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PEGylation of the antimicrobial peptide nisin A: problems and perspectives

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Abstract

Nisin is a natural antimicrobial peptide produced by *Lactococcus lactis* and widely employed as food preservative. Its low solubility in neutral aqueous solutions, its instability at physiological pH and its rapid breakdown by proteolytic enzymes has limited its use for processed foods (processed cheese, milk and derivatives, canned vegetables). The conjugation to poly(ethylene glycol) (PEG) could improve its solubility and protect it towards enzymes present in non optimally processed food. We report the synthesis of a PEG–nisin conjugate, and the microbiology assays against some bacterial cell lines.

Keywords: Nisin; Poly(ethylene glycol); Conjugation

1. Introduction

Nisin is a small, amphiphylic peptide (34 aminoacid residues) produced by some strains of *Lactococcus lactis* [1] and belonging to the family of lantibiotics [2], a group of antimicrobial peptides which is characterised by the presence of uncommon aminoacids, that are formed in a post-translational modification process. Serine and threonine residues are dehydrated to produce the unsaturated forms dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Cysteine residues react through specific addition reactions to such unsaturated amino acids, forming the lanthionine and β-methyllanthionine residues [3,4] that name the lantibiotic family.

The thioether bridges of the lanthionines force the peptide into a five cycles structure (Fig. 1). The structure of nisin has been investigated since 1949 [5–7], but it was only in 1971 that, using aminoacid sequencing and cyanogen bromide cleavage, Gross and Morell [3] could elucidate its complete primary structure.

Nisin is very active against a broad range of Gram-positive micro-organisms. [8,9] Although the exact mechanism of antibacterial activity is not fully understood, it is believed that nisin interact with the bacterial membrane, favouring the formation of pores through which a rapid efflux of ions and cytosolic solutes (aminoacids and nucleosides principally) takes place [9–11] (Fig. 2). The pores formed are non-selective and short-lived. Pore size varies as a consequence of molecules joining and leaving the complex structure in a dynamic process [12]. In a recent paper [13], the authors suggest that the pore-formation process is triggered by the docking of nisin to the lipid-bound cell wall precursor lipid II. The C-terminal part of nisin is then assumed to translocate across the membrane and into the cytosolic compartment (Fig. 2).

Several nisin–lipid II complexes are then assumed to assemble in a barrel-shaped structure limiting a cavity (pore) through which ions and cytosolic solutes migrate under concentration and/or charge gradient effects.

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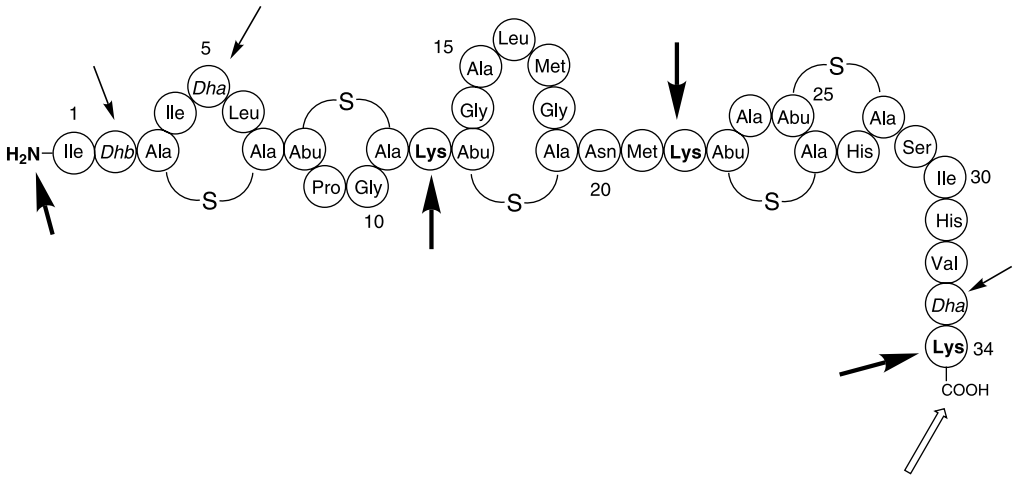


Fig. 1. Schematic representation of the primary structure of Nisin A. Ala-*S*-Ala represents lanthionine, Abu-*S*-Ala β-methyllanthionine, Dha dehydroalanine, and Dhb dehydrobutyrine. Arrows indicate possible sites of PEGylation (←, amino groups; ⇐, carboxylic group; ←, double bonds).

The subsequent membrane depolarisation leads to the inhibition of several biosynthetic processes, and in particular to peptidoglycan formation. This in turn leads to cell death either directly or by sensitisation to other cytotoxic agents.

