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Complete List of Authors:	Babos, Fruzsina; MTA-ELTE, Research Group of Peptide Chemistry Szarka, Eszter; Eötvös Loránd University, Department of Immunology Nagy, György; Buda Hospital of Hospitaller Brothers of St. John, Majer, Zsuzsa; Eötvös Loránd University, Laboratory of Chiroptical Structure Analyzes Sármay, Gabriella; Eötvös Loránd University, Department of Immunology Magyar, Anna; MTA-ELTE, Research Group of Peptide Chemistry Hudecz, Ferenc; Hungarian Academy of Science, Research Group for Peptide Chemistry

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The role of *N*- or *C*-terminal biotinylation in autoantibody recognition of citrullin containing filaggrin epitope peptides in Rheumatoid arthritis

Fruzsina Babos^{‡,†}, Eszter Szarka[§], György Nagy[†], Zsuzsa Majer[⊥], Gabriella Sármay[§],
Anna Magyar[‡], Ferenc Hudecz^{*‡,†}

[‡]Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Pázmány Péter sétány 1/A, H-1117 Budapest, Hungary

[§]Department of Immunology, Eötvös Loránd University, Budapest, Hungary

[†]Buda Hospital of Hospitaller Brothers of St. John, Frankel Leó út 54, H-1023 Budapest, Hungary

[⊥]Laboratory of Chiroptical Structure Analyzes, Insitute of Chemistry, Eötvös Loránd University, Budapest, Hungary

[†]Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary

*Corresponding author:

Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Pázmány Péter sétány 1/A, H-1117 Budapest, Hungary; Phone: (+36)-1-372-2828; Fax: (+36)-1-372-2620

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 (³⁰⁶SHQESTXGX³²⁴GRS³²⁴) 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 (³⁰⁶SHQESTXGX³²⁴GRS³²⁴) 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 doesn't 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

1
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3 manifest. Therefore a specific and sensitive (serological) marker, which is presented very
4 early in the disease, is still needed.

5 Various autoantibodies have been detected in sera from RA patients², and were found
6 to recognize citrullinated proteins or peptides: the so called anti-citrullinated protein/peptide
7 antibodies (ACPA)³. In this report we are dealing with the filaggrin (filament-aggregating
8 protein), which is one of the possible targets of these antibodies. The possibility that citrulline
9 (Cit) residues might be present in the epitopes was investigated by Schellekens⁴. Several
10 peptides were synthesized and an epitope region consisting of nineteen amino acids:
11 ³⁰⁶SHQESTRGRSRGRSGRS³²⁴ was found, which contained five arginine residues that
12 might be target of citrullination. Numerous analogues were synthesized substituting arginine
13 residues into citrulline or glutamine, glutamic acid, alanine and ornitin. The results suggested
14 that not the absence of arginine, but the presence of citrulline specifically rendered the peptide
15 autoantigenic.
16

17 The best binding was obtained by the peptide once-substituted analogue at the 312
18 position: ³⁰⁶SHQESTXGRSRGRSGRS³²⁴ (X= citrulline) (4). The cyclic derivative of this
19 peptide – SHQ(CESTXGRSRGRC)_{cyclo}SGRS³²⁴ – was introduced as target antigen first to
20 detect antibodies by ELISA in the clinical practice⁵.
21

22 By systematic change of the arginine residues to citrulline an applying multipin
23 ELISA approach with the RA patients' sera⁶ we have identified an analogue, in which three
24 Arg residues at positions 312, 314, 316 are replaced by Cit:
25 ³⁰⁶SHQESTXGXSGRSGRSGRS³²⁴. Furthermore, by *N*- and *C*-terminal shortening of the
26 peptide sequence the minimum epitope peptide ³¹¹TXGRS³¹⁵ was determined⁷.
27

28 To detect the epitope binding we have designed a novel group of peptide conjugates
29 with biotin. The biotin-avidin interaction, applied in ELISA to anchor the peptides to the
30 plates, is one of the strongest non-covalent interactions known and it is stable toward a variety
31 of harsh conditions^{8,9}. Biotin-labeled peptides are widely used for example in affinity
32 purification¹⁰, FRET-based flow cytometry¹¹, solid-phase immunoassays¹² and receptor
33 localization¹³.

34 The biotin, as label is most frequently attached directly to the *N*-terminal amino
35 group¹⁴⁻¹⁶, and in only a few cases to the *C*-terminal¹⁷ of the B-cell epitope peptides.
36 According to our knowledge no systematic studies were reported on the effect of the position
37 of biotin on the antibody recognition of a peptide epitope. The distance between the epitope
38 binding site and the biotin could also influence the interaction between the partners. To avoid
39 this several linker types were developed and were utilized in the biotinylation methods:
40 aminohexanoic acid (biotin LC, aminocaproic acid)¹⁸⁻²⁰, tetrapeptide – SGSG²¹ or β-alanine²²
41 was incorporated. Interestingly, essentially no data are available on the analysis of the effect
42 of spacer moiety between biotin and B-cell epitope on antibody binding properties.
43

44 The aim of the present work was to answer interesting and very important questions:
45 how can the position of the biotin influence the antibody recognition and how far should
46 have been located the biotin from the minimum filaggrin epitope. We also examined that if
47 the secondary structure is influenced by the biotin's position. Based on our preliminary
48 results⁷ we have synthesized a new set of *N*- and *C*-terminal biotin conjugates of peptides with
49 Arg or Cit using various linkers between the epitope core and the biotin (Figure 1) to test the
50 availability of the epitope as well as the specificity of antibody binding when the peptide
51 conjugates are coupled to neutravidin coated plates. We were also interested to clarify the role
52 of the secondary structure in antibody binding by ECD.
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54 Here we report on our findings that autoantibodies derived from RA patients recognize
55 specifically both filaggrin epitope region (³⁰⁶SHQESTXGXSGRSGRSGRS³²⁴) and short
56 epitope core (³¹¹TXGRS³¹⁵) peptides (where X = Cit). Our data indicate that the positioning of
57 the biotin label within a peptide sequence can markedly influence the antibody binding, but
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3 the length of the linker has essentially no effect on the recognition. Based on ECD
4 experiments we demonstrate that the Arg/Cit change does not influence the solution
5 conformation of the peptide analogues. However, the presence and position of the biotin
6 moiety has a pronounced effect on the conformation of the 5-mer epitope core peptides, while
7 it doesn't alter the secondary structure of the 19-mer epitope region peptides.
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10 EXPERIMENTAL PROCEDURES

