Bioorganic & Medicinal Chemistry 18 (2010) 3703–3706

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Multiple glycosylation of de novo designed α-helical coiled coil peptides

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ARTICLE INFO

Article history:
Received 18 January 2010
Revised 18 March 2010
Accepted 25 March 2010
Available online 27 March 2010

Keywords:
Glycopeptide
Post-translational modification
Helix
Solid phase synthesis

ABSTRACT

The aim of this study was to investigate the influence of multiple O-glycosylation in α-helical coiled coil peptides on the folding and stability. For this purpose we systematically incorporated one to six β-galactose residues into the solvent exposed positions of a 26 amino acid long coiled coil helix. Surprisingly, circular dichroism spectroscopy showed no unfolding of the coiled coil structure for all glycopeptides. Thermally induced denaturations reveal a successive but relative low destabilization of the coiled coil structure upon introduction of β-galactose residues. These first results indicate that *O*-glycosylation of the glycosylated variants is easily tolerated by this structural motif and pave the way for further functional studies.

1. Introduction

The α-helical coiled coil structure is an ubiquitous protein folding motif with diverse biological functions. It is represented in motor proteins as myosin and kinesin, in cytoskeletal proteins such as cortexillin and keratin, in DNA-binding proteins as GCN4, and it plays a key role during HIV fusion.[1,2]

Most proteins can be post-translationally modified by glycosylation. This reversible, covalent omnipresent modification can have great impact on the structure and function of proteins, ranging from changes in solubility, oligomerization and conformation, subcellular localization or binding, to interactions with other proteins.[3,4] Glycosylation is essential for a number of fusion proteins to fold into a trimeric state, which is again necessary for the approximation of the pathogen to the cell surface of the host cell.[2] However, the prediction of these changes is still challenging. De novo designed coiled coils have been proven to mimic protein structures and are therefore useful tools to elucidate protein structure and function. This study addresses the influence of multiple O-glycosylation on the conformation and stability of α-helical coiled coil peptides in order to understand the complex mechanisms involved in protein folding.

Coiled coils consist of two to seven α-helices wrapped around each other in a slight left-handed superhelical twist.[5] The primary structure is characterized by a periodicity of seven residues, the so-called heptad repeat, which is commonly denoted $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ (Fig. 1). Positions *a* and *d* are typically occupied by nonpolar residues that form the first recognition domain by hydrophobic core packing. Charged amino acids in positions *e* and *g* form the second recognition motif by interhelical electrostatic interactions. Polar residues are often found in the remaining heptad repeat positions *b*, *c* and *f*, which are solvent exposed. These positions are not directly involved in helix oligomerization and thus well suited for modifications such as glycosylation.[6] β-Galactose was introduced by glycosylation of serine's hydroxyl side chains in positions *b*, *c*, and *f*. Here, we used β-galactose as an easily accessible residue that serves as a model to study the impact of *O*-linked sugars on coiled coil peptide folding. All peptides were synthesized by conventional solid phase peptide synthesis (SPPS) following the Fmoc-protecting strategy and a building block approach to introduce the *O*-linked β-galactose.

Table 1 shows the sequences of the investigated peptides. Basis peptide *B* contains two additional serine residues (in positions 13 and 14) in comparison to peptide *A* to make possible an extension of glycosylated sites up to six galactose residues per 26mer of peptide. The conformation and stability of all peptides were investigated by circular dichroism spectroscopy.

2. Results and discussion

2.1. Oligomerization of unglycosylated and highly glycosylated peptide *B*

Analytical ultracentrifugation experiments were performed to exclude that differences in stability arise from a change in the olig-

Abbreviations: Abz, *o*-aminobenzoic acid; CD, circular dichroism; DBU, diazabicyclo[5,4,0]-undec-7-ene; DIC, diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; ESI-MS, electospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; (Gal-)S, β-D-galactopyranosyl-L-serine; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; SPPS, solid phase peptide synthesis; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane.