For its nanomolar-range antimicrobial activity, its virtual lack of toxicity for humans, and its exceptional resistance to thermal treatments, nisin is widely employed as a natural preservative in the dairy and the canning industries. In Italy it has received approval for use in cheese, processed cheese, canned vegetables and confectionery creams at 100–500 IU of nisin per g of food product.

Further informations on nisin structure elucidation and antimicrobial activity, as well as its wide use in food industry worldwide, can be found in the last review by Clarkson and Delves-Broughton [14].

As nisin is poorly soluble in neutral and mild alkaline aqueous media, and it is easily degraded by proteolytic enzymes, we felt that conjugation of the peptide with a non-toxic, non-immunogenic and FDA-approved polymer such as poly(ethylene glycol) (PEG), in analogy to other protein–PEG conjugates already reported in literature (asparaginase, adenosine deaminase, interferon alpha, G-CSF, superoxide dismutase to name but a few), should improve its applicability as food preservative.

2. Chemistry

Nisin structure has four amino residues (three lysine side-chains and one α amino) and only one carboxylic function (the C-terminal one). From the model pro-

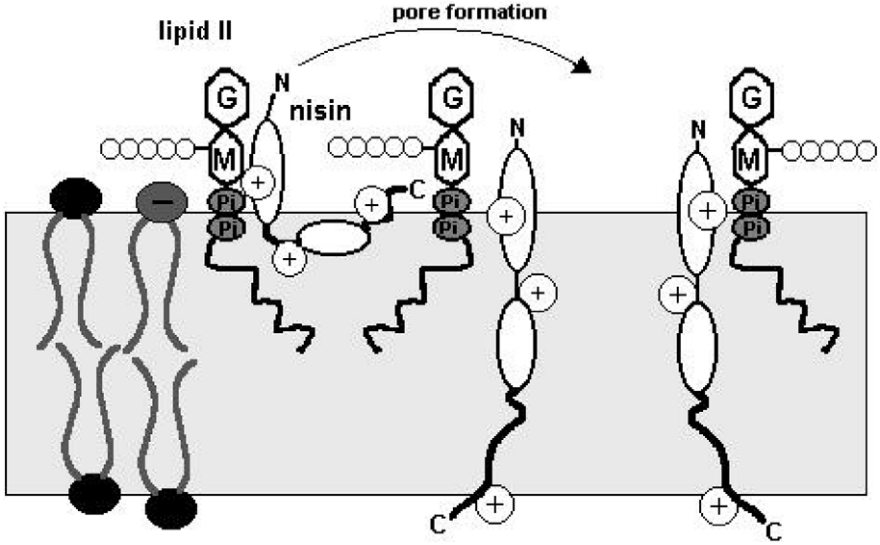


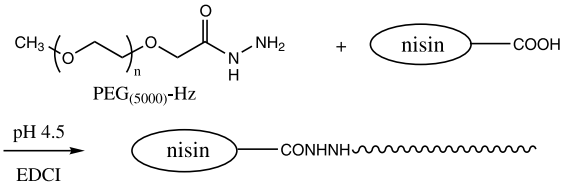
Fig. 2. Model for the lipid II-mediated nisin pore formation (from Reference [13]).

posed by Wiedemann et al. [13] it is clear that the carboxylic function has to migrate through the cell membrane for pore formation. The C-terminal portion of the protein is also rather flexible if compared with the N-portion, allowing higher accessibility to the carboxylic moiety. As PEG is known to easily cross cellular membranes, due to its high flexibility and anphiphilic properties, we planned to address the –COOH function as a suitable candidate for PEGylation. Furthermore, the presence of one single –COOH group would in principle lead to unambiguous conjugation.

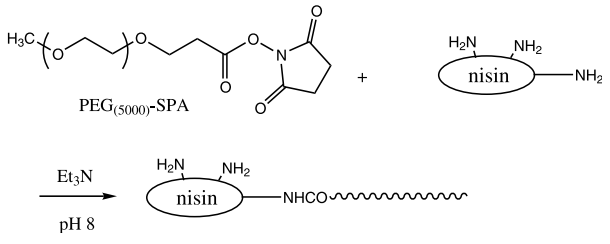
We attempted several conjugation conditions using PEG–hydrazide (m.w. 5000 Da), as at acidic pH this should protonate the nisin amino functions avoiding their participation in the reaction (Scheme 1).