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12
13 **Materials.** All amino acid derivatives and resins (MBHA, Rink Amide MBHA) and coupling
14 agents (*N,N'*-diisopropylcarbodiimide (DIPCI), *N*-diisopropyl-ethylamine (DIEA)) and
15 scavengers (triisopropylsilan (TIS)) were purchased from IRIS Biotech GmbH (Marktredwitz,
16 Germany) or Reanal (Budapest, Hungary), (benzotriazole-1-
17 yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from
18 NovaBiochem (Läufelfingen, Switzerland). Scavenger, coupling agents, and cleavage
19 reagents (*p*-cresol, 1-hydroxybenzotriazole (HOBt), piperidine, trifluoroacetic acid (TFA),
20 and hydrogen fluoride (HF)) were Fluka products (Buchs, Switzerland). Solvents for synthesis
21 (dichloromethane (DCM), *N,N'*-dimethylformamide (DMF) and diethylether) were purchased
22 from Molar Chemicals (Budapest, Hungary). Dimethylsulfoxide (DMSO) was from Sigma-
23 Aldrich Kft (Budapest, Hungary) and HPLC-grade acetonitrile (MeCN) and methanol
24 (MeOH) were from Merck Kft (Budapest, Hungary). Biotin and biotinyl-6-aminohexanoic
25 acid were Fluka products (Buchs, Switzerland) and the 4,7,10-trioxa-1,13-tridecane-diamino-
26 succinic acid (Ttds) was synthesized by us as described before^{23,24}. Rabbit anti human IgG
27 HRPO conjugate was purchased from Dako Agilent Technologies Company (Glostrup,
28 Denmark).
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32 **Synthesis of Free Peptides and *N*-terminal Biotinylated Peptide Conjugates.** The non-
33 biotinylated and the *N*-terminal biotinylated 5-mer and 19-mer derivatives listed in Table 1
34 were built up on Rink Amide-4-methylbenzhydrylamine (Rink Amide-MBHA) resin (0.56
35 mmol/g coupling capacity) using Fmoc/^tBu chemistry. Amino acids were coupled as Fmoc
36 derivatives by the DIC/HOBt coupling method using a 3 molar excess over the resin capacity
37 in DMF. The side chain of Glu residue was blocked with a *tert*-butyl ester protecting group
38 and of His- and Gln residues were blocked with trityl group. *Tert*-Butylether group was
39 applied for the protection of the OH function of Ser and Thr side chains, while the *tert*-
40 butyloxycarbonyl group was used for blocking the ϵ -amino group of Lys residue and
41 pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) group for the guanidino function of Arg.
42 The *N*-terminal Fmoc group was removed by treatment of the resin-bound peptide with
43 piperidine/DMF (30:70, v/v) mixture for 3 + 17 min at room temperature. The success of the
44 coupling was controlled by ninhydrin reaction²⁵. After removal of the last Fmoc group, the *N*-
45 terminus was acetylated by acetic anhydride and DIEA (5:5 eqv.) or biotinylated (peptide :
46 biotin derivative : HOBt : PyBOP : DIEA = 1:3:3:3:10) in DMF/DMSO (25:75, v/v). After
47 washing, the peptide-resin was dried in a desiccator. The peptides were cleaved from the resin
48 with a mixture of TFA-water-TIS (95:2.5:2.5, v/v/v) at room temperature for 2 hrs. The crude
49 products were purified by semipreparative RP-HPLC and the purified compounds were
50 characterized by analytical RP-HPLC and ESI-ion trap mass spectrometry. The yields were
51 50% to the resin capacity.
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56 **Synthesis of *C*-terminal Biotinylated Peptide Conjugates.** The *C*-terminal biotinylated 5-
57 mer and 19-mer conjugates listed in Table 1 were built up on 4-methylbenzhydrylamine
58 (MBHA) resin (0.7 mmol/g coupling capacity) using Fmoc/^tBu chemistry. Amino acids were
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3 coupled as described above. An additional Lys (using Fmoc-Lys(Boc)-OH) was built into the
4 C-terminus of the peptide to conjugate easily the biotin derivatives to its ϵ -amino group. After
5 removal of the Boc protecting group from the side chain the ϵ -NH₂ group of Lys was
6 biotinylated (peptide : biotin derivative : HOBt : PyBOP : DIEA = 1:3:3:3:10) in
7 DMF/DMSO (25 : 75, v/v). After washing, the remaining part of the sequence was built up by
8 Fmoc strategy. The Fmoc group was removed by treatment of the resin-bound peptide with
9 piperidine/DMF (30:70, v/v) mixture for 3 + 17 min. The success of the coupling was
10 controlled by ninhydrin reaction²⁵. After removal of the last Fmoc group, the N-terminus was
11 acetylated by acetic anhydride and DIEA (5:5 eqv.) and after washing with DMF, methanol
12 and diethylether, the peptide-resin was dried in a desiccator. The peptides were cleaved from
13 the resin with anhydrous HF in the presence of *p*-cresol (HF-*p*-cresol 5 mL : 0.2 g) at room
14 temperature. The crude products were purified by semipreparative RP-HPLC and the purified
15 compounds were characterized by analytical RP-HPLC and mass spectrometry (Table 5). The
16 yields were 40% to the resin capacity.
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20 **Synthesis of C-terminal Ttds-containing and Biotinylated Peptide Conjugates.** The C-
21 terminal biotinylated and Ttds-containing 5-mer and 19-mer conjugates listed in Table 1 were
22 synthesized as described above, but with an additional Lys residue (using Boc-Lys(Fmoc)-
23 OH) on the C-terminus of the peptide. After removal of the Fmoc protecting group from the
24 ϵ -NH₂ function of Lys, Ttds linker was coupled as Fmoc-Ttds form by the DIC/HOBt coupling
25 method using a 4 molar excess over the resin capacity in DMF. After washing, the Fmoc
26 protecting group was cleaved from the Ttds residue and biotin derivatives were coupled
27 (peptide : biotin derivative : HOBt : PyBOP : DIEA = 1:3:3:3:10) in DMF/DMSO (25:75,
28 v/v). After washing and removing of the Boc protecting group by TFA, the remaining part of
29 the sequence was built up by Fmoc strategy. The Fmoc group was removed by treatment of
30 the resin-bound peptide with piperidine/DMF (30:70, v/v) mixture for 3 + 17 min. The
31 success of the coupling was controlled by ninhydrin reaction²⁵. After removal of the last Fmoc
32 group, the N-terminus was acetylated by acetic anhydride and DIEA (5:5 eqv.) and after
33 washing, the peptide-resin was dried in a desiccator. The peptides were cleaved from the resin
34 with anhydrous HF in the presence of *p*-cresol (HF-*p*-cresol 5 mL : 0.2 g). The crude products
35 were purified by semipreparative RP-HPLC and the purified compounds were characterized
36 by analytical RP-HPLC and mass spectrometry. The yields were 35% to the resin capacity.
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40 **High-Performance Liquid Chromatography (HPLC).** Analytical RP-HPLC was performed
41 on a Knauer (H. Knauer, Bad Homburg, Germany) system using a Phenomenex Jupiter C18
42 column (250 mm × 4.6 mm) with 5 μ m silica (300 Å pore size) (Torrance, CA) as a stationary
43 phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1%
44 TFA in water) and eluent B (0.1% TFA in acetonitrile-water (80:20, v/v)) was used at a flow
45 rate of 1 mL/min. Peaks were detected at $\lambda = 220$ nm. The crude products were purified on a
46 semipreparative Vydac 218 TP C18 column (250 mm × 10 mm) with 10 μ m silica (300 Å
47 pore size). The flow rate was 4 mL/min. The same eluents with a linear gradient of 20-60% B
48 in 40 min were applied.
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51 **Electrospray Ionization Mass Spectrometry (ESI-MS).** Electrospray ionization mass
52 spectrometry was performed with a Bruker Daltonics Esquire 3000+ mass spectrometer
53 (Bremen, Germany), operating in continuous sample injection mode at 4 μ L/min flow rate.
54 Samples were dissolved either in a mixture of acetonitrile - water (1:1, v/v) or 50% MeOH,
55 1% AcOH in water. Mass spectra were recorded in positive ion mode in the *m/z* 50-3000
56 range.
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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 between $\lambda = 180$ -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 40mM 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 anti-human IgG (1:2000 dilution), conjugated to horse radish peroxidase, for an hour at 37°C, signal was developed by tetramethyl benzidin (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 4M 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. Cut off 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 cut off 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 cut off 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 nineteenmer epitope region peptide (³⁰⁶SHQESTXGXSSXGRSGRSGS³²⁴) 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 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 oligoethylenglycol 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 ($^{306}\text{SHQESTRGRSRGRSGRSGS}^{324}$ 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 amid 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 case of the *C*-terminal biotinylated derivatives repetition of the conjugation was needed. There can be a sterical reason of this difficulty because the ϵNH_2 group of the lysine is not enough far 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 anti-cyclic citrullinated 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 ($^{306}\text{SHQESTXGXSGRSGRSGS}^{324}$) of filaggrin the *N*-terminal biotinylated 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 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 towards 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 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**) cut off value was 1.352, the specificity was 98% and the sensitivity was 51%. In case of the epitope region peptide (**p18**) the cut off 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 methods²⁶, 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 $p<0.0001$ and 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 detail²⁹. 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 represents also 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 $\lambda = 190$ nm³¹. 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 poly-proline 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 non-covalently³⁴.

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3 In solution the conformation of the biotinyl-6-aminohexanoic containing derivatives
4 and the non-biotinylated peptides were studied by ECD spectroscopy. Spectra were recorded
5 in water and in TFE, which is considered as ordered structure promoting solvent³⁵.

