pH-triggered peptide self-assembly into fibrils: a potential peptide-based subunit vaccine delivery platform

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Abstract

Background: Peptide-based subunit vaccines require strong adjuvants (immunostimulant) for therapeutic potency. There is strong demand for the discovery of new safe adjuvants and vaccine delivery systems that can be produced in a highly controlled manner. The pH dependent self-assembly of isopeptide derivatives into fibrils has been reported. Appropriately designed fibrils may have adjuvanting potency.

Methods: Isopeptides conjugated to B-cell peptide epitopes (antigens) were synthesized. Properties of the resultant conjugates and their ability to undergo O-N intramolecular acyl migration reaction were analysed by HPLC, circular dichroism, and transmission electron microscopy.

Results: The conjugates were converted to “parent” peptides via the pH-triggered O-N acyl migration reaction. The parent peptides aggregated into fibrils. The differences in conjugates composition resulted in different fibril morphology. Aggregation of produced peptides induced desired secondary conformation of antigens (peptide epitopes).

Conclusions: The isopeptide approach presented herein may serve as a new self-adjuvanting delivery system for peptide-based vaccines. Such vaccines could be stable when stored in a non-aggregative form, and then converted on demand to the active form in pH-controlled manner. The use of a vaccine that is solely composed of endogenous components may reduce vaccine-associated side effects.

Keywords: O-N acyl migration reaction, isopeptides, peptide-based vaccine, fibrils, peptide conformation, nanotechnology, adjuvant, delivery system

Introduction

The development of new vaccines is often hampered by significant obstacles: vaccine heterogeneity, toxicity of additives (eg., immunostimulant), side effects (autoimmune and allergic responses), stability, and difficulties with characterization and manufacturing processes [1]. Most of these obstacles can be overcome by the design of a peptide subunit vaccine that contains the minimal microbial components necessary to stimulate an appropriate immune response. Purified, fully characterized synthetic peptides can be produced using modern solid phase peptide synthesis (SPPS). However, peptides alone are poorly immunogenic and need a strong adjuvant (immunostimulant) to elicit the desired immune response [2-4]. Many adjuvants are poorly characterized complex mixtures derived from bacteria. These adjuvants are associated with significant toxicity or poor stimulating activity, thus few are licensed for human use [5]. There is strong demand for the discovery of safe, new adjuvants and delivery systems that can be produced in a highly controlled manner [6,7]. Several approaches have been reported to overcome adjuvant-associated side effects [1,6]. We have extensively used exogenous lipids and polymers to form self-adjuvanting delivery systems that obviate the use of toxic adjuvants [8-13]. Recently, it has been reported that self-assembled peptides exclusively composed of endogenous amino acids were able

to stimulate immune responses [14-16].

Peptide self-assembly into extracellular matrices has been studied extensively [17], and the self-assembly of O-acyl isopeptides-polymer conjugates was recently reported [18,19]. O-acyl isopeptides were originally used for the synthesis of peptides that contained difficult sequences. In this method a native amide bond at a serine or threonine residue was isomerized to form an ester bond. Upon the completion of peptide synthesis, the native peptide bond was restored via an O-N intramolecular acyl migration reaction [20-23]. O-N acyl isomerization (Figure 1) allowed drastic changes to the properties of the peptide.

Herein, we describe the application of an O-N intramolecular acyl migration reaction to the design of a potential vaccine delivery system which is able to self-assemble to form fibrils. Appropriately designed O-N isopeptides were conjugated by azide alkyne cycloaddition with B-cell peptide epitopes. The conjugates were only able to adopt β -sheet conformation and self-assemble to form fibrils upon pH-triggered intramolecular acyl migration, and their O-acyl isoforms were stable in the non-aggregative form.

Methods

General

Protected L-amino acids were obtained from Novabio-chem

Figure 1. O–N Intramolecular acyl migration reaction in peptides containing hydroxyl amino acids.