Extremely poor yields even when using large molar excess of PEG hydrazide were unexpected, but suggested a difficult access to the carboxylic group despite the postulated flexibility of the C-terminal portion of the protein. When we gave a closer look at the pore-formation mechanism, we realised that a more convenient PEGylation site could be the N-terminal function: this was apparently involved in lipid II binding, but remained outside the cell membrane during pore formation.

As we had experience in PEGylation of α versus ε-lysine amino groups, playing on the differences in nucleophilic and kinetic properties at different pH conditions [15], we decided to acylate the α amino groups using the reagent PEG–succynimidyl propionamide (PEG-SPA, m.w. 5000 Da); the solvent of choice proved to be dimethyl sulfoxide (Scheme 2). The coupling reaction was carried out in presence of triethylamine to maintain the mixture mildly basic (pH 8), and using different amounts of PEG-SPA (1, 2 and 4 equiv. with respect to nisin).



Scheme 1. Synthesis of PEG–nisin conjugate via PEG hydrazide (PEG-Hz).



Scheme 2. Synthesis of PEG–nisin conjugate via PEG succinimidyl succinate (PEG-SPA).

The formation of a new peak eluting at 24.3 min was attributed to the new PEG–nisin conjugate (Fig. 3b; nisin eluted at 19.4 min, Fig. 3a).

3. Experimental procedures

3.1. Materials and methods

mPEG-Hz, m.w. 5000 Da, and mPEG-SPA, m.w. 5000 Da, were purchased by Shearwater Polymers (Huntsville, AL). Nisin was provided as pure sample by Aplin & Barrett Ltd., Beaminster, Dorset, England. Alternatively, a commercial sample containing approximately 1000 U/mg (NMB Biologicals, Cambridge, England) was purified by RP-HPLC by a modification of a reported method [16].

Solvents and reagents were purchased from Aldrich and Fluka Chemie.

HPLC gel filtration analyses were performed on a JASCO HPLC system (880-PU pump, JASCO 830-RI refractive index detector) coupled to a Shimadzu C-R4A Chromatopac integrator; stationary phase: Superdex 75 analytical Gel filtration column and Superose^{TM} semi-preparative column; mobile phase aq. Na_2HPO_4 0.1 M, pH 5.2.

RP-HPLC were performed on a Gilson HPLC system equipped with a Vydac C18 Analytical column (20 × 1.5 cm).

MALDI-TOF mass Spectroscopy measures were performed on a Hewlett–Packard HP MALDI-TOF spectrophotometer, using sinapinic acid as matrix.

Protein concentrations in solution were calculated using the BIO-RAD Protein Assay protocol based on the colour change of Coomassie brilliant blue G-250 dye and UV spectrophotometric reading of absorbance at 595 nm [17].

3.2. Chemistry

3.2.1. Synthesis of PEG (5000 Da)–nisin conjugate via PEG-Hz

Citrate buffer (3 ml, pH 4.5) was mixed with 1 ml of DMF. Nisin (10 mg, 0.003 mmol) was dissolved in this mixture, followed by EDCI (1 mg, 0.003 mmol) and PEG-Hz (149 mg, 0.03 mmol, 10 equiv.). The resulting mixture was stirred at room temperature for 12 h, following the reaction by RP-HPLC (column Vydac-C18, eluent A = H_2O mQ grade, 0.05% TFA; eluent B = CH_3CN, 0.05% TFA). After 12 h the HPLC chromatogram did not detect any product.

