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Investigating the Effects of O-GlcNAc Modifications in Parkinson's Disease Using Semisynthetic α -Synuclein

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Abstract

α -Synuclein is a small aggregation-prone protein associated with Parkinson's disease (PD). The protein's biochemical and biophysical properties can be heavily influenced by various types of posttranslational modification (PTMs) such as phosphorylation, ubiquitination, and glycosylation. To understand the site-specific effects of various PTMs have on the protein and its aggregation, obtaining a homogeneous sample of the protein of interest with the specific modification of interest is key. Expressed protein ligation (EPL) has emerged as robust tool for building synthetic proteins bearing site-specific modifications. Here, we outline our approach for building α -synuclein with site specific O-GlcNAc modifications, an intracellular subtype of glycosylation that has been linked to the inhibition of protein aggregation. More specifically, we provide specific protocols for the synthesis of α -synuclein bearing an O-GlcNAc modification at threonine 72, termed α -synuclein(gT72). However, this general approach utilizing two recombinant fragments and one synthetic peptide is applicable to other sites and types of modifications and should be transferable to various other protein targets, including aggregation prone proteins like tau and TDP-43.

Keywords

α -synuclein; Posttranslational modification; Synthetic protein chemistry; Expressed protein ligation; O-GlcNAc

1 Introduction

α -Synuclein is a small protein of 140 amino acids (14 kDa) [1] that is found at a fairly high concentration of 50 μ M in presynaptic terminals [2]. This protein is composed of different domains: an N-terminal lysine rich domain that binds to membranes (residues 1–60), the nonamyloid component (NAC) domain that is hydrophobic and is the driving force in the aggregation process (residues 61–95), and an acidic C-terminal domain (residues 96–140) [3]. Monomeric α -synuclein is largely unstructured in solution but will bind to the membranes where the protein adopts an α -helical structure important for its vesicle

trafficking function [4–7]. In a process that is still poorly understood, α -synuclein can also form β -sheet rich amyloid aggregates that are toxic to cells and commonly found in the brains of patients suffering from Parkinson's disease and other synucleinopathies [8]. Although this aggregation process is understandably complicated, it can be broken down into a concentration-dependent two-step process: a nucleation step that yields small oligomers/seeds and a subsequent extension step that leads to the initial seeds being extended into larger, toxic fibrils [9].

α -Synuclein has been found to bear various posttranslational modifications (PTMs) in vivo, raising the possibility that these modifications may play either beneficial or deleterious roles in the initiation and/or progression of synucleinopathies [10, 11]. This includes O-GlcNAc modifications that have been found at nine different sites from a variety of in vivo proteomics studies (Fig. 1a) [12–16], including several within the NAC region, raising the question of whether they have an effect on the protein's aggregation properties. O-GlcNAcylation is the addition of the monosaccharide *N*-acetylglucosamine to serine and threonine residues of intracellular proteins (Fig. 1b) [17, 18]. Several lines of genetic and biochemical evidence support a role for O-GlcNAc in preventing neurodegeneration. Neuron-specific knockout of the enzyme responsible for adding O-GlcNAc, O-GlcNAc transferase, results in neurodegeneration and neuron cell death [19, 20], and the levels of this modification have been shown to be lower in Alzheimer's disease patients [21, 22]. Additionally, enzymatic O-GlcNAcylation of recombinant tau in vitro reduces its aggregation [23, 24], and increasing the amounts of O-GlcNAcylation slows neurodegeneration in a mouse model of Alzheimer's disease [23]. Recently, we have shown that site-specific O-GlcNAcylation at four different sites in the NAC domain of α -synuclein generally results in inhibition of its aggregation with interesting site-specific differences [25–27]. Importantly, this general inhibitory role for O-GlcNAcylation was confirmed by enzymatic modification of recombinant α -synuclein [28], but this resulted in a heterogeneous mixture of different O-GlcNAc modifications that were not separated.