(Läufelfingen, Switzerland), Reanal (Budapest, Hungary) or Mimotopes (Melbourne, Australia). Rink amide MBHA resin, *N,N'*-dimethylformamide (DMF), dichloromethane (DCM), methanol, *N,N'*-diisopropylethylamine (DIPEA), piperidine and trifluoroacetic acid (TFA) were obtained from Merck (Hohenbrunn, Germany). 1-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) was purchased from Mimotopes (Melbourne, Australia). All other reagents were obtained at the highest available purity from Sigma-Aldrich (Castle Hill, NSW, Australia). ESI-MS was performed on a Perkin-Elmer-Sciex API3000 instrument with Analyst 1.4 software (Applied Biosystems/MDS Sciex, Toronto, Canada). Analytical RP-HPLC was performed on an Agilent instrument with a 1 mL min⁻¹ flow rate and detection at 214 nm. Separation was achieved by running gradient mode of 0–100% solvent B over 40 min with 0.1% TFA/H₂O as solvent A and 90% acetonitrile/0.1% TFA/H₂O as solvent B on a Vydac analytical C18 column (218TP54; 5 mm, 4.6 x 250 mm). Purification was carried out by preparative RP-HPLC using a Waters Delta 600 system with a 10 mL min⁻¹ flow rate. Compounds were detected at 230 nm. Separations were performed with solvent A and solvent B on a Vydac preparative C18 column (218TP1022; 10 mm, 22 x 250 mm). A Zetasizer Nano ZP instrument (Malvern Instruments, UK) with DTS software was used for particle size measurements. Sizes were analyzed using a non-invasive back scatter system. Measurements were taken at 25°C with a scattering angle of 173° using disposable cuvettes.

Synthesis

Boc-Thr(Fmoc-Val)-OH

The isodipeptide unit was prepared as previously described [24–26].

Isopeptide 1

Isopeptide **1** was synthesized using Fmoc-chemistry on Rink amide MBHA resin by SPPS. Fmoc-amino acids (3.2 equiv.) activated with HBTU (3.0 equiv.) and DIPEA (3.2 equiv.) were coupled to the peptide resin (2 x 2 h). The isodipeptide unit Boc-Thr(Fmoc-Val)-OH (3.2 equiv.) activated with HBTU (3.0 equiv.) and DIPEA (3.2 equiv.) was coupled to the peptide resin (3 h + overnight). Amino acids were coupled in the following

order: Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Val-OH, Boc-Thr (Fmoc-Val)-OH, Fmoc-Val-OH, Fmoc-Val-OH, Boc-Thr (Fmoc-Val)-OH, Fmoc-Val-OH. Each Fmoc deprotection was performed with 20% piperidine (1 x 10 min, 1 x 20 min). Compound **1** was cleaved from the resin using a solution of TFA/triisopropylsilane/water (95:2.5:2.5) for 4 hours. The resultant compound was purified by preparative HPLC using a C18 column with a gradient of solvent B. HPLC analysis (C18 column): *t_R* = 15.83 min, purity > 95%. Yield: 27%. ESI-MS: *m/z* 871.8 (calc 871.1) [M+H]⁺; 436.7 (calc 436.5) [M+2H]²⁺; MW: 871.08.

Isopeptide 2

Isopeptide **2** was synthesized using the above SPPS procedure, with the further addition of Boc-Thr(Fmoc-Val)-OH and Fmoc-Val-OH before cleavage from the resin.

HPLC analysis (C18 column): *t_R* = 16.66 min, purity > 95%. Yield: 37%. ESI-MS: *m/z* 1172.2 (calc 1171.4) [M+H]⁺; 586.2 (calc 586.2) [M+2H]²⁺; 391.2 (calc 391.1) [M+3H]³⁺; MW: 1170.44.

Isopeptide 3

Isopeptide **3** was synthesized in the same manner as isopeptide **1**, with the addition of Fmoc-Gly followed by azidoacetic acid at the N-terminus of the peptide. HPLC analysis (C18 column): *t_R* = 17.76 min, purity > 95%. Yield: 23%. ESI-MS: *m/z* 1011.9 (calc 1012.2) [M+H]⁺; 506.4 (calc 506.6) [M+2H]²⁺; MW: 1011.18.