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

9
10 The ECD spectra of pentapeptides representing the epitope core without biotin
11 (³¹¹TXGRS³¹⁵ vs. ³¹¹TRGRS³¹⁵) indicate the presence of conformer-mixture composed of
12 mainly unstructured conformers (Figure 5a) exhibiting at $\lambda \sim 222$ nm a weak and near $\lambda \sim 196$
13 nm a strong negative band. The spectra of the *N*-terminal biotin conjugates (*BiotLC*-
14 ³¹¹TXGRS³¹⁵ vs. *BiotLC*-³¹¹TRGRS³¹⁵) are characterized by two negative bands; a weaker at
15 $\lambda \sim 222$ nm and a stronger one near $\lambda \sim 198$ nm as well as with, a weak positive band at $\lambda \sim$
16 243 nm and intense positive ECD values at $\lambda \sim 180$ nm. These ECD curves represent higher
17 ordered structures: in the conformational mixture a significant amount of β turns (mainly β I-
18 III turn) nearby the extended conformers appeared (Figure 5b). The presence of a positive
19 band at $\lambda \sim 243$ nm is indicative for the biotin moiety³⁴. The spectra of the *C*-terminal
20 biotinylated epitope peptides (³¹¹TXGRS³¹⁵K(*BiotLC*) vs. ³¹¹TRGRS³¹⁵K(*BiotLC*)) are
21 characteristic for PPII type helix and/or unstructured conformation (Figure 5c). There are two
22 broad positive bands at $\lambda \sim 239$ nm and 215 nm and a strong negative band at $\lambda \sim 195$ nm,
23 where the band $\lambda \sim 239$ nm as the biotin's band could be also recognized.

24
25 The comparison of spectral patterns of the three pentapeptide-pairs, the similar shapes
26 indicate, that the arginine/citrulline change in a given pair does not influence significantly
27 their conformation in water. However, small, but marked differences in the spectral
28 characteristics (position and intensities of the positive bands) between the *N*- and *C*-terminally
29 biotinylated peptides could well be documented.

30
31 Next we have compared the ECD spectra of the above compounds in TFE and
32 observed essential no differences in spectral features between the Arg- and Cit-containing
33 analogues (data not shown). The spectra of citrulline containing free and biotin conjugated
34 pentapeptides in TFE are presented in Figure 6. The ECD spectrum of biotin free epitope core
35 peptide (³¹¹TXGRS³¹⁵) showed predominantly β I-III turn conformation (Figure 6b: black
36 color) with characteristic C-type spectrum³⁶: a weak, blue-shifted helix spectrum with a
37 positive band at $\lambda = 186$ nm and two negative bands at $\lambda = 224$ nm and at $\lambda = 199$ nm. The
38 spectrum of the *N*-terminal biotinylated derivative (*BiotLC*-³¹¹TXGRS³¹⁵) (Figure 6b: red
39 color) may represent significant amount of β II turn conformers: there is a strong positive
40 band at $\lambda = 190$ nm and a negative band near $\lambda = 223$ nm and appeared at $\lambda = 243$ nm the
41 biotin's positive band. The spectrum of the *C*-terminal biotinylated pentapeptide
42 (³¹¹TXGRS³¹⁵K(*BiotLC*)) represents a mixed set of conformers composed of β II turn (or γ
43 turn) and unstructured conformation (Figure 6b: blue color) with two positive bands at $\lambda =$
44 243 nm (a broad, very weak) and at $\lambda = 196$ nm (stronger one) and a negative band at $\lambda = 225$
45 nm. The biotin's band at $\lambda = 243$ nm could be also recognized. Like in the case of spectra
46 recorded in water, we found marked differences in the spectral pattern (position and intensity
47 of the bands) between the *N*- and *C*-terminally biotinylated peptides.

48
49 The ECD spectra of the free and biotin conjugated epitope core peptides
50 (³¹¹TXGRS³¹⁵) recorded in water and also in TFE (Figure 6a,b) suggest that conformation of
51 the compounds are very much dependent on the presence as well as position of the biotin-
52 moiety in both solvents.

53
54 Next we have studied and compared the ECD spectra of the peptide-pairs covering the
55 epitope region of filaggrin (³⁰⁶SHQESTXGXSXGRSGRSGS³²⁴) in water and also in TFE. It
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is worth to mention that this 19-mer peptides comprise the $^{311}\text{T}\underline{\text{X}}\text{GRS}^{315}$ epitope core peptide studied above.

In water we found minor difference (stronger negative band near $\lambda \sim 197$ nm) between the 19-mer peptide with Arg residues as compared to the one, which contained 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 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) (Figures 7a vs. 7c) and the presence of biotin at the N-terminus resulted only limited changes in the population of conformers (appearance of some ordered structure nearby PPII and unordered conformation) (Figures 7a vs. 7b). 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. Comparison of 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 case of each derivatives nearby random structure. The composition of conformers was confirmed with CDNN deconvolution-program³⁷.

The results of the ECD study of the epitope region peptide ($^{306}\text{SHQEST}\underline{\text{X}}\underline{\text{G}}\underline{\text{X}}\underline{\text{S}}\underline{\text{X}}\text{GRSGRSGS}^{324}$) recorded in water and also in TFE (Figures 8a vs. 8b) 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 on the presence and position of biotin. This suggests that conformation of these compounds are very 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 interaction³⁸. 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 hormone³⁹. The biotin-peptide conjugate (LHRH⁶⁻¹⁰) was able to inhibit the binding of anti-LHRH¹⁻¹⁰ antisera to LHRH¹⁻¹⁰ as detected by ELISA. Recently a new biotin-avidin mediated competitive ELISA was developed to detect residues of tetracyclines in milk (100 $\mu\text{g/L}$) using biotin labeled rabbit anti-sheep IgG⁴⁰. In another report Asp-hemolysin-related N-terminally biotinylated synthetic peptides were examined by flow cytometry that supposed to be potent inhibitors of platelet-activating factor¹⁶. Interestingly only one report described presents details about N- and C-terminally biotinylation¹⁷. 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 either on the N-terminal, or on the C-terminal. The rational

of the design of these conjugates were not explained. These conjugates were studied for monoclonal and polyclonal antibody binding by ELISA.

Seemingly no systematic studies were 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 ($^{311}\text{TXGRS}^{315}$) and both the *C*- or *N*-terminally biotinylated epitope region peptide ($^{306}\text{SHQESTXGXSGRSGRSGS}^{324}$) 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 details. Results clearly show that the arginine/citrulline change has no significant influence on the solution conformations neither of the 5-mer epitope nor of 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 of 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 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 case of the 19-mer epitope region peptides the position of biotin has almost no influence on the secondary structure of the conjugates.

Taken together the results of the indirect ELISA and ECD experiments 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

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3 from the secondary structure studies. The conformation of the epitope region peptide
4 ($^{306}\text{SHQESTXGX}\underline{\text{SX}}\text{GRSGRSGS}^{324}$) does not change markedly due to the different
5 biotinylation site, while the results of the ECD experiments suggest that in case of the 5-mer
6 epitope core ($^{311}\text{TX}\underline{\text{GRS}}^{315}$) the conformation influenced very much by the presence and
7 position of the biotin. Thus, these factors drastically could affect the accessibility of the
8 epitope core, the detection of the antibody binding phenomenon.
9

10 11 12 CONCLUSIONS

13 Novel *N*- or *C*-terminally biotinylated RA-specific peptide conjugates corresponding
14 to the minimum epitope core or the epitope region of filaggrin with or without different linker
15 moieties were prepared. The new compounds were tested by indirect ELISA using
16 NeutrAvidin coating and the solution conformation of peptides in water and in ordered
17 structure promoting solvent (TFE) was assessed by ECD. Data discussed above indicate that
18 in case of the long 19-mer epitope region peptide ($^{306}\text{SHQESTXGX}\underline{\text{SX}}\text{GRSGRSGS}^{324}$) the
19 primary structure determines predominantly the recognition by RA-specific autoantibodies.
20 We assume that in this case the biotinylation essentially does not influence the binding since
21 the minimum epitope core ($^{311}\text{TX}\underline{\text{GRS}}^{315}$) is located in the middle of the sequence, in a
22 distance from both *N*- or *C*-terminal. In contrast, in case of the 5-mer epitope core peptide
23 conjugate: both the primary and the secondary structure may affect the antibody binding.
24 Furthermore, in the present study we proved that the positioning of the biotin label on a
25 peptide sequence can profoundly influence its recognition by antibodies thus the sensitivity of
26 an ELISA. Based on the results of this we propose an approach how to identify the best
27 analogue of an epitope peptide of a given sequence for binding studies: biotinylate peptides
28 both *N*-terminal and *C*-terminal, incorporate spacers of different lengths and types, and screen
29 to determine the optimal combination of these elements to develop an efficient and sensitive
30 antibody binding assay.
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33 34 35 AUTHOR INFORMATION