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doi:10.1016/j.bmc.2010.03.061

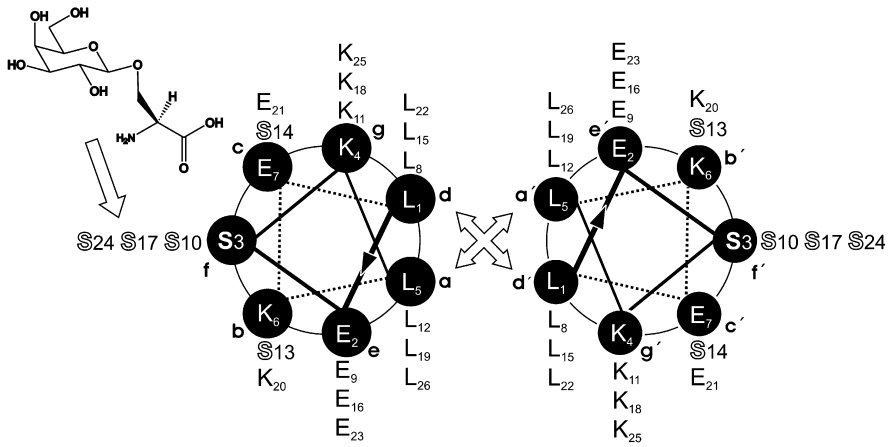

Figure 1. Helical wheel representation of the basis peptide *B* coiled coil dimer with the indicated glycosylation sites at positions 3, 10, 13, 14, 17, and 24.

Table 1
Sequences of the coiled coil-based model peptides

Peptide	Sequence
A	Abz–LESKLKELESKLKELESKLKELESKL-OH
A-10	Abz–LESKLKELE**(Gal-)S**KLKELESKLKELESKL-OH
A-17	Abz–LESKLKELESKLKELE**(Gal-)S**KLKELESKL-OH
A-10/17	Abz–LESKLKELE**(Gal-)S**KLKELE**(Gal-)S**KLKELESKL-OH
B	Abz-LESKLKELESKLSSLESKLKELESKL-OH
B-17	Abz-LESKLKELESKLSSLE**(Gal-)S**KLKELESKL-OH
B-10/13/14	Abz-LESKLKELE**(Gal-)S**KL**(Gal-)S(Gal-)S**LESKLKELESKL-OH
B-10/13/14/17	Abz-LESKLKELE**(Gal-)S**KL**(Gal-)S(Gal-)S**LE**(Gal-)S**KLKELESKL-OH
B-3/10/13/14/17/24	Abz-LE**(Gal-)S**KLKELE**(Gal-)S**KL**(Gal-)S(Gal-)S**LE **(Gal-)S**KLKELE**(Gal-)S**KL-OH

omerization state due to the glycosylation. Peptide oligomerization was determined for the unglycosylated control peptide *B* and for the extensively glycosylated peptide *B-3/10/13/14/17/24* which contains six galactose residues. Sedimentation molar mass distributions of both peptides were compared. The molecular weights of these peptides were determined from integration of the diffusion corrected molar mass distribution from sedimentation velocity experiments (Fig. 2). Table 2 shows that the observed molecular weights match the values of monomer and dimer for peptide *B* (M:

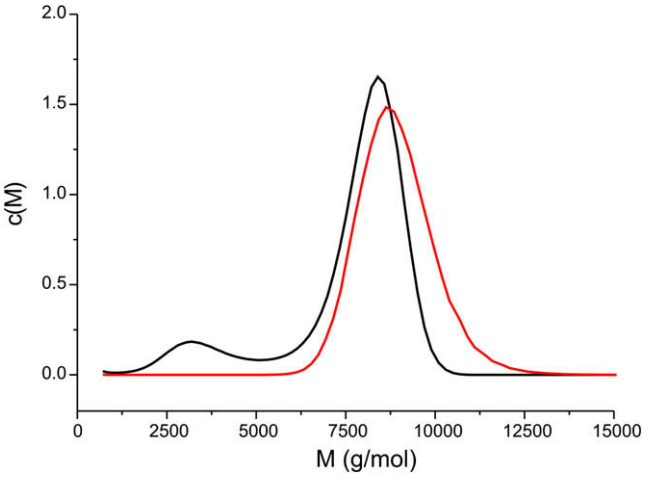

Figure 2. Molar mass distribution c(M) from sedimentation velocity analysis. Samples containing 100 μM peptide B (black line) and 100 μM peptide *B-3/10/13/14/17/24* (red line) in 100 mM phosphate buffer pH 7.4, respectively.