Isopeptide 4

Isopeptide **4** was synthesized in the same manner as isopeptide **2**, with the addition of Fmoc-Gly followed by azidoacetic acid at the N-terminus of the peptide. HPLC analysis (C18 column): *t_R* = 18.60 min, purity > 95%. Yield: 18%. ESI-MS: *m/z* 1311.2 (calc 1311.5) [M+H]⁺; 656.3 (calc 656.3) [M+2H]²⁺; 438.0 (calc 437.8) [M+3H]³⁺; MW: 1310.54.

Peptide epitope 5

Peptide **5** (J14) was synthesized as previously reported [27].

Peptide epitope 6

Peptide **6** (88/30 with Gly spacer on C-terminus) was synthesized using standard Fmoc-SPPS in a similar manner to peptide **1**, but without the use of the isodipeptide building

block. HPLC analysis (C18 column): t_R = 17.60 min, purity > 95%. ESI-MS: m/z 1172.3 (calc 1172.3) $[M+2H]^{2+}$; 782.0 (calc 781.9) $[M+3H]^{3+}$; 586.7 (calc 586.6) $[M+4H]^{4+}$; 469.6 (calc 469.5) $[M+5H]^{5+}$; MW: 2342.57.

Peptide epitope 7

Peptide **7** (CHCC(O)HNCH₂C(O)J14) was synthesized in the same manner as peptide **5**, with the addition of a glycine spacer at the N-terminus followed by a propiolic acid (4.0 equiv.) activated with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 4.0 equiv.) was coupled (2 x 2 h) to the peptide. HPLC analysis (C18 column): t_R = 19.58 min, purity > 95%. ESI-MS: m/z 1732.7 (calc 1732.5) $[M+2H]^{2+}$; 1156.2 (calc 1156.6) $[M+3H]^{3+}$; 866.9 (calc 867.7) $[M+4H]^{4+}$; 693.8 (calc 693.6) $[M+5H]^{5+}$; 578.2 (calc 578.2) $[M+6H]^{6+}$; 496.8 (calc 495.7) $[M+7H]^{7+}$; 434.1 (calc 433.9) $[M+8H]^{8+}$; MW: 3462.91.

Peptide epitope 8

Peptide **8** was synthesized in the same manner as peptide **6**, with the addition of a glycine spacer at the N-terminus, followed by a propiolic acid. HPLC analysis (C18 column): t_R = 18.00 min, purity > 95%. ESI-MS: m/z 1227.4 (calc 1226.8) $[M+2H]^{2+}$; 818.3 (calc 818.2) $[M+3H]^{3+}$; 614.1 (calc 613.9) $[M+4H]^{4+}$; MW: 2451.65.

Conjugate (9) of compounds 3 and 7

The conjugation was performed as previously described [27,28]. Briefly, compounds **3** (2.05 mg, 1.65×10^{-3} mmol) and **7** (7.41 mg, 1.65×10^{-3} mmol) were dissolved in DMF (1 mL) and copper wires were added (70 mg). The reaction mixture was protected from light and stirred at 50 °C for 3 hours under nitrogen atmosphere. The resultant compound was purified by preparative HPLC using a C18 column with a gradient of solvent B. HPLC analysis (C18 column): t_R = 19.76 min, purity > 95%, Yield: 36%. ESI-MS: m/z 1119.9 (calc 1119.5) $[M+4H]^{4+}$; 895.9 (calc 895.8) $[M+5H]^{5+}$; 746.9 (calc 746.7) $[M+6H]^{6+}$; 640.2 (calc 640.1) $[M+7H]^{7+}$; 560.4 (calc 560.3) $[M+8H]^{8+}$; 498.4 (calc 498.1) $[M+9H]^{9+}$; 448.6 (calc 448.4) $[M+10H]^{10+}$; MW: 4474.08.