36 *Corresponding author:

37 Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd
38 University, Pázmány Péter sétány 1/A, H-1117 Budapest, Hungary; Phone: (+36)-1-372-
39 2828; Fax: (+36)-1-372-2620

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51

52 53 54 ASSOCIATED CONTENT

55 * S Supporting Information

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

1
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3 ACPA: anti-citrullinated peptide/protein antibodies
4 BiotLC: biotinyl-6-aminohexanoic acid (longchain biotin)
5 Boc: ^tButyloxycarbonyl
6 BSA: bovine serum albumin
7 CCP: cyclic citrullinated peptide
8 DCM: dichloromethane
9 DIEA: *N*-diisopropyl-ethylamine
10 DMF: *N,N'*-dimethylformamide
11 DMSO: dimethylsulfoxide
12 ECD: electronic circular dichroism
13 ELISA: enzyme-linked immunosorbent assay
14 ESI-MS: electrospray ionization mass spectrometry
15 Fmoc: fluorenylmethoxycarbonyl
16 HF: hydrogenfluoride
17 HOBt: 1-hydroxybenzotriazole
18 HRPO: horse radix peroxidase
19 MeOH: methanol
20 OD: optical density
21 PAD: peptidyl arginine deiminase
22 PBS: phosphate buffered saline
23 PyBOP: benzotriazole-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate
24 RA: Rheumatoid arthritis
25 RP-HPLC: reverse phase high-performance liquid chromatography
26 SLE: systemic lupus erythematosus
27 TFA: trifluoroacetic acid
28 TFE: trifluoroethanol
29 TIS: triisopropylsilan
30 Ttds: 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid
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TABLES

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

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

^a RP-HPLC chromatograms and mass spectra are shown in the Supporting Information (S1-S24).

^b Column: 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.

^c Bruker Daltonics Esquire 3000+ ion trap mass spectrometer.

* X = 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.

Table 2. The interpretation of the OD ratio values of the conjugates

Peptide pairs	mean OD ratio			p-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*
p10/p9	35.21	0.95	0.61	p<0.001*
p12/p11	23.98	n.d.	0.40	p<0.001*
p16/p15	3.55	0.52	0.92	p<0.05**
p18/p17	2.26	0.41	0.64	p<0.05**
p20/p19	2.08	0.43	0.59	p<0.05**
p22/p21	2.37	0.42	0.72	p<0.05**

*Comparison 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).

**The 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).

FIGURES

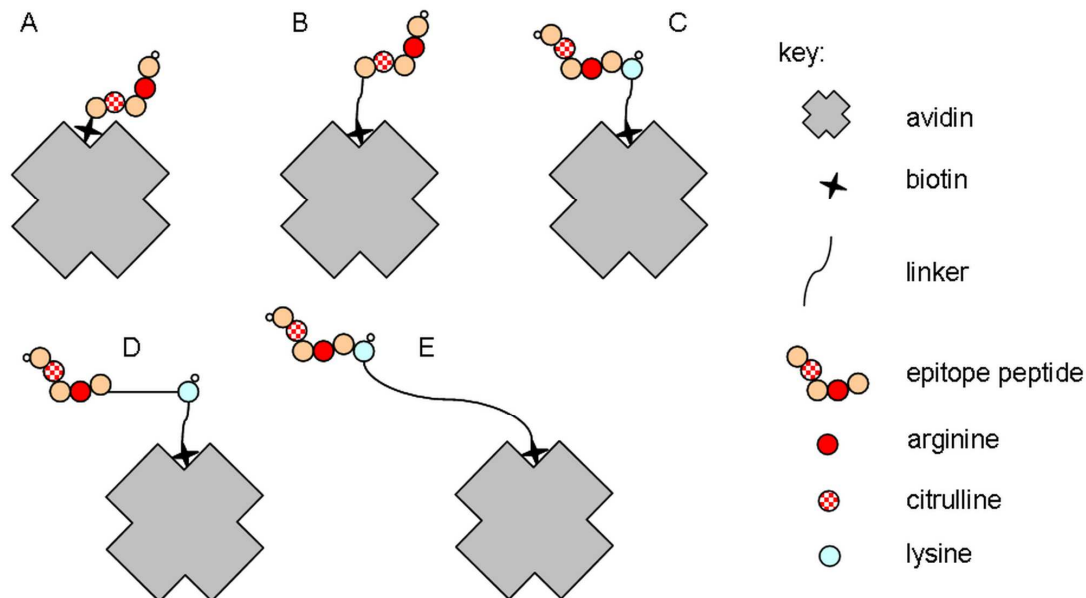


Fig. 1 Schematic presentation of the *N*- and *C*-terminal biotinylated forms. (A) *N*-terminal biotin conjugated peptide, (B) *N*-terminal biotinyl-6-aminohexanoic acid conjugated peptide, (C) *C*-terminal biotinyl-6-aminohexanoic acid conjugated peptide, (D) *C*-terminal biotinyl-6-aminohexanoic acid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

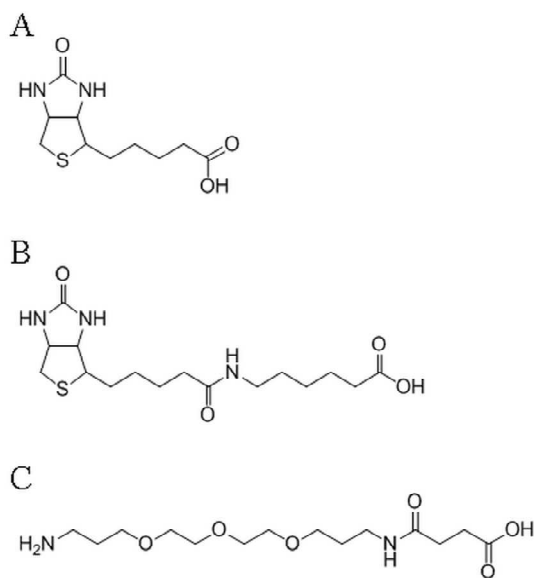


Fig. 2 Biotin derivatives and linker: (A) *N*-(+)-biotin, (B) *N*-(+)-biotinyl-6-aminohexanoic acid = *BiotLC*, (C) 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid = *Ttds*

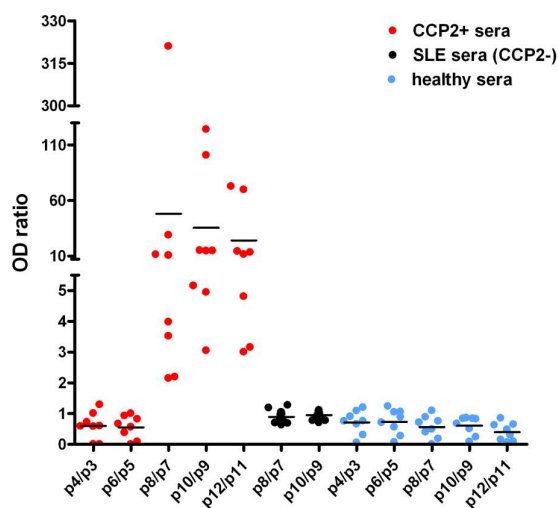


Fig. 3 Binding of antibodies from RA sera to peptides containing the minimum epitope ($^{311}\text{T}\underline{\text{X}}\text{GRS}^{315}$) of filaggrin as detected by indirect ELISA. The peptides were biotinylated either at the *N* terminal (**p3-p6**) or the *C* terminal (**p7-p12**) position. The 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).