Table 2
Molecular mass distribution from sedimentation velocity experiments and respective surface areas in percent

Peptide	M_w[a] peak 1	M_w[a] peak 2
B	3510 (11%)	8206 (82%)
B-3/10/13/14/17/24	—	8905 (96%)

[a] In g/mol.

3109 g/mol), in which the dimer fraction is much bigger. For peptide *B-3/10/13/14/17/24* (M: 4081 g/mol) sedimentation velocity experiments reveal an exclusive dimeric structure. As both peptides show a dimeric structure as major compound, glycosylation has a negligible influence on the oligomerization state of the peptide.

2.2. Conformation and stability of the glycosylated coiled coil peptides

The impact of introduced *O*-linked β-galactose residues on the folding and stability of the coiled coil peptides was investigated by circular dichroism spectroscopy. Surprisingly, characteristic α-helical coiled coil CD-spectra with two minima at 208 and 222 nm were observed for all variants (Fig. 3). Temperature induced denaturation showed a successive but unexpectedly low impact on the coiled coil structure upon introduction of up to twelve β-galactose residues per coiled coil dimer (Fig. 3). Decreased melting temperature of peptide *B* compared to peptide *A* can be assigned to the replacement of glutamate and lysine at positions 13 and 14 by serine, which has a lower helix propensity.[7]

Most likely the destabilizing effect of glycosylation can be attributed to the sterical demand of the sugar moiety. Calculated volumes[8] reveal that single glycosylation increases the serine side chain volume dramatically from 26.1 to 168.7 Å. Furthermore, carbohydrate hydroxyl groups can be involved in hydrogen bonding to the backbone amides. These hydrogen bonds compete with the stabilizing intramolecular hydrogen bonding pattern in α-helices. However, hydrogen bonding has only a high impact on structural stability in hydrophobic environment and plays a minor role in solvated positions.[9] Since the galactose residues are incorporated into the solvent exposed face of the coiled coil helices, hydrogen bonding to the peptide backbone will have a negligible effect on peptide conformation in our study.

Up to date, only few studies dealing with the incorporation of carbohydrates in coiled coil or helix-loop-helix motifs have been undertaken and they have only assigned the aspect of single sub-