Key to our ability to prepare site-specifically O-GlcNAcylated α -synuclein was the application of protein ligation techniques. More specifically, we took advantage of expressed protein ligation (EPL), an extension of native chemical ligation (NCL). NCL takes advantage of a reaction between a peptide thioester and peptide or protein bearing an N-terminal cysteine to create a native amide bond [29]. More specifically, the first step of an NCL reaction is a reversible transthioesterification between a C-terminal thioester of one peptide and an N-terminal cysteine residue of the other. Once this intermediate formed, an S-N acyl shift results in the native amide bond. While this technology can be used successfully for the generation of site-specifically modified proteins, the major drawback is the size limitation of solid phase peptide synthesis (SPPS). The solution to this problem, termed expressed protein ligation (EPL) [30], involves the generation of recombinant protein-thioesters, which occur naturally during protein splicing. Protein splicing is a posttranslational process where an intein fragment is removed from the sequence, which results in an amide bond formation between the two flanking segments or exteins. While the biological role of inteins is not clear, the splicing mechanism of inteins can be used to generate protein thioesters. Proteins of interest are recombinantly expressed as simple N-terminal fusion to an intein, which can be cleaved in the presence of exogenous thiols,

yielding a recombinant C-terminal protein thioester. This protein thioester can then participate in a ligation reaction. Expressed protein ligation allows the incorporation of synthetic peptides that contain PTMs to be incorporated into proteins. Our route involves a protein thioester, a synthetic O-GlcNAc-modified peptide and a protein fragment generated from *E. coli*.

The following protocol describes the use of EPL to synthesize α -synuclein with a single O-GlcNAc modification at threonine 72, or α -synuclein(gT72), as schematized in Fig. 2a. Specifically, O-GlcNAc modified threonine [31] is first incorporated into a synthetic peptide thioester (A) using SPPS on the Dawson thioester resin. Next, peptide A undergoes a ligation reaction with recombinant fragment B, obtained by heterologous expression in *E. coli*. The N-terminal thiazolidine of the resulting product is then removed to give intermediate D, which readily undergoes a second ligation reaction with protein thioester C to yield full-length O-GlcNAcylated α -synuclein. α -Synuclein contains no native cysteines residues. Therefore, the cysteines required for our synthesis can be transformed into the native alanine residues by a final desulfurization reaction. Importantly, we have applied this same general strategy to O-GlcNAc modifications at threonines 72, 75, and 81 or serine 87 by using the different ligation sites highlighted in Fig. 2b.

This protocol is representative of the synthesis of site-specific O-GlcNAcylated α -synuclein, but should be applicable toward the synthesis of other synthetic proteins bearing site specific PTMs. Specifically, we describe the incorporation of O-GlcNAc-modified threonine into peptides by using SPPS. Following the ligation reaction between the peptide (A) and C terminal fragment (B) of α -synuclein, expressed from *E. coli*, the ligation product 1 (D) is ligated to the N terminus of α -synuclein (C) that is expressed from *E. coli*. Finally, desulfurization of full length α -synuclein is conducted to convert cysteine to alanine (its native sequence) (Fig. 1b).

2 Materials

Prepare all solutions using ultrapure water (18 M Ω) H₂O at 25 °C. Dispose of waste appropriately.

2.1 Reagents

1. Growth Medium: LB (Luria–Bertani–Miller) broth [25 g/L]. This medium is used to generate recombinant proteins.
2. Growth Medium: TB (Terrific Broth) [50 g/L plus 8 mL of 50% glycerol]. This medium is used to generate recombinant proteins.
3. Antibiotic Stock Solution: 100 mg/mL ampicillin. Store at –20 °C. The final concentration in media is 100 μ L/mL.
4. Antibiotic Stock Solution: 50 mg/mL kanamycin solutions. Store at –20 °C. The final concentration 50 μ L/mL.
5. Lysis buffer for C-terminal fragment expression: 500 mM sodium chloride (NaCl), 100 mM Tris, 10 mM β -mercaptoethanol (β ME, added fresh), 1 mM

ethylenediaminetetraacetic acid (EDTA), pH 8.0. This buffer can be stored at 4 °C for ~2 months.