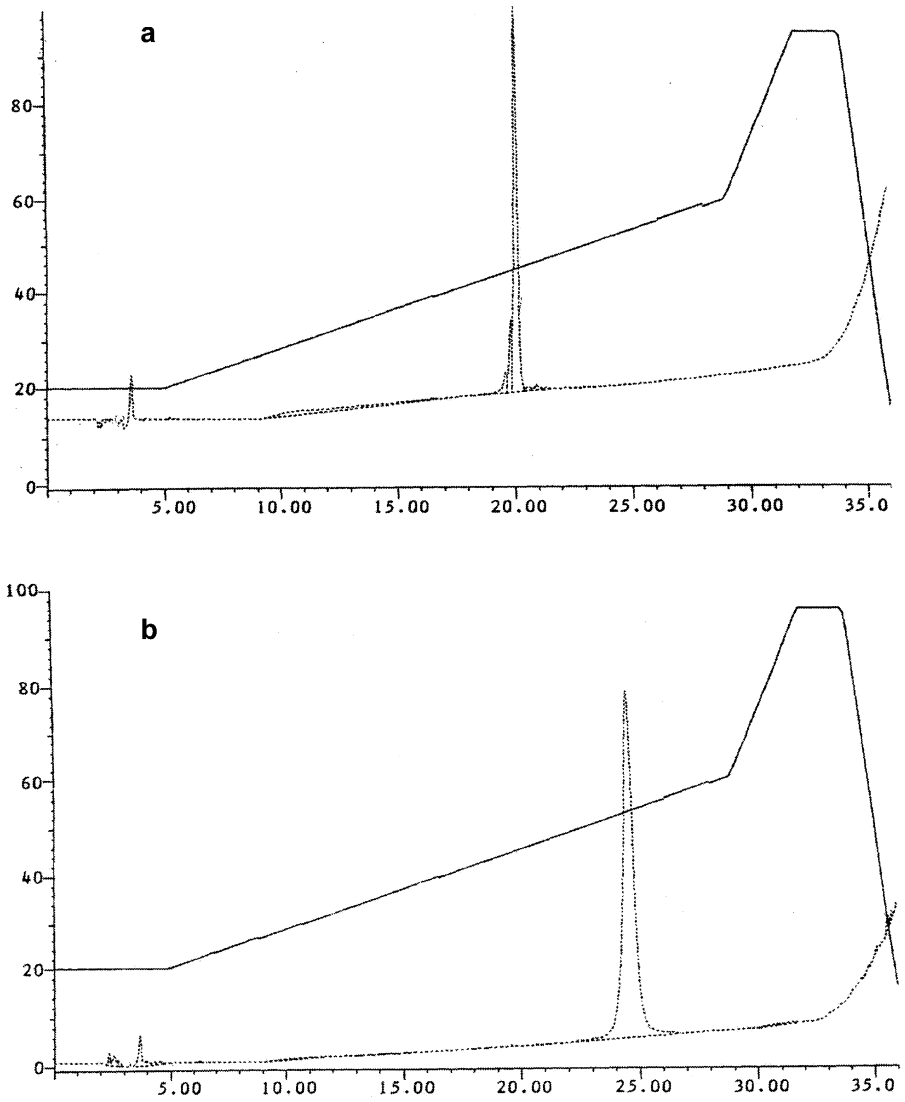


Fig. 3. RP-HPLC chromatogram of (a) PEG–nisin conjugate, and (b) nisin (Vydac C18 analytical column; eluent A, H_2O; eluent B, CH_3CN, 0.05% TFA. Gradient: 0–5′ 20% B; 30′ 80% B; 30′–32′ 80% B; 35′ 20% B).

3.2.2. Synthesis of PEG (5000 Da)–nisin conjugate via PEG-SPA

Nisin (2 mg, 0.0006 mmol) was dissolved in 2.5 ml of DMSO, gently heating to help complete dissolution. Once nisin fully dissolved, 7.8 mg (2 equiv.) of PEG-SPA (5000 Da) were added portionwise, keeping the mixture under vigorous stirring, and Et_3N was added until pH 8 was obtained. At regular intervals aliquots of the reaction mixture (200 μl) were collected, treated with 1 mg of H–Gly–OEt to quench the unreacted PEG-SPA and injected into a RP-HPLC system (Vydac C18 column, eluent A = H_2O mQ grade, 0.05% TFA; eluent B = CH_3CN, 0.05% TFA. Gradient: 0–5′ 20% B; 30′ 80% B; 30′–32′ 80% B; 35′ 20% B). The formation of a new peak eluting at 24.3 min was attributed to the new PEG–nisin conjugate.

Separation of native from PEGylated nisin was performed using a Superdex 75 analytical gel-filtration column, eluent aq. NaH_2PO_4 pH 5.2 and, on semi-preparative scale, a Superose^{TM} 12 semipreparative column (same eluent).

The fractions positive to iodine assay were pooled and freeze-dried.

MALDI-TOF spectroscopy analysis gave a broad peak centred at 8000 Da, consistent with a mono-PEGylated species.

3.3. Biological data

3.3.1. Determination of conjugated protein content

The BIO-RAD method was used to determine the amount of conjugated protein in the product obtained from gel-filtration purification.

A calibration curve of adsorbance versus concentration at 750 nm was made preparing solutions of nisin in water (pH 4) at scalar concentrations (from 112.6 to 10

μg/ml). To 100 μl of each solution were added 0.125 ml of reagent A and 1 ml of reagent B. The solution of PEG–nisin was similarly prepared dissolving 0.92 mg (accurately weighed) in 5 ml of water (pH 4). To 100 ml of the solution was added 0.125 ml of reagent A and 1 ml of reagent B and adsorbance was read at 750 nm.