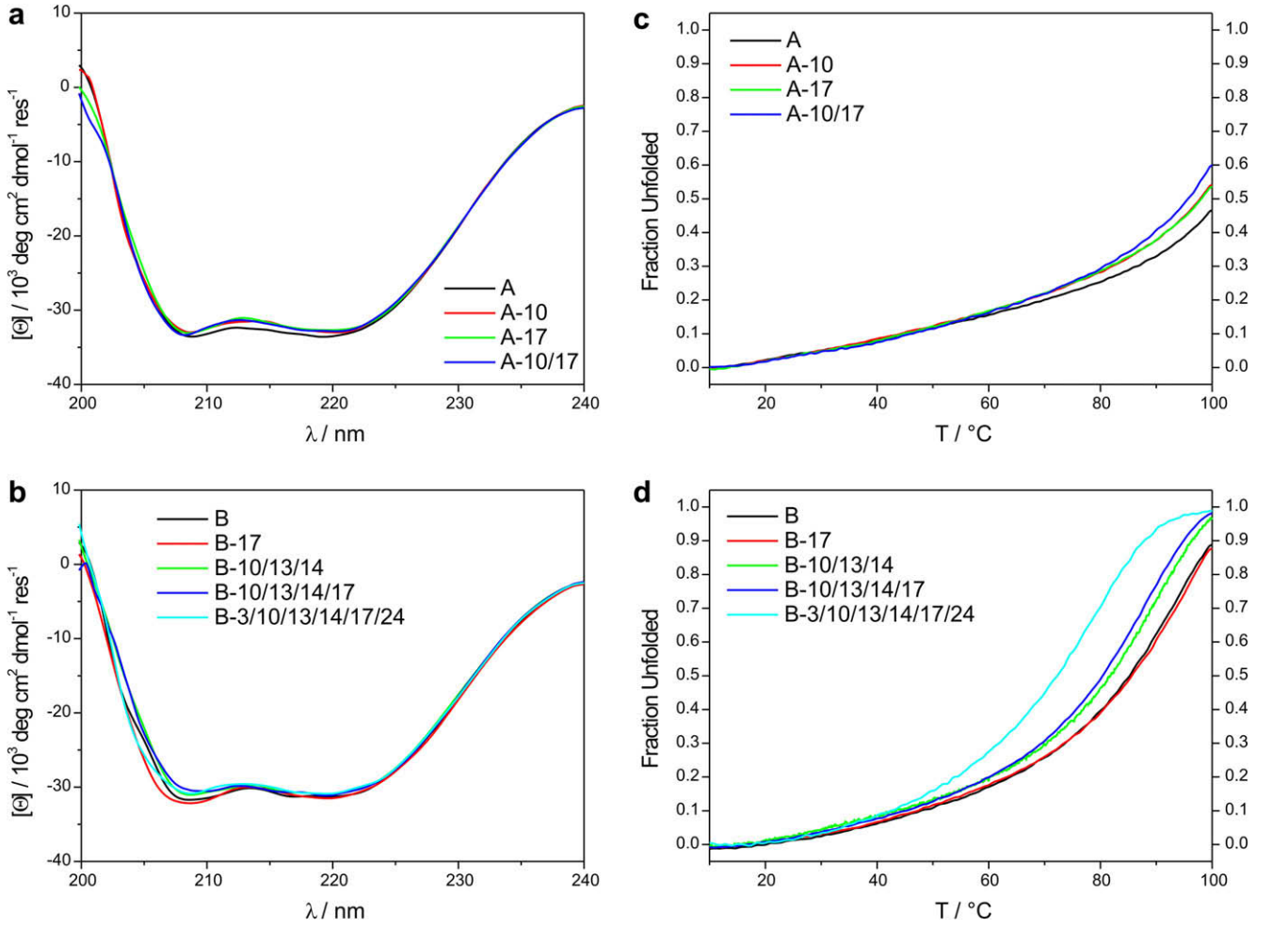

Figure 3. CD-spectra of peptides and corresponding glycopeptides *A* (a) and *B* (b). Thermal denaturation curves of peptides and corresponding glycopeptides *A* (c) and *B* (d). All peptides were dissolved in 100 mM phosphate buffer pH 7.4 containing 0.025% NaN_3.

stitution.[10–13] However, the investigation of multiple glycosylation is of major importance, since most protein-carbohydrate interactions—including the recognition of many bacteria and viruses by their host—are of multivalent nature.[2] Mehta et al have shown that the introduction of a single monosaccharide into position *d* of the hydrophobic core already led to an expected unfolding of the native coiled coil structure by perturbing the close interhelical side chain packing. However, herein presented results show that multiple O-glycosylations with the monosaccharide β-galactose in solvent exposed positions are well tolerated in coiled coil structures. This finding correlates well with the observation that N-glycosylation sites NXS/T are concentrated in the positions *b*, *c*, and *f* of the α-helical coiled coil domain of the glycoprotein laminin.[14] Based on these first results further studies are being preformed to elucidate the impact of more complex biologically relevant carbohydrate moieties.