6. Lysis buffer for N-terminal fragment expression: 50 mM NaH₂PO₄, 300 mM NaCl, 5 mM imidazole, 2 mM tris(2-carboxyethyl)phosphine (TCEP) (freshly added), pH 8.0 with 2 mM PMSF (freshly added). This buffer can be stored at 4 °C for ~2 months.
7. Thiolysis buffer for protein thioester generation: 150 mM monosodium phosphate (NaH₂PO₄), 150 mM sodium 2-mercaptoethanesulfonate (MESNa), pH 7.4. This buffer should be prepared fresh.
8. Phenylmethylsulfonyl fluoride (PMSF) solution: 200 mM PMSF dissolved in iso-propanol. This stock solution should be prepared fresh.
9. Wash buffer for N-terminal fragment purification: 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 2 mM TCEP (freshly added), pH 7.4. This buffer can be stored at 4 °C for ~2 months.
10. Elution buffer for N-terminal fragment purification: 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, 2 mM TCEP (freshly added), pH 7.8. This buffer can be stored at 4 °C for ~2 months without the addition of TCEP.
11. VA-061 stock solution for desulfurization: 200 mM Va-061 dissolved in methanol. This stock solution should be prepared fresh.
12. MPAA stock solution for protein ligation reactions: 250 mM 4-Mercaptophenylacetic acid (MPAA), 3M guanidine, 300 mM phosphate pH 7.4. This stock solution can be stored at –20 °C for ~1 month.
13. TCEP stock solution: 300 mM TCEP, 3 M guanidine, 300 mM phosphate pH 7.4. This stock solution can be stored at –20 °C for ~1 month.
14. Ligation buffer: 6 M guanidine, 300 mM phosphate, pH 7.4. This buffer should be prepared fresh.
15. Desulfurization buffer: 6 M guanidine, 300 mM phosphate, 300 mM TCEP, pH 7.4. This buffer should be prepared fresh.
16. HPLC buffer A: 0.1% trifluoroacetic acid (TFA) in water.
17. HPLC buffer B: 0.1% trifluoroacetic acid (TFA), 10% acetonitrile, 90% water.
18. Dichloromethane.
19. *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU).
20. *N,N*-Diisopropylethylamide (DIEA).
21. Fmoc-protected amino acids.
22. Boc-Thz-OH.
23. Hydrazine hydrate solution: 80% hydrazine hydrate v/v in methanol (MeOH).

24. p-Nitrophenyl chloroformate.
25. Cleavage cocktail solution: 95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% water.
26. Peptide precipitation solution: diethyl ether.
27. Fmoc-deprotection solution: 20% v/v piperidine in DMF.
28. Dawson Dbz AM resin.
29. IPTG solution: 1 M isopropyl β -D-1-thiogalactopyranoside in H₂O.
30. Ethanethiol.
31. Tertbutylthiol.

2.2 Plasmids

1. pET42b vector: NdeI and SpeI restrictions sites for introducing C-terminal protein fragments (Fig. 2, fragments B).
2. pTBX1 vector: NdeI and Bpu10I restrictions sites for introducing N-terminal protein fragments as in-frame, 5' genetic fusions to the AvaE intein (Fig. 2, fragment C).

2.3 Equipment

1. Temperature controlled incubator/shaker for protein expression.
2. Water bath temperature controlled incubator.
3. Temperature controlled centrifuge.
4. Rocking platform to gently introduce mixing into solution.
5. High performance liquid chromatography (HPLC) for separation of various species.
6. Mass Spectrometer for peptide and protein characterization.
7. Lyophilizer.
8. Bio-Spin disposable chromatography column.
9. Gas dispersion tube, large, fritted.

3 Methods

3.1 C-terminal Protein Fragment Expression (B, Fig. 2)

The C-terminal protein fragment is obtained by heterogeneous expression in *E. coli*. During this expression the initiator methionine is endogenously removed from the recombinant protein, resulting in an N-terminal cysteine residue that can react with metabolites in the *E. coli* host. After purification, these modifications are removed by methoxyamine treatment to generate a free N-terminal cysteine required for protein ligation.

