that the biotin has no interaction with the peptide skeleton. Comparing the ECD spectra of the above compound pairs in TFE, we detected no major alterations in the pattern between the Arg- and Cit-containing 19-mer peptides (data not shown).

In TFE the spectral features of the ECD spectra of the 19-mer compounds without or with biotin (**p14**, **p16**, **p18**) (Figure 8b) reflect the presence of conformer mixture with a high populations of ordered (mainly helical) structures (a strong positive band at $\lambda \sim 190$ nm, two negative bands $\lambda \sim 207$ nm, and $\lambda \sim 220$ nm); namely, α -helix, β -sheet, and β -turn are presented in different populations in the case of the nearby random structure of each derivative. The composition of conformers was confirmed with CDNN deconvolution-program.³⁷

The results of the ECD study of the epitope region peptide ($^{306}\text{SHQESTXGXSGRSGRS}^{324}$) recorded in water and also in TFE (Figure 8a vs b) indicate that the spectral pattern of the compounds (**p14**, **p16**, **p18**) essentially do not vary. Consequently these compounds could adopt similar 3D

structure as detected by ECD regardless of the presence and position of biotin. This suggests that conformation of these compounds are much less dependent on the presence and/or position of the biotin-moiety.

DISCUSSION

Biotin is a frequently used label moiety, which plays an important role in various analytical methods, predominantly in immunoassays due to the strong biotin–avidin interaction.³⁸ Several biotinylation methods have been developed and the biotin peptide/protein conjugates are widely used. For example Zeng et al. synthesized *N*-terminal biotinylated peptides with a 6-aminohexanoic acid residue spacer for the examination of a B-cell epitope of the luteinizing hormone releasing hormone.³⁹ The biotin–peptide conjugate (LHRH^{6–10}) was able to inhibit the binding of anti-LHRH^{1–10} antisera to LHRH^{1–10} as detected by ELISA. Recently a new biotin–avidin mediated competitive ELISA was developed to detect residues of tetracyclines in milk (100 µg/L) using biotin labeled rabbit antiserum IgG.⁴⁰ In another report Asp-hemolysin-related *N*-terminally biotinylated synthetic peptides were examined by flow cytometry that were supposed to be potent inhibitors of platelet-activating factor.¹⁶ Interestingly only one report described presents details about *N*- and *C*-terminally biotinylation.¹⁷ For an epitope mapping study overlapping peptides of 10 amino acid residues covering the β -amyloid peptide 1–42 were prepared. One subset of peptides was biotinylated at the *C*-terminus, while the other set was biotinylated at the *N*-terminus. Unfortunately, the same oligopeptide was not labeled on the *N*-terminal or on the *C*-terminal. The rationale of the design of these conjugates was not explained. These conjugates were studied for monoclonal and polyclonal antibody binding by ELISA.

Seemingly no systematic studies have been reported so far on the effect of the position of biotin on the antibody recognition or on the comparison of the different biotinylation methods.

In this report we describe the synthesis of *N*- and *C*-terminal biotin–peptide conjugates corresponding to a 19-amino-acid-long epitope region and also to a 5-amino-acid-long epitope derived from filaggrin with or without spacer structure. With this new set of peptides and their biotin conjugates we have studied how the position of the biotin and the distance between the biotin and the epitope core could influence the (auto)antibody recognition.

Serum samples from RA patients, from healthy subjects and from SLE patients, were comparatively examined. The results indicate that the binding of the *C*-terminally biotinylated epitope peptide (³¹¹TXGRS³¹⁵) and both the *C*- or *N*-terminally biotinylated epitope region peptide (³⁰⁶SHQESTXGXSGRSGRSGS³²⁴) is specific for RA. In sharp contrast with these results polyclonal antibodies derived from RA patients did not recognize the 5-mer peptide epitope conjugate with *N*-terminal biotin.

In order to understand differences/similarities observed in the binding experiments, the secondary structure of free and biotin conjugated peptides was studied in detail. Results clearly show that the arginine/citrulline change has no significant influence on the solution conformations of the 5-mer epitope or the 19-mer epitope region peptide in water and even in ordered structure promoting TFE.

These studies suggest that the conformational properties of the *N*- and *C*-terminally biotinylated epitope core pentapeptide conjugates differ substantially. The type of secondary structure elements in the conjugate with *C*-terminal biotin resembles a mixture of PPII type helix and/or unstructured conformers (in

water) or a mixture of β II turn (or γ turn) and unstructured conformers (in TFE) and characteristically differs from conjugate with *N*-terminal biotin, which has a conformer mixture of a significant amount of β turns, mainly β I–III turn (in water) or β II turn conformers (in TFE).