Conjugate (10) of compounds 3 and 8

Conjugation was performed as described for compound **9**. HPLC analysis (C18 column): t_R = 18.61 min, purity > 95%, Yield: 37%. ESI-MS: m/z 1732.5 (calc 1732.4) $[M+2H]^{2+}$; 1156.6 (calc 1155.3) $[M+3H]^{3+}$; 867.0 (calc 866.7) $[M+4H]^{4+}$; 693.7 (calc 693.6) $[M+5H]^{5+}$; 578.4 (calc 578.1) $[M+6H]^{6+}$; MW: 3462.83.

Conjugate (11) of compounds 4 and 7

Conjugation was performed as described for compound **9**. HPLC analysis (C18 column): t_R = 19.85 min, purity > 95%, Yield: 38%. ESI-MS: m/z 1593.0 (calc 1592.1) $[M+3H]^{3+}$; 1194.5 (calc 1194.4) $[M+4H]^{4+}$; 955.9 (calc 955.7) $[M+5H]^{5+}$; 797.1 (calc 796.6) $[M+6H]^{6+}$; 682.9 (calc 682.9) $[M+7H]^{7+}$; 597.9 (calc 597.7) $[M+8H]^{8+}$; 531.7 (calc 531.4) $[M+9H]^{9+}$; 478.2 (calc 478.3) $[M+10H]^{10+}$; MW: 4773.45.

Conjugate (12) of compounds 4 and 8

Conjugation was performed as described for compounds **9**. HPLC analysis (C18 column): t_R = 18.82 min, purity > 95%, Yield: 31%. ESI-MS: m/z 1255.5 (calc 1255.1) $[M+3H]^{3+}$; 941.8 (calc 941.6) $[M+4H]^{4+}$; 753.4 (calc 753.7) $[M+5H]^{5+}$; 628.2 (calc 628.0); MW: 3762.19.

O-N intramolecular acyl migration

Conversion of isopeptides **9-12** into the parent peptide **13-16** was based on previously published methods [26]. Briefly, the isopeptide (**9**, **10**, **11**, or **12**; 5 μ L at 10 mM in DMSO) was added to phosphate buffered saline (PBS, 495 μ L, pH 7.4) and the mixture was shaken at RT. At the desired time points (0h, 1h, 2h, etc.), 60 μ L samples were taken from the mixture, diluted with an equal volume of DMSO and directly analysed by HPLC. HPLC was performed with a linear gradient of solvent B (0–100%, 40 min). The results were analysed at 214 nm.

CD analysis

CD spectra were measured on a JASCO (Tokyo, Japan) J-710 spectropolarimeter using a quartz cuvette with a 1 mm path length at 23 °C. The conjugate samples were analysed either freshly dissolved in water or in buffer (pH = 7.4) upon completion of O-N acyl migration. CD spectra of isopeptides **9-12** were measured at 0.1 mM concentration. To avoid suspension formation, CD spectra of peptides **13-16** were measured immediately after migration reaction at 0.05 mM concentration.

Transmission electron microscopy (TEM)

A sample of the conjugate was added to glow discharged carbon coated 200 mesh grids for 3 min and then wicked off with filter paper. Pictures were taken from a JEM-1010 transmission electron microscope (JEOL Ltd., Japan) operated at 80 kV.

Results and discussion

In recent years, nanotechnology has been widely applied to the study of new adjuvant formulations and vaccine delivery systems [1,29,30]. The ability of vaccines (or their delivery systems) to self-assemble in nanoscale was found to be important for inducing immune responses. For example, it was demonstrated that small nanoparticles (20-40 nm) are preferable for the induction of strong humoral immunity. The self-adjuvanting activity of nanofibers, several micrometres in length fibrils with widths of around 15 nm, was also reported [14]. These fibrils carrying model peptide were able to elicit high IgG titers without adjuvant in mice. We proposed that an appropriately designed O-acyl isopeptide could be used as a β -sheet-based fibril precursor with potential self-adjuvanting activity. Valine-rich peptides conjugated to poly(ethylene oxide) have previously been reported to be able to self-assemble into fibrils [18,19]. Thus, β -sheet precursor isopeptides **1-4**, which were rich in valine moieties,

Scheme 1. Structure of control O-N isopeptides 1-4, peptide epitopes 5-8, synthesis of O-N isopeptides 9-12 and fibrillizing peptides 13-16.