3. Materials and methods

3.1. Synthesis of N^{α}-(9-fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine

The synthesis of the building block for SPPS followed the instructions of Kihlberg and co-workers (1).[15] Galactosepentaacetate (2.73 g, 7 mM) and Fmoc-L-Ser-OH (2.89 g, 8.37 mM) were dissolved in 70 ml ACN and $BF_3{\cdot}Et_2O$ (2.64 ml, 21 mM) was added slowly under nitrogen atmosphere. After 1 h stirring at room temperature the reaction mixture was diluted with DCM and washed with 1 M HCl (3 × 50 mL) and H_2O (2 × 50 mL). The organic phase was dried with $MgSO_4$ and the solvents were evaporated. The crude product was purified by preparative reversed phase HPLC equipped with a Luna™ C8 column (10 μ, 250 × 21.20 mm, Phenomenex). Eluation solvents were ACN/0.1% TFA and water Millipore/0.1% TFA. The flow rate was 20 mL min^{-1} and a gradient of 5% to 70% ACN in 30 min was used. In each run 250 mg raw product was dissolved in 1 mL ACN and injected. ACN and H_2O of the combined fractions were evaporated. The product was dissolved in DCM, which was evaporated immediately to give a white powder (2.18 g, 47% yield). ESI-MS $(M+H)^+$ calcd 658.2136, obsd 658.2140.

3.2. Peptide synthesis, purification, and characterization

Peptides were synthesized using a SyroXP-I peptide synthesizer (Multi-SynTech GmbH) on a 0.05 mM scale according to standard Fmoc/tBu chemistry. Preloaded Fmoc-Leu–Wang residue (0.64 mmol/g; Novabiochem®-Merck) was used for all peptides. For standard couplings a fourfold excess of amino acid and coupling reagents TBTU/HOBt as well as an eightfold excess of DIEA relative to resin loading was used. All couplings were preformed as double couplings (30 min). The coupling mixture contained 0.23 M $NaClO_4$ to prevent on-resin aggregation. Glycosylated amino acid was introduced manually. Fmoc-Ser(β-D-Gal$(Ac)_4$)-OH was activated by means of DIC/HOAt without the addition of base to prevent racemisation and deprotection of the sugar protecting groups. The molar

excess of Fmoc-Ser(β-D-Gal(Ac)$_4$)-OH and coupling reagents was reduced to 1.5-fold for the first and 0.5-fold for the second coupling and reaction time was extended to three hours. A mixture of DBU and piperidine (2% each) in DMF was used for Fmoc deprotection (4 × 5 min). Peptides were cleaved from resin by treatment with 2 mL TFA/TIPS/H_2O (90:9:1) for three hours. The resin was washed twice with TFA (1 mL) and DCM (dry, 1 mL) and excess solvent was removed by evaporation. The peptides were precipitated with cool diethyl ether, centrifuged, and dried in vacuum. Glycopeptides were deacetylated by the treatment with sodium methanolate (20 mM in methanol, pH 11) over night. Thereafter the pH was adjusted to 4 by addition of concentrated acetic acid and the solvent was evaporated. Purification of all peptides was carried out by preparative reversed phase HPLC equipped with a Luna™ C8 (10 μ, 250 × 21.20 mm, Phenomenex) column. Eluation solvents were ACN/0.1% TFA and water Millipore/0.1% TFA. The flow rate was 20 mL min^{-1}. Purified peptides were characterized by analytical HPLC and ESI-MS.

3.3. Concentration determination

Peptide concentrations were estimated by UV spectroscopy using the absorption maximum at 320 nm of *o*-aminobenzoic acid (Abz), which was attached to each N-terminus. A calibration curve was recorded using different concentrations of H_2N-Abz-Gly-OH·HCl. After concentration determination peptide solutions were diluted to yield 100 μM peptide.