On the other hand, ECD spectra recorded in water as well as in TFE demonstrate only minor differences in the conformational features of the epitope region 19-mer peptide conjugates with *C*- or *N*-terminal biotin. The secondary structure elements in both of the conjugates resemble PPII type helix and/or unstructured conformers (in water) or conformer mixture predominated by ordered structures (α -helix, β -sheet, and β -turn) (in TFE).

Data from the ECD studies suggest that the position of biotin in the 5-mer epitope peptide has a pronounced effect of secondary structure in solvents as studied, while in the case of the 19-mer epitope region peptides, the position of biotin has almost no influence on the secondary structure of the conjugates.

If the results of the indirect ELISA and ECD experiments are taken together, two important features of antibody binding to synthetic peptide antigens corresponding to filaggrin are shown. First, as expected, only the citrulline containing peptides can be recognized by the CCP2 positive RA sera; however, the conformation data unraveled that the Arg/Cit change in the peptide sequence does not modify soundly the secondary structure of the peptide conjugates. It means that the primary structure of the epitope by modification of charges (e.g., replacement of three positively charged guanidino group of three Arg residues by neutral side chains of Cit) predominantly determines the antibody recognition and the contribution of the type of secondary structure could only be limited to the antibody binding.

The second feature is that while in case of the filaggrin epitope region 19-mer conjugates there was no difference in the antibody recognition of the *N*- or *C*-terminal biotinylated derivatives, the position of biotin in the epitope core 5-mer conjugates significantly alters antibody binding. These findings are in harmony with observations derived from the secondary structure studies. The conformation of the epitope region peptide (³⁰⁶SHQESTXGXSGRSGRSGS³²⁴) does not change markedly due to the different biotinylation site, while the results of the ECD experiments suggest that in the case of the 5-mer epitope core (³¹¹TXGRS³¹⁵) the conformation was influenced very much by the presence and position of the biotin. Thus, these factors could drastically affect the accessibility of the epitope core and the detection of the antibody binding phenomenon.

CONCLUSIONS

Novel *N*- or *C*-terminally biotinylated RA-specific peptide conjugates corresponding to the minimum epitope core or the epitope region of filaggrin with or without different linker moieties were prepared. The new compounds were tested by indirect ELISA using NeutrAvidin coating and the solution conformation of peptides in water and in ordered structure promoting solvent (TFE) was assessed by ECD. Data discussed above indicate that in the case of the long 19-mer epitope region peptide (³⁰⁶SHQESTXGXSGRSGRSGS³²⁴) the primary structure determines predominantly the recognition by RA-specific autoantibodies. We assume that in this case the biotinylation essentially does not influence the binding since the minimum epitope core (³¹¹TXGRS³¹⁵) is located in the middle of the sequence, in a distance from both *N*- or *C*-terminal. In contrast, in the case of the 5-mer epitope core peptide conjugate, both the primary and secondary structures may affect the antibody

binding. Furthermore, in the present study we proved that the positioning of the biotin label on a peptide sequence can profoundly influence its recognition by antibodies, and thus the sensitivity of an ELISA. Based on these results, we propose an approach to identify the best analogue of an epitope peptide of a given sequence for binding studies: biotinylate peptides both N-terminal and C-terminal, incorporate spacers of different lengths and types, and screen to determine the optimal combination of these elements to develop an efficient and sensitive antibody binding assay.

■ ASSOCIATED CONTENT

■ Supporting Information

RP-HPLC chromatograms and ESI-MS spectra of the peptides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACPA, anticitrullinated peptide/protein antibodies; BiotLC, biotinyl-6-aminohexanoic acid (longchain biotin); Boc, ^tButyloxycarbonyl; BSA, bovine serum albumin; CCP, cyclic citrullinated peptide; DCM, dichloromethane; DIEA, *N*-diisopropyl-ethylamine; DMF, *N,N'*-dimethylformamide; DMSO, dimethylsulfoxide; ECD, electronic circular dichroism; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; HF, hydrogenfluoride; HOBt, 1-hydroxybenzotriazole; HRPO, horseradish peroxidase; MeOH, methanol; OD, optical density; PAD, peptidyl arginine deiminase; PBS, phosphate buffered saline; PyBOP, benzotriazole-1-yloxy)-trispyrrolidinophosphonium hexafluorophosphate; RA, Rheumatoid arthritis; RP-HPLC, reverse phase high-performance liquid chromatography; SLE, systemic lupus erythematosus; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TIS, triisopropylsilan; Ttds, 4,7,10-trioxo-1,13-tridecane-diamino-succinic acid

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