Figure 2. HPLC charts of pH-triggered conversion of isopeptide **9** via O-N intramolecular acyl migration into peptide **13** at (a) 0 h; (b) 1 h; (c) 2 h; (d) 1 day in PBS (pH = 7.4).

were designed (**Scheme 1**). B-cell peptide epitopes derived from Group A Streptococcus (GAS) M-protein were chosen as model antigens.

A chimeric peptide, J14 (KQAEDKVKASREAKKQVEKA-LEQLEDKVK) contains the minimal protective epitope (in italic) enclosed within yeast helical “promoting” sequence from yeast protein CGN4. This peptide is derived from a conserved fragment of Group A streptococcal M-protein that has a tendency to adopt a helical conformation and has been widely used for the development of peptide based vaccines [31–33]. The second B-cell epitope, 88/30 (DNGKAIYERARERALQELGP), was derived from the N-terminal hyper variable region of GAS M-protein, and has a tendency to form random coil rather than an ordered conformation. This epitope has also been used for the development of a vaccine against GAS [34–36]. The peptides were synthesized by SPPS. Due to coupling difficulties, glycine was introduced as a linker (the first C-terminal amino acid) during synthesis of 88/30 peptide on the resin. The isopeptides were modified with an azide moiety by coupling azidoacetic acid to their N-termini,

while the peptide epitopes were modified with the addition of a propiolic acid to the N-termini. A glycine spacer was used between the azide or alkyne moiety and the peptides to reduce steric hindrance around these functional groups. Peptide epitopes **7** and **8** were conjugated to isopeptide **3** and **4** by 1,3-dipolar copper catalyzed alkyne-azide cycloaddition (CuAAC or click) reaction to form four conjugates **9–12** (**Scheme 1**). It was found that the isopeptides were completely stable at 4°C for at least 3 weeks. In general, isopeptides in the solid form are very stable; stability at 4°C for up to two years has previously been reported [26,37].

The ability to convert isopeptides **9–12** to their “native” form (**13–16**) was examined in PBS (pH = 7.4) and monitored by HPLC. The conversion of isopeptide **9** to peptide **13** is illustrated in (**Figure 2**) as an example of this reaction. It is well documented that isopeptides are stable at acidic pH but undergo relatively quick and irreversible isomerization into the parent peptide (**Figure 1**) under basic pH [22,37]. In the case of isopeptide **9**, both isodipeptide units are required to undergo O-N acyl migration reactions to form the desired peptide **13**

(Scheme 1). At physiological pH (7.4) 97% of **9** had converted to **13** after two hours. After one day of reaction, no compound with an ester was detected. In contrast, 97% of the isopeptide **10** was converted into fully “migrated” peptide **14** after one day, while after 3 days of reaction only 83% and 53% of conversion was detected for peptides **15** and **16**, respectively. Extension of the reaction time above 3 days did not result in further conversion. This observation can be easily explained by the fact that both **15** and **16** possessed 3 isodipeptide units which need to undergo the O-N migration reaction to form the final product. Indeed, several partially migrated byproducts were detected by HPLC, which were unable to undergo further O-N acyl migration reactions, most likely due to steric hindrance associated with β -sheet formation and extensive product fibrillization (data not shown). In all cases, the migration product formed a milky suspension, in contrast to the completely soluble starting isopeptides.