From the previously built calibration curve the amount of nisin in the PEGylated sample (expressed as free protein) was calculated to be 5.25% (expressed as free nisin), indicating the presence of some unreacted PEG in the mixture. As separation of the unreacted PEG was troublesome, and possibly incompatible with protein integrity, we decided to test the full mixture for in vitro antibiotic activity, while the nisin concentration in the product was evaluated with the Bio-Rad assay.

3.3.2. Bacterial strains, media and growth conditions

We used for this study one Gram-negative (*Pseudomonas aeruginosa* ATCC 27853) and four Gram-positive bacterial strains (*Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 19433, and a clinical isolate of *Listeria monocytogenes*). Bacteria were grown over night at 37 °C in Mueller Hinton (MH) Broth (Difco). After growth each cell suspension was adjusted with MH to a final optical density of 0.5 McFarland and 200 μl of this suspension were then diluted in 5 ml of MH.

3.3.3. Minimal inibitory concentration (MIC) determination

Serial dilution in MH of nisin and PEG–nisin were performed in a 96-wells plate and added with 1 volume (50 μl) of bacterial suspension to obtain a final concentration of nisin or PEG–nisin of 500; 250; 125; 62.5; 31.2; 15.6 IU/ml. Plates were incubated at 37 °C for 24 h. Each well was scored for presence (+) or absence (−) of visible bacterial growth (Table 1). The experiment was repeated twice.

4. Results and discussion

In the present work we attempted the conjugation of the natural antimicrobial protein nisin to poly(ethylene glycol) (PEG). From the proposed mechanism of transmembrane migration and pore formation, we devised two possible sites of PEGylation: the terminal carboxylic group and the α-amino group. The first was migrating through the cell membrane after lipid II—nisin interaction, while the N-terminal amino group was always facing the extracellular compartment. The attempts to conjugate nisin with PEG-Hz, expressely studied for –COOH binding, failed despite the theoretical flexibility of the C-terminal portion of the molecule and subsequent accessibility of the function.

Table 1
Antibacterial activity of nisin and PEG–nisin towards different Gram-positive and Gram-negative bacteria

Bacteria	Gram	MIC (IU/ml)	
		Nisin	PEG–nisin
S. aureus 25923	+	250	> 500
E. faecalis 19433	–	500	> 500
P. aeruginosa 27853	–	> 500	> 500
L. monocytogenes	+	250	> 500
S. aureus 29213	+	250	> 500

MIC, minimum inhibition concentration, in IU/ml (expressed as free protein).

Despite the low solubility of nisin in aqueous media, we were able to find a suitable protocol for the introduction of PEG chains at the amino residues of nisin, by means of the active ester PEG-SPA. Playing on the pH conditions, and on the PEG/nisin ratios, we were able to produce a mono-PEGylated species. The conjugation product was isolated from the native protein, although some amount of unreacted PEG could not be eliminated and the mixture PEG/PEG–nisin had been used for antibacterial activity after titration of protein content with the Bio-Rad assay. Although sequence analysis was not performed, from literature [15] we knew that, given the mildly basic conditions employed, PEG-SPA was mainly directed towards the α-amino group.

We performed a biological assay to determine the MIC of nisin and PEG–nisin for four Gram-positive and one Gram-negative bacterial strains. Data reported in Table 1 show that, as expected, nisin was only active against Gram-positive bacteria, although at fairly high doses. To our surprise no antibacterial activity could be measured for the PEGylated protein. It is not uncommon that PEGylated proteins retain fractions of their original activity, due to interaction of the PEG chains with active sites, or modification of the protein folding, or change in solubility. However, the total loss of activity can only be explained taking into account the peculiar mechanism of antibacterial activity of nisin, that penetrate the cell membrane forming a pore (Fig. 2) and allowing solutes to freely distribute within the cytosolic environment. For this activity to take place, it is necessary that the delicate equilibrium of lipophlicity/hydrophilicity of the native protein is respected. The introduction of PEG chains heavily alter this fragile equilibrium and, ultimately, can be responsible for the lack of activity registered for the PEG conjugate.

Furthermore, in the postulated formation of a lipid II–nisin complex prior to internalisation and pore formation, it has been noted [13] that the N-terminal segment of the lantibiotic is essential for binding, with the ε-amino groups of lysine side-chains interacting with pyrophosphate groups of lipid II. This might explain the loss of activity if one (or more) ε-amino group is

acylated instead of the N-terminal one. The use of different PEGs, that can bind to other moieties within the protein, or that can alkylate the amino groups without affecting the charge (e.g. PEG–aldehyde, PEG–thioimidate [18]) is currently under investigation in our laboratories, and might address the loss of activity.

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