1. Transform BL21 (DE3) chemically competent *E. coli* with pET42b plasmid encoding the protein fragment onto a kanamycin-containing LB plate.
2. Inoculate 50 mL LB media containing kanamycin (50 μ L/mL) with a single colony to initiate a starter culture for recombinant expression. Incubate at 37 °C with constant agitation of 250 rpm for 12–16 h.
3. Inoculate 300 mL TB media with 3 mL of the starter culture, 50 μ L/mL kanamycin and incubate at 37 °C till OD₆₀₀ 0.7–1. Add final concentration of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubate at room temperature (RT) with constant agitation of 250 rpm for 18 h.
4. Harvest bacteria by centrifugation at 6000 $\times g$, 4 °C for 30 min. Discard the media.
5. Resuspend the bacterial pellet in 10 mL cold lysis buffer.
6. Lyse cells by incubating the sample at 80 °C for 10 min. Mix the contents of the tube every minute by inversion.
7. Incubate the sample at room temperature for 30 min.
8. Add freshly prepared phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM and incubate on ice for 30 min.
9. Centrifuge at 7000 $\times g$, 4 °C for 30 min.
10. Collect the supernatant by decanting and adjust its pH to 3.5 (*see* Note 1).
11. Incubate supernatant on ice for 30 min.
12. Centrifuge at 7000 $\times g$, 4 °C for 30 min.
13. Collect the supernatant by decanting and dialyze it against 2 L of degassed 1% acetic acid at 4 °C overnight (*see* Note 2).
14. Centrifuge protein solution at 7000 $\times g$, 4 °C for 30 min.
15. Reveal the N-terminal cysteine residue by adding methoxyamine hydrochloride to a final concentration of 250 mM and adjusting the pH to 4.0. Incubate at room temperature over night on a rocking platform.
16. Purify protein fragment using reverse-phase liquid chromatography (HPLC). Identify the desired protein fragment by MS.
17. Lyophilize the C-terminal fragment and store as a solid at –20 °C.

3.2 O-GlcNAcylated Peptide (A, Fig. 2)

The O-GlcNAc-modified threonine is incorporated into a synthetic peptide that contains the thioester that is necessary for NCL reaction. The ligation sites are located at the junction of glycine alanine or two alanine residues (Fig. 2b), where one alanine is mutated to a cysteine for NCL reaction. The last residue on the N-terminal portion of the peptide is Boc-Thz-OH. This thiazolidine ring is opened by methoxyamine to generate cysteine that is later necessary for NCL.

1. Add Dawson Dbz AM resin, 0.1 mmol scale, to a 5 mL disposable reaction vessel with frit for SPPS (*see* Note 3).
2. Incubate resin in 3–4 mL of dichloromethane (DCM) for 20 min, wash once with dimethylformamide (DMF), and incubate resin in 3–4 mL of DMF for an additional 20 min (*see* Note 4).
3. Incubate 2 × 20 min in 3–4 mL of 20% piperidine in DMF.
4. Wash the resin with DMF (*see* Note 4).
5. Activate amino acid by dissolving 5 equivalents (eq) of the appropriate *N*-Fmoc-protected amino acid, 5 eq of HBTU, 5 eq of *N,N*-diisopropylethylamine (DIEA) in 3–4 mL of DMF. Incubate for 4–6 min.
6. Incubate resin and preactivated amino acid (from **step 6**) for 1 h (*see* Note 5).
7. Wash the resin with DMF and repeat the **steps 3–7** to iteratively couple the remaining amino acids in the peptide sequence.
8. Couple O-GlcNAcylated threonine or serine amino acids at the appropriate positions (*see* Note 6). Dissolve 2 eq of pentafluorophenyl (PFP) O-GlcNAcylated threonine or serine in 4 mL of DMF and add to the growing peptide in the reaction vessel. Incubate overnight.
9. Couple Boc-Thz-OH using the reagents in **step 5** as the final amino acid.
10. Incubate peptide with hydrazine hydrate (80% v/v in methanol (MeOH)) twice for 45 min to remove the *O*-acetate groups on the O-GlcNAc.
11. Activate Dawson linker with treatment of 5 eq *p*-nitrophenyl chloroformate in 3–4 mL of DCM for 1 h, followed by 5 eq of DIEA in 3–4 mL of DMF for 30 min.
12. Cleave peptide with 7 mL of cleavage cocktail (95:2.5:2.5 trifluoroacetic acid (TFA)/H₂O/triisopropylsilane) room temperature for 3 h.
13. Use a Bio-Spin disposable chromatography column to filter cocktail cleavage solution into a clean tube.
14. Precipitate crude peptide solution in cold diethyl ether (1:10 cleavage solution to diethyl ether) at –80 °C overnight.
15. Centrifuge at 7000 × *g*, 4 °C for 30 min and air dry the peptide pellet.
16. Completely dissolve peptide pellet in a mixture of HPLC buffers A and B and then lyophilize.
17. Dissolve the solid peptide in 8 mL of thiolysis buffer and incubate the mixture at room temperature for 1 h.
18. Purify the peptide on C18 semipreparative column and characterize the peaks by MS.
19. Lyophilize the peptide and store as a solid at –20 °C.