CD analysis was performed to determine the change in secondary structure of peptides before and after O-N acyl migration (Figure 3). As a general rule for CD spectroscopy of peptides, a noticeable double minimum around 208 and 222 nm indicate an α -helical structure, while a single minimum at 204 nm is characteristic of random coil, and single minimum at 217 nm indicates β -sheet. Isopeptides **9-12** adopted a random conformation regardless of the peptide epitopes incorporated into the conjugates. Interestingly, after O-N acyl migration, the resulting peptide showed a more organized secondary structure. Conjugates that possessed J14 showed rather helical and β -sheet conformations with shallow minimum at 208 and 216-219 nm for both **13** and **15**. This outcome can be explained by the predisposition of J14 to form a helical structure and the valine-rich peptide to form a β -sheet (Figure 3a). Indeed, in the case of the conjugates that possessed the 88/30 epitope, only the induction of a β -sheet-like conformation was detected

after migration, with a single minimum at 220-221 nm on the CD spectrum (Figure 3b). This data suggested that upon commencement of the pH-triggered intramolecular O-N acyl migration reaction, the initial isopeptides were converted to poorly soluble products which have a strong tendency to self-assemble via the formation of β -sheets. This method induced formation of helical structures at a similar degree to that previously observed with self-assembled and self-adjuvanting nanoparticles [13,27,38].

Formation of a milky solution after migration suggested that β -sheet conformation induce aggregation and fibril formation, in similar manner to other β -sheet peptides [39]. To investigate this hypothesis, TEM images of peptides **13-16** were recorded. As shown in (Figure 4), extensive fibril formation was detected. However, while conjugate **13** formed typical long fibrils with diameter around 10 nm, it was difficult to observe individual fibrils in the TEM image of compound **14**. Both conjugates **15** and **16** formed similar short aggregated fibrils with a rod-like shape. Thus, the aggregation behaviour and shape of the fibrils produced depended more strongly on the number of isodipeptide units in the conjugate than on the nature of the peptide epitope.

We demonstrated that isopeptides conjugated to peptide epitopes were able to undergo pH-triggered O-N acyl migration and self-assemble into fibrils. This process induced the desired conformational properties of the peptide epitopes (eg., helicity of the J14 peptide). These conjugates can be stored in their isopeptide form as a precursor of a peptide-based vaccine and be transformed into the fibril-like structure just before administration *in vivo*. Alternatively, an isopeptide can be administered and undergo conversion to “parent” peptide *in vivo*, thus acting as the prodrug of a vaccine. The ability to convert an isopeptide to the parent peptide under cellular media conditions has been already confirmed in the case of

Figure 4. TEM images of compound (a) 13; (b) 14; (c) 15; (d) 16 in PBS (bar 200 nm). Samples were stained with 2% of uranyl acetate.

amyloid peptide A β 1–42 [40].

Conclusions

New self-adjuvanting delivery systems are vital for the development of safe and effective peptide-based vaccines. It has been shown that fibrils may be an effective delivery system; therefore we examined the ability of isopeptides to self-assemble into fibrils via the pH-triggered O-N intramolecular migration reaction. The conjugates of valine-rich isopeptides and B-cell peptide epitopes (which served as an antigen) were synthesized, and their ability to form fibril-like structures was confirmed. Antigens in these conjugates were able to adopt the desired “native” conformation. The approach presented herein may serve as a new delivery system for peptide-based vaccines. An isopeptide-based vaccine could be stored in a non-aggregative form, and then converted to its active form prior to administration. This would circumvent the storage related over-aggregation, precipitation, and changes in other properties of fibril-based vaccines. In addition, very limited toxicity of such a vaccine could be expected because it is composed solely of endogenous components.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MS generated the idea, designed and coordinated the research, analysed and compiled the data, and wrote the manuscript. JK synthesized and analysed the conjugates. ZZ performed and analysed the TEM experiment. IT coordinated the research and wrote the manuscript. The final manuscript was read and approved by all of the authors.

There won't be any further changes in the authorship which includes either addition or removal of author's details and Mariusz Skwarczynski will be sole responsible person for all the communications and proceedings that are needed to be done with the publisher (According to the necessity of the publisher) on behalf of all the authors.

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