3.4. Circular dichroism

Peptides were dissolved in phosphate buffer (100 mM, pH 7.4, 0.025% NaN_3). CD-spectra were recorded on a Jasco-715 spectropolarimeter at 20 °C using 0.1 cm Quartz Suprasil® cuvettes (Hellma). Thermal induced denaturations were preformed by recording the CD-signal at 222 nm upon heating from 10 to 100 °C (3 °C/min). Elipticity was normalized to concentration (c/mol L^{-1}), number of residues (n = 27, including the N-terminal label Abz) and path length (l/cm) using Eq. 1 where θ_{obs} is the measured ellipticity in millidegrees and [θ] the mean residue ellipticity in 10^3 deg cm^2 dmol^{-1} residue^{-1}. All measurements were repeated three times and the average was calculated.

$$[\theta] = \frac{\theta_{obs}}{10,000 \cdot l \cdot c \cdot n} \quad (1)$$

3.5. Analytical ultracentrifugation

Prior to AUC analysis, the proteins were extensively dialyzed against phosphate buffer (100 mM, pH 7.4) using a SpectraPor®-Membran (MWCO; Carl Roth GmbH). Analytical ultracentrifugation (AUC) was performed on a XL-I (Beckman-Coulter, Palo Alto, CA) ultracentrifuge at 25 °C applying the UV–vis absorption optics at 318 nm and using standard 12 mm double sector center pieces. Sedimentation velocity experiments were performed at 60,000 rpm and overall peptide concentrations of 100 μM. The samples were dissolved in 100 mM phosphate buffer at pH 7.4. The partial specific volume of the samples was determined in a density oscillation tube (DMA 5000, Anton Paar, Graz) to be 0.611 ml g^{-1} for peptide *B* and 0.715 ml g^{-1} for peptide *B-3/10/13/14/17/24*. The buffer density (ρ = 1.010934 g/ml) and buffer viscosity (η = 0.009243 P), both at 25 °C, were calculated using the freeware program SEDNTERP.[16,17] The sedimentation velocity data were evaluated using the program SEDFIT[18] yielding the sedimentation molar mass distributions shown in Figure 2.

Acknowledgment

We thank the DFG (SFB 765, 'Multivalency as Chemical Organization and Action Principle: New Architectures, Functions, and Applications') for financial support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.061.

References and notes

1. Parry, D. A. D.; Bruce Fraser, R. D.; Squire, J. M. *J. Struct. Biol.* **2008**, *163*, 258.
2. Dutch, R. E.; Jardetzky, T. S.; Lamb, R. A. *Biosci. Rep.* **2000**, *20*, 597.
3. Varki, A. *Glycobiology* **1993**, *3*, 97.
4. Dwek, A. D. *Chem. Rev.* **1996**, *96*, 683.
5. Woolfson, D. N. *Adv. Protein Chem.* **2005**, *70*, 79.
6. Pagel, K.; Koksch, B. *Curr. Opin. Chem. Biol.* **2008**, *12*, 730.
7. Chou, P. Y.; Fasman, G. D. *Biochemistry* **1974**, *13*, 222.
8. Zhao, Y. H.; Abraham, M. H.; Zissimos, A. M. *J. Org. Chem.* **2003**, *68*, 7368.
9. Scheike, J. A.; Baldauf, C.; Spengler, J.; Albericio, F.; Pisabarro, M. T.; Koksch, B. *Angew. Chem., Int. Ed.* **2007**, *46*, 7766.
10. Mehta, S.; Meldal, M.; Ferro, V.; Duus, J. Ø.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1997**, *9*, 1365.
11. Vijayalekshmi, S.; George, S. K.; Andersson, L. K.; Kihlberg, J.; Baltzer, L. *Org. Biomol. Chem.* **2003**, *1*, 2455.
12. Andersson, L. K.; Dolphin, G. T.; Kihlberg, J.; Baltzer, L. *J. Chem. Soc., Perkin Trans. 2* **2000**, *3*, 459.
13. Andersson, L.; Stenhagen, G.; Baltzer, L. *J. Org. Chem.* **1998**, *63*, 1366.
14. Beck, K.; Hunter, I.; Engel, J. *FASEB J.* **1990**, *4*, 148.
15. Kihlberg, J.; Elofsson, M.; Salvador, L. A. *Methods Enzymol.* **1997**, *289*, 221.
16. http://www.rasmb.bbri.org.
17. Lebowitz, J.; Lewis, M. S.; Schuck, P. *Protein Sci.* **2002**, *11*, 2067.
18. Schuck, P. *Biophys. J.* **2000**, *78*, 1606.