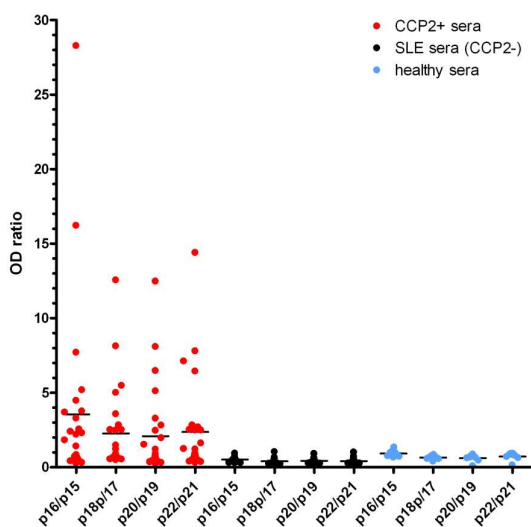


Fig. 4 Binding of anti-citrullinated peptide antibodies (ACPA) to peptides containing the epitope region ($^{306}\text{SHQEST}\underline{\text{X}}\underline{\text{G}}\underline{\text{X}}\underline{\text{S}}\underline{\text{X}}\text{GRSGRSGS}^{324}$) of the filaggrin. The red drops represent the OD ratio 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).

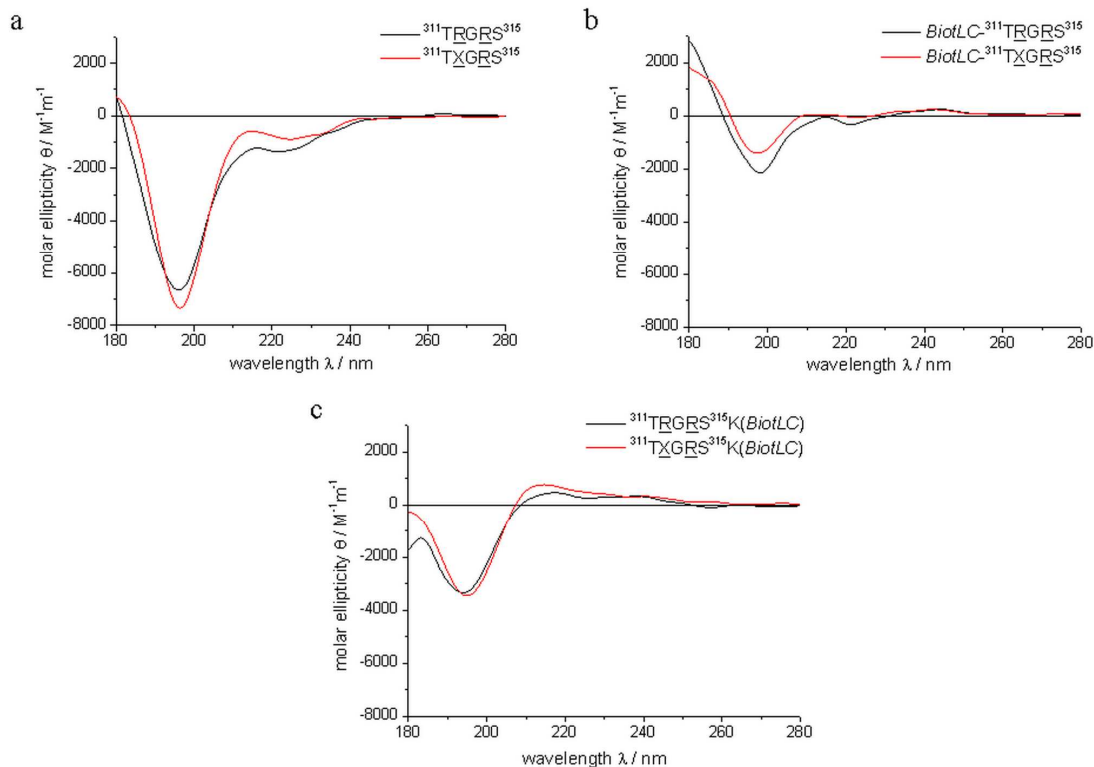


Fig. 5 ECD spectra of the $^{311}TXGRS^{315}$ epitope core peptide and its biotin conjugates in aqueous solution. (a) ECD spectra of the biotin free peptides (**1**, **2**), (b) ECD spectra of the N-terminal biotinylated conjugates (**5**, **6**), (c) ECD spectra of the C-terminal biotinylated conjugates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

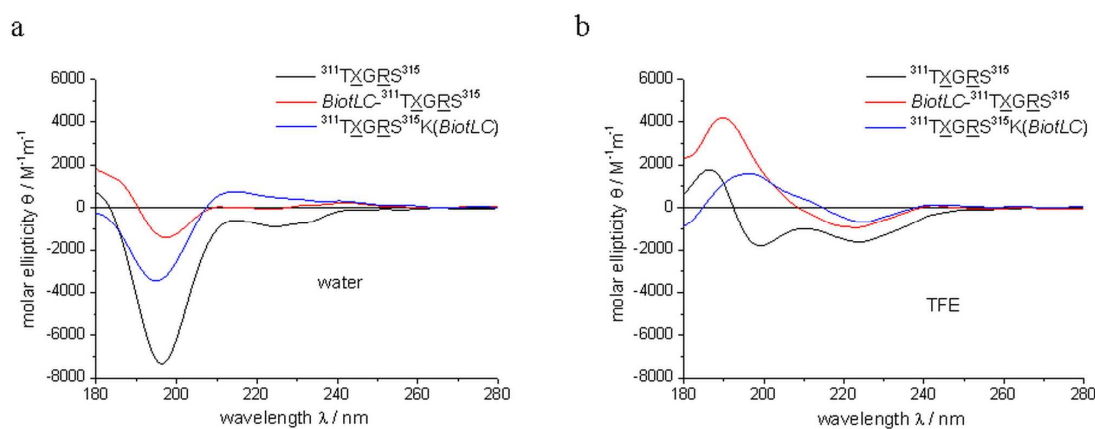


Fig. 6 ECD spectra of the $^{311}TXGRS^{315}$ flaggrin epitope core and its biotin conjugates (a) in water and (b) in trifluoroethanol.