3.3 N-terminal Protein Fragment Expression (C, Fig. 2)

The C-terminal protein fragment is obtained by heterogeneous expression as an N-terminal fusion to the AvaE intein in *E. coli*. After an initial purification, the intein can be cleaved by the addition of exogenous thiols to yield the N-terminal protein fragment as a C-terminal thioester for subsequent ligation reactions.

1. Transform BL21 (DE3) chemically competent *E. coli* with pTBX1 plasmid encoding the protein fragment onto an ampicillin-containing LB plate (100 μ L/mL).
2. Inoculate 50 mL LB media containing ampicillin (100 μ L/mL) with a single colony to initiate a starter culture for recombinant expression. Incubate at 37 °C with constant agitation of 250 rpm for 12–16 h.
3. Inoculate 300 mL TB media with 3 mL of the starter culture, ampicillin (100 μ L/mL) and incubate at 37 °C till OD₆₀₀ 0.7–1. Add final concentration of 0.5 M isopropyl β -D-1-thio-galactopyranoside (IPTG) and incubate at room temperature (RT) with constant agitation of 250 rpm for 18 h.
4. Harvest bacteria by centrifugation at 6000 $\times g$, 4 °C for 30 min. Discard the media.
5. Resuspend the cell pellets in 10 mL cold lysis buffer.
6. Lyse cells with a tip sonicator (35% amplitude, 30 s on/off, total time of 12 min) (*see Note 7*).
7. Centrifuge at 7000 $\times g$, 4 °C for 30 min.
8. Collect the supernatant by decanting.
9. Add 1 mL of Ni-AGA Agarose resin to a Bio-Spin disposable chromatography column.
10. Rinse the Ni-AGA Agarose resin with water (10 mL) and then 2 $\times$ 15 mL with wash buffer for N-terminal fragment purification.
11. Add the protein supernatant to the Ni-AGA agarose resin and incubate at 4 °C for 1 h.
12. Wash the matrix 3X with 10 mL of wash buffer for N-terminal fragment purification.
13. Elute the protein 3X with 4 mL of elution buffer for N-terminal fragment purification.
14. Combine the elution fractions and dialyze them against 1L of 0.5 Dulbecco's phosphate-buffered saline (DPBS) at 4 °C overnight (*see Note 2*).
15. Transfer protein solution to a clean tube and incubate the purified protein-intein fusion with 250 mM MESNa. This step will allow the intein to produce a protein thioester that can be intercepted with MESNa, resulting in the cleavage of the intein and generation of the N-terminal fragment thioester.

16. Adjust pH to 7.4 and incubate the reaction at RT overnight.
17. Purify protein on C4 semipreparative HPLC column and characterize protein peaks by MS.
18. Lyophilize the N-terminal protein fragment and store as a solid at -20°C .

3.4 Ligation Reaction 1: O-GlcNAc Peptide (69–90) (A, Fig. 2) and C-terminal Fragment (91–140) (B, Fig. 2)

1. Completely dissolve the lyophilized C-terminal protein fragment (B, Fig. 2) (1.6 mM) in the ligation buffer by bath sonication and then add this solution to a fresh tube containing O-GlcNAc peptide fragment A (2 mM). Repeat bath sonication to dissolve both components.
2. Add MPAA and TCEP from the stock solutions to a final concentration of 30 mM each.
3. The reaction pH should be within a 7.0–7.4 range. If necessary, raise the pH with 1 M NaOH.
4. Place reaction tube on the rocking platform.
5. Monitor reaction by HPLC. Take 1 μL of the reaction mixture and add to 20 μL of TCEP stock solution. Mix and inject (21 μL) into HPLC analytical C18 column. Run gradient 0–70% B over 60 min and characterize peaks by MS.
6. When reaction is complete, add 250 mM methoxyamine, adjust the pH of the reaction to 4.0 and incubate on the rocking platform at room temperature overnight. This will remove the N-terminal thiazolidine protecting-group and reveal the N-terminal cysteine of ligation product one (D, Fig. 2).
7. Run HPLC, collect all peaks and identify the desired peak (fragment D) by MS.
8. Before the purification, add a small amount of solid TCEP (~ 1 mg per 100 μL) to the reaction and incubate the tube at room temperature on the rocking platform for 10 min. This will reduce any disulfide bonds that may have formed.
9. Purify reaction on HPLC C18 semi-prep column.
10. Lyophilize the ligation product (D) and store as a solid at -20°C .