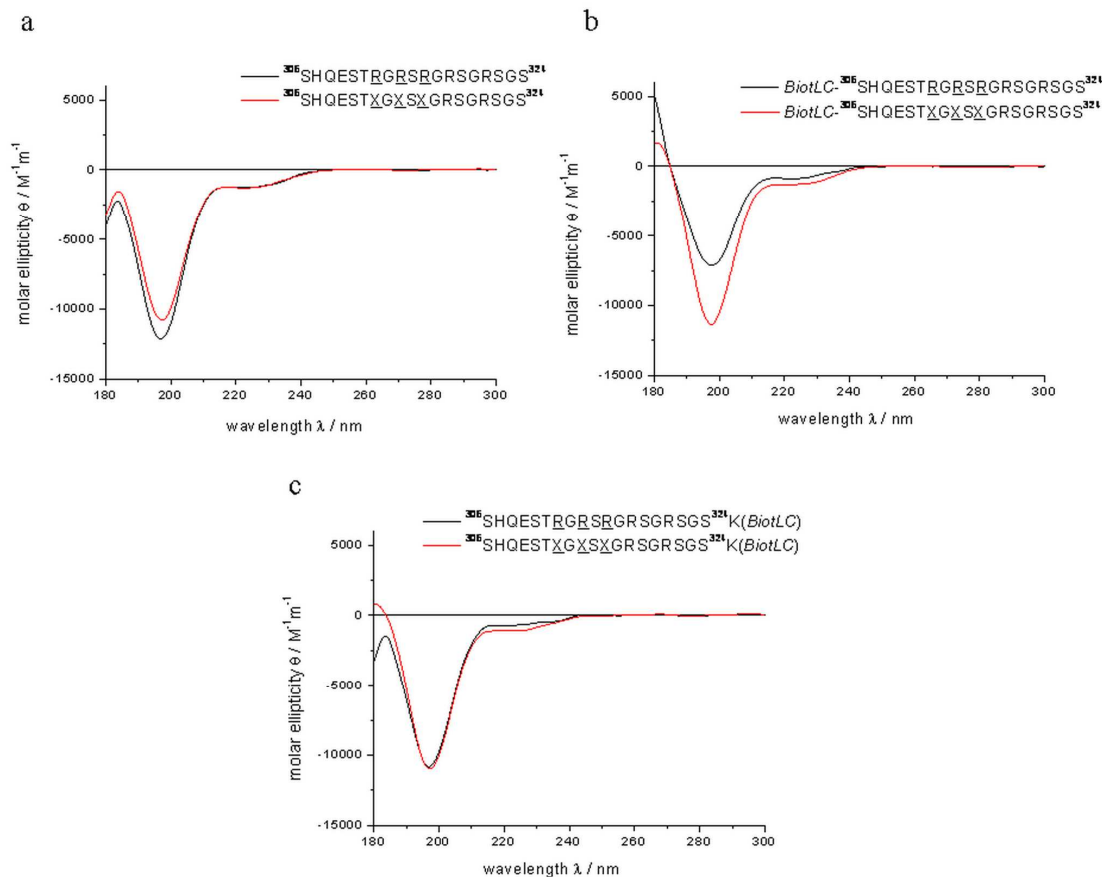


Fig. 7 ECD spectra of the $^{306}\text{SHQESTXGXSSXGRSGRSGS}^{324}$ filaggrin epitope region peptide in water. (a) ECD spectra of the biotin free peptides (13,14), (b) ECD spectra of the *N*-terminal biotinylated derivatives (15,16), (c) ECD spectra of the *C*-terminal biotinylated derivatives (17,18).

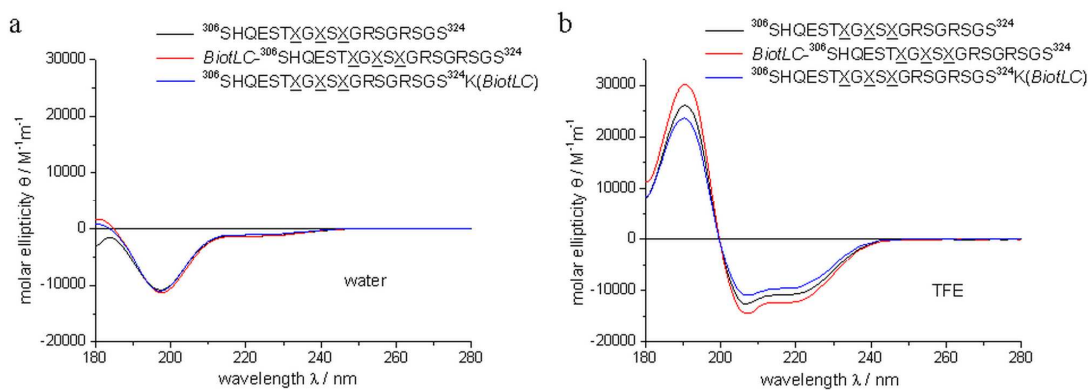


Fig. 8 ECD spectra of the $^{306}\text{SHQESTXGXSSXGRSGRSGS}^{324}$ filaggrin epitope region and its biotin conjugates (a) in water and (b) in TFE.

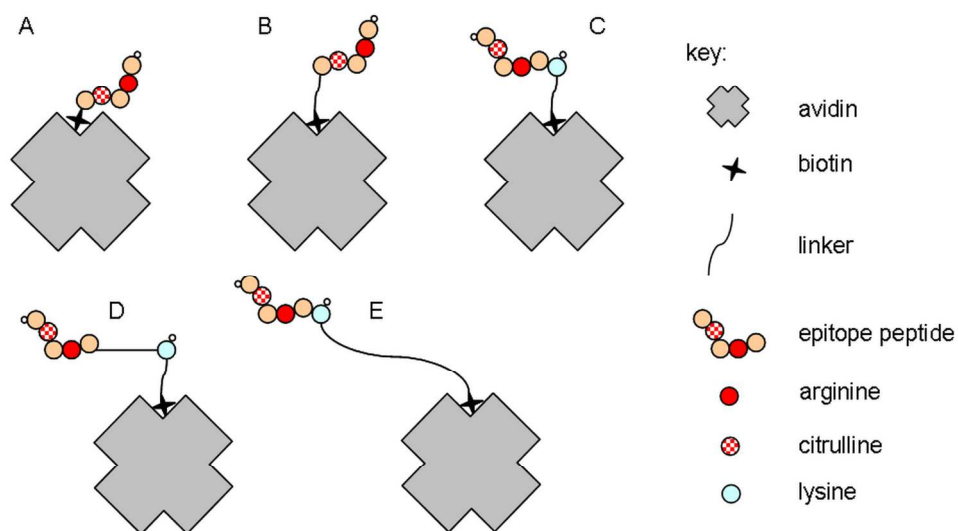


Fig. 1 Schematic presentation of the *N*- and *C*-terminal biotinylated forms. (A) *N*-terminal biotin conjugated peptide, (B) *N*-terminal biotinyl-6-aminohexanoic acid conjugated peptide, (C) *C*-terminal biotinyl-6-aminohexanoic acid conjugated peptide, (D) *C*-terminal biotinyl-6-aminohexanoic acid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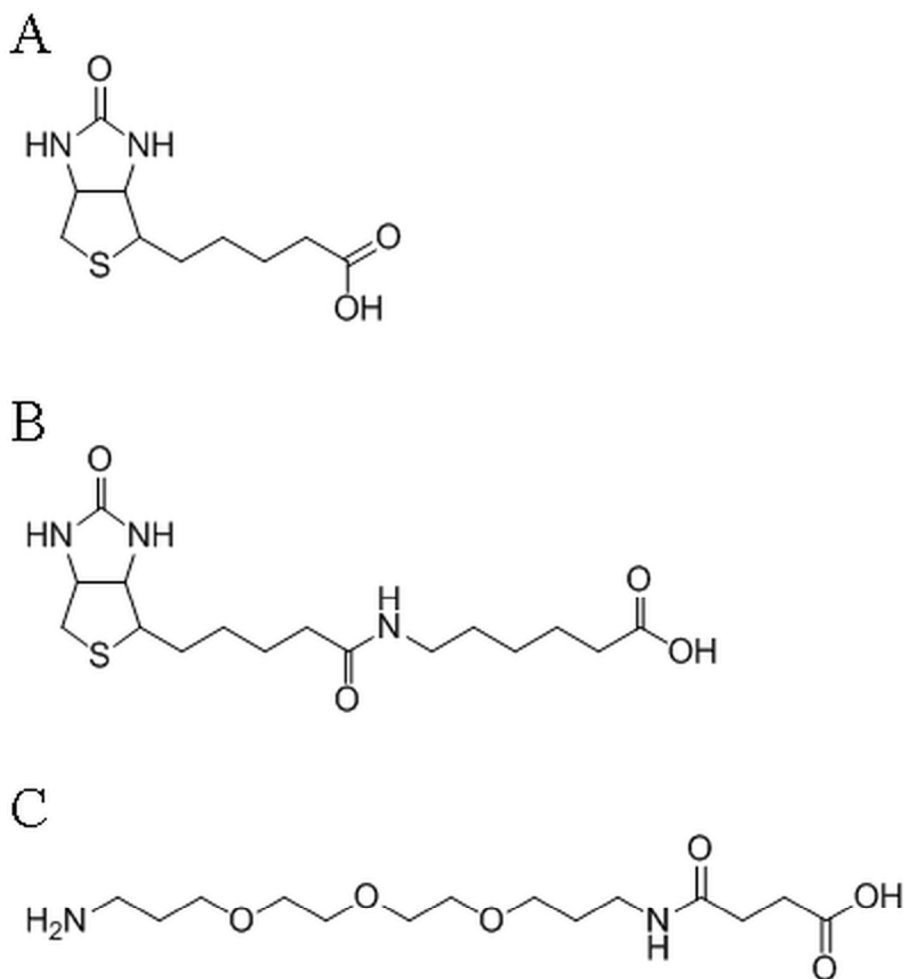


Fig. 2 Biotin derivatives and linker: (A) N-(+)-biotin, (B) N-(+)-biotinyl-6-aminohexanoic acid = *BiotLC*, (C) 4,7,10-trioxa-1,13-tridecane-diamino-succinic acid = *Ttd*
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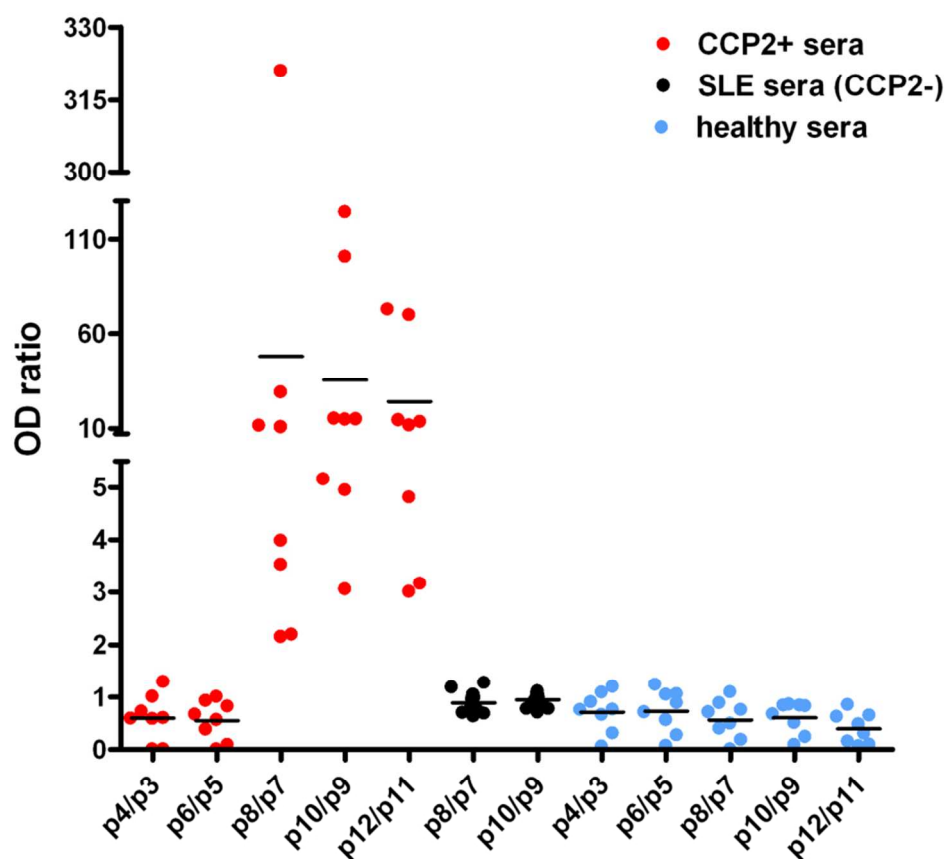


Fig. 3 Binding of antibodies from RA sera to peptides containing the minimum epitope ($^{311}\text{TXGRS}^{315}$) of flaggrin as detected by indirect ELISA. The peptides were biotinylated either at the N terminal (**p3-p6**) or the C terminal (**p7-p12**) position. The red drops represent the OD ratio 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).
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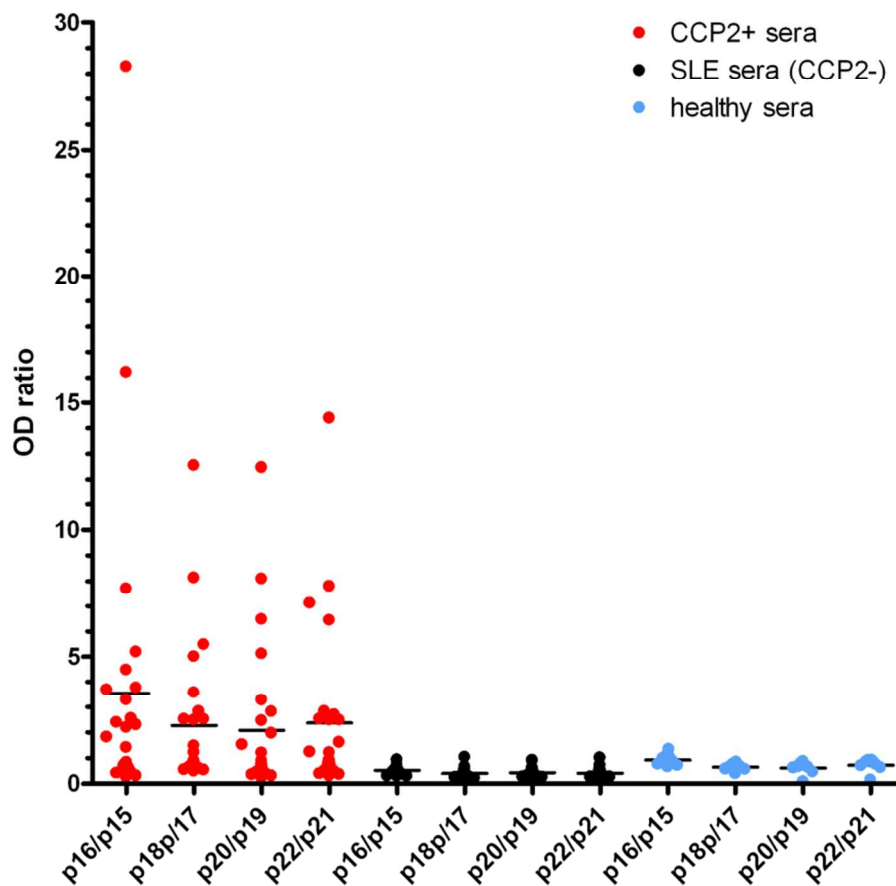


Fig. 4 Binding of anti-citrullinated peptide antibodies (ACPA) to peptides containing the epitope region (306 SHQESTXGXSXGRSGRSGS 324) of the filaggrin. The red drops represent the OD ratio of the peptides tested with the CCP2 positive sera. Sera from healthy subjects were used as control (blue drops) and SLE sera were used as disease control (black drops).

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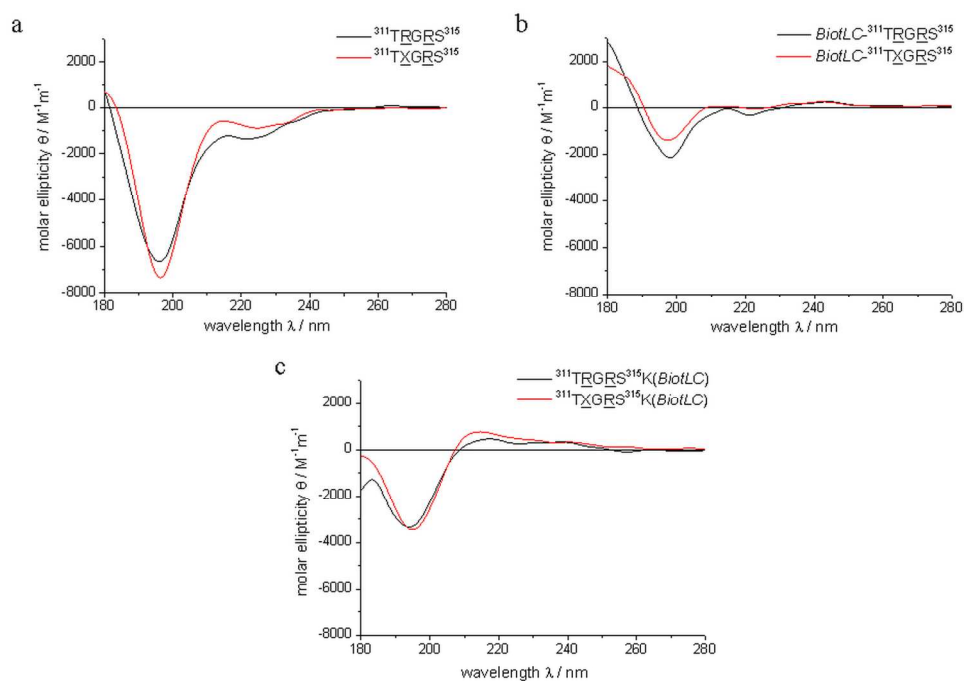