3.5 Ligation Reaction 2: N-Terminal Fragment (1–68) (C, Fig. 2) and Ligation-Product D (69–140) (Fig. 2)

1. Completely dissolve the ligation-product D (2 mM) and the N-terminal fragment C (8 mM) in ligation buffer by bath sonication.
2. Add MPAA and TCEP from the stocks to a final concentration of 30 mM each.
3. The reaction pH should be within a 7.0–7.4 range. If necessary, raise the pH with 1 M NaOH.
4. Place reaction tube on the rocking platform.

5. Monitor reaction by HPLC. Take 1 μ L of the reaction mixture and add to 20 μ L of TCEP stock solution. Mix and inject into HPLC analytical C18 column. Run gradient 0–70% B over 60 min and characterize peaks by MS.
6. When reaction is complete, add a small amount of solid TCEP (~1 mg per 100 μ L) to the reaction and incubate the tube at room temperature on the rocking platform for 10 min. This will reduce any disulfide bonds that may have formed.
7. Purify reaction on HPLC C18 semi-prep column.
8. Lyophilize the ligation product and store as a solid at -20°C .

3.6 Desulfurization of Ligation Product (1–140)

1. Dissolve ligation product (~0.75 mg/mL) in desulfurization buffer.
2. Transfer the solution to a round bottom flask and place the reaction in a water bath at 37°C .
3. Add 2.5% v/v ethanethiol and 10% v/v tertbutylthiol and place the reaction flask under a nitrogen atmosphere by first evacuating the air under reduced pressure and replacing it with nitrogen gas.
4. Stir the reaction for 10 min at 37°C .
5. Add VA-061 radical initiator to the reaction to give a final concentration of 2 mM.
6. Stir the reaction at 37°C overnight.
7. Monitor the progression of the reaction by HPLC (0–70% over 60 min) and purify the final product on C4 semi-prep HPLC column.
8. Lyophilize the final product and store as a solid at -20°C .

4 Notes

1. Lowering the pH to 3.5 results in the precipitation of endogenous *E. coli* proteins while the C-terminal protein fragment remains in solution.
2. To dialysis protein sample, use traditional dialysis membrane tubing with a molecular weight cutoff of 3500 Da.
3. For the peptide synthesis use reaction syringe with a filter frit.
4. Wash peptide by shaking the syringe in 3–4 mL of the appropriate solvent for 1 min, followed by extrusion of the solvent through the frit. Repeat this washing 4 times with additional DMF or DCM.
5. Couple first amino acid twice for 30 min (second time prepare fresh preactivation solution). Arginine and proline should be coupled twice for 45 min. Isoleucine, valine, and threonine should be coupled once for 1.5 h.
6. Do not add HBTU or DIEA to the PFP O-GlcNAcylated amino acid during preactivation step because PFP already acts as an activator of the carboxylate.

7. During tip sonication place the sample on the ice to prevent temperature increase of protein supernatant.

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Fig. 1.

O-GlcNAcylation of α -synuclein. (a) α -Synuclein has been found to be O-GlcNAcylated at nine different positions from in vivo proteomics studies. (b) O-GlcNAc is the reversible addition of the monosaccharide N-acetylglucosamine to serine and threonine residues of intracellular proteins

Fig. 2. Synthesis of O-GlcNAcylated α -synuclein. **(a)** Synthetic route of α -synuclein(gT72). **(b)** The primary sequence of human α -synuclein with the different O-GlcNAcylation sites (blue) that we have prepared and the ligation sites (red) that we have utilized