Fig. 5 ECD spectra of the $^{311}\text{TXGRS}^{315}$ epitope core peptide and its biotin conjugates in aqueous solution. (a) ECD spectra of the biotin free peptides (**1**, **2**), (b) ECD spectra of the N-terminal biotinylated conjugates (**5**, **6**), (c) ECD spectra of the C-terminal biotinylated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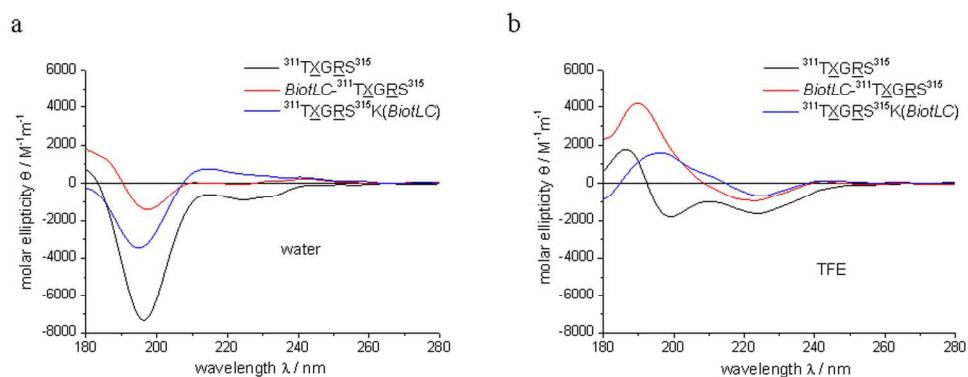


Fig. 6 ECD spectra of the $^{311}\text{TXGRS}^{315}$ filaggrin epitope core and its biotin conjugates (a) in water and (b) in trifluoroethanol.
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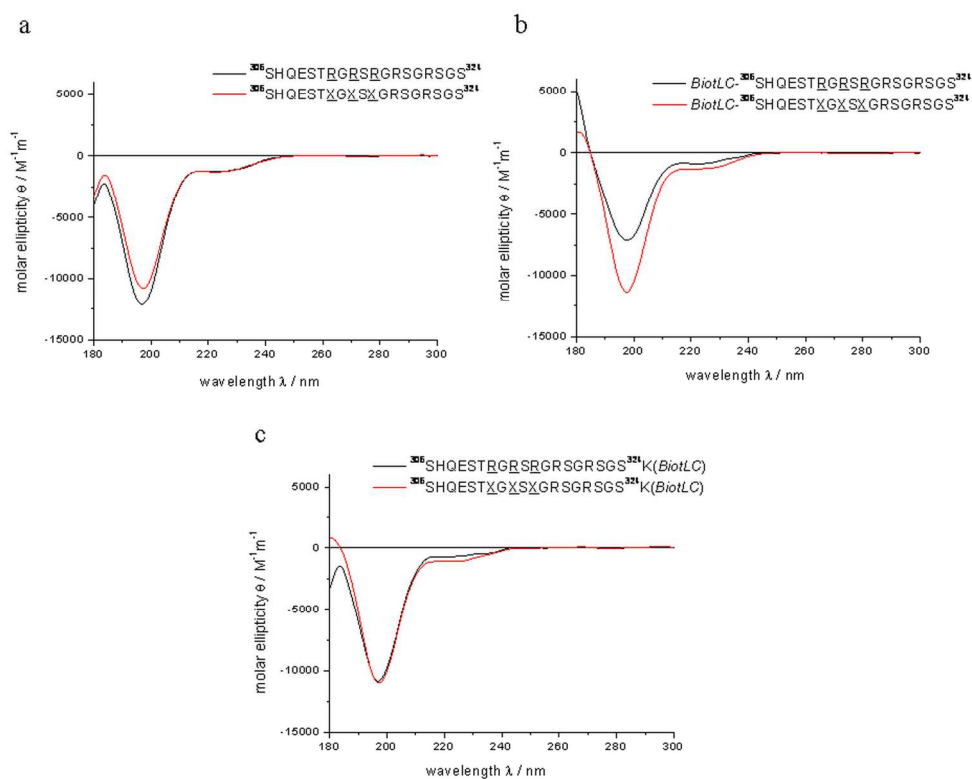


Fig. 7 ECD spectra of the $^{306}\text{SHQESTXGX SXGRSGRSGS}^{324}$ filaggrin epitope region peptide in water. (a) ECD spectra of the biotin free peptides (**13,14**), (b) ECD spectra of the N-terminal biotinylated derivatives (**15,16**), (c) ECD spectra of the C-terminal biotinylated derivatives (**17,18**).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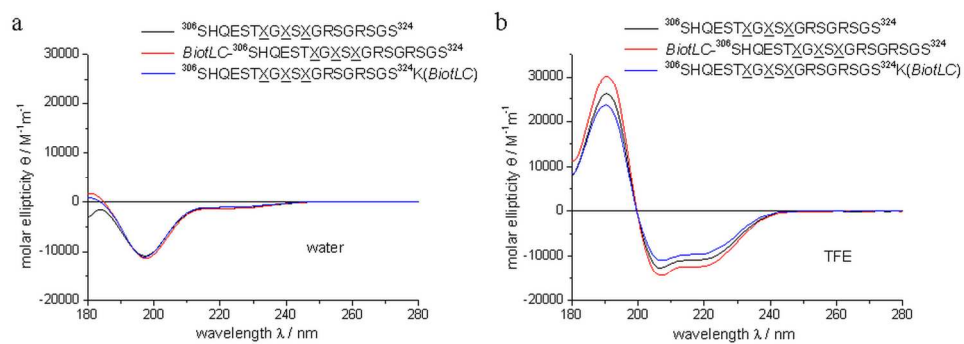


Fig. 8 ECD spectra of the $^{306}\text{SHQESTXGX SXGRSGRSGS}^{324}$ flaggrin epitope region and its biotin conjugates (a) in water and (b) in TFE.
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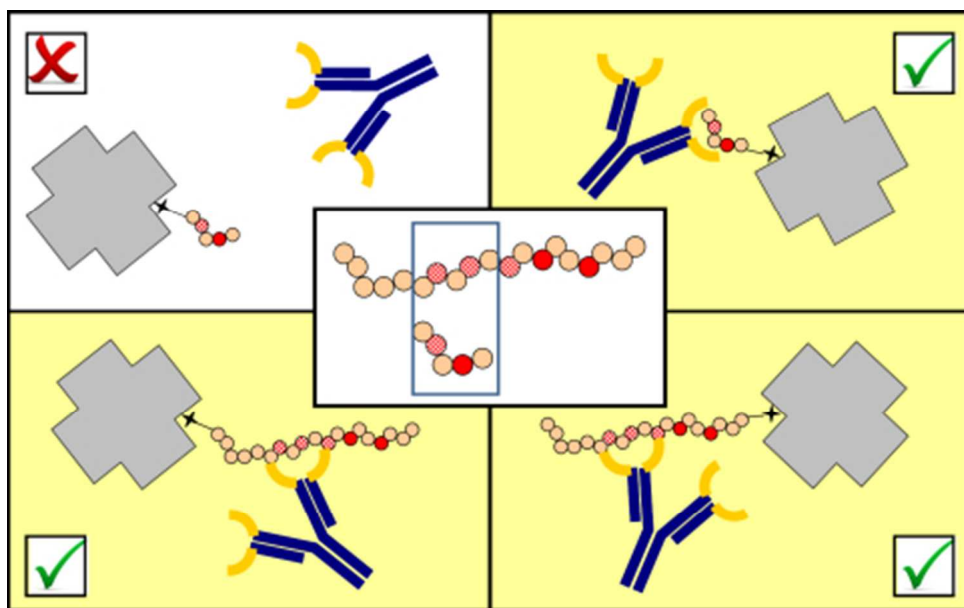


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