Application Note
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Structural Analysis of Glucagon in Aqueous and Organic Solvents Using Microfluidic Modulation Spectroscopy

- ○ Biosimilars
- ○ mAbs
- ○ ADCs
- ○ AAVs
- ○ Ligand Binding
- ☑ Protein/Peptide Analysis
- ○ VLPs
- ○ Nucleic Acid
- ○ Fusion Proteins
- ○ Enzyme Analysis

- ☑ Aggregation
- ⬢ Quantitiation
- ☑ Structure
- ☑ Stability
- ☑ Similarity

Abstract

Peptide drugs such as glucagon often face solubility and stability challenges that necessitate the use of organic solvents like dimethyl sulfoxide (DMSO), particularly in early discovery and screening stages. However, structural characterization of peptides in DMSO is difficult for most conventional biophysical techniques due to solvent interference. In this study, we applied Microfluidic Modulation Spectroscopy (MMS) to quantify the secondary structure of glucagon in increasing concentrations of DMSO and at varying pH conditions. MMS revealed a progressive reduction in intermolecular β-sheet content and an increase in α-helical and turn structures with increasing DMSO concentration, indicating a shift toward monomeric, helical conformations. Lowering the pH further enhanced α-helix formation and suppressed aggregation-prone β-sheets. These results highlight the ability of MMS to deliver high-resolution, quantitative secondary structure data for peptides in organic solvent environments, providing critical insights for early-stage peptide drug development where organic solvents like DMSO are frequently required.

Introduction

Glucagon is a peptide hormone essential for glucose homeostasis and has growing applications in emergency hypoglycemia treatment and metabolic disease therapies. However, its therapeutic use is challenged by instability and aggregation tendencies under aqueous conditions.[1] Understanding how solvents and pH influence glucagon's secondary structure is critical for improving formulation and storage strategies.

Analyzing peptide conformation in the presence of organic solvents such as DMSO presents a unique challenge for traditional biophysical techniques, as solvent absorption and interference can obscure spectral signals. This limitation has hindered the ability to accurately characterize peptides in formulation-relevant conditions that require organic co-solvents for solubility or stability.

Microfluidic Modulation Spectroscopy (MMS) offers a highly sensitive, label-free approach to analyzing protein and peptide conformation in native and formulated conditions. By leveraging infrared (IR) spectroscopy with real-time buffer referencing, MMS enables quantitative secondary structure determination even in complex solvent environments, including aqueous/organic mixtures and low pH conditions.

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Introduction, continued

This capability is especially important for therapeutic peptides like glucagon, where understanding structural behavior in both aqueous and organic solvents can guide downstream formulation strategies that minimize aggregation and extend shelf life. Here, we used MMS to monitor glucagon's structural changes in response to increasing DMSO concentrations and under different pH conditions.

Methods

Glucagon (1-29, human, powder, purity > 99.8%) was purchased from MedChemExpress (NJ, USA). For the DMSO gradient experiment, glucagon was directly dissolved in DI water and 10%, 20%, 30%, 40%, 50% DMSO:water mixture, and finally 100% DMSO at 1 mg/mL. The 100% DMSO sample was briefly heated in a warm water bath and sonicated to aid in dissolution. The pH for these samples were measured to be pH 5-5.2. For the pH experiment, the glucagon in water sample and the glucagon in 50% DMSO sample were adjusted to pH 2 using HCl. The exact same amount of HCl was spiked into their respective buffer reference. The same solvents were used as the buffer reference for the respective sample. The differential absorbance spectra of each sample against its buffer reference were measured across the Amide I band region (1712 – 1588 cm^{-1}) using the Aurora. Triplicate measurements were collected and averaged for each sample.

The data were analyzed using the RedShiftBio Analytics software package.[2] First, the raw differential absorbance was converted to absolute absorbance by normalizing concentration and optical path length. For samples in aqueous buffers, the displacement of the peptide molecules in solvent is considered due to water absorption. Thus, the raw differential absorbance spectra were also corrected using corresponding buffer spectra to generate absolute absorbance. For samples in 100% DMSO, the molecular displacement can be neglected as DMSO is effectively transparent in the Amide I band. The second derivatives of the absolute absorbance spectra are automatically generated along with the "similarity plot" by inverting and baseline subtracting the second derivative plot. Gaussian curve fitting is then applied to the similarity plot to generate the higher-order structure (HOS) bar chart.

The secondary structure assignments are shown in Table 1.

Table 1: Gaussian curve fit settings and HOS structural elements designations, adopted from Dong et al.[3,4]

Wavenumber (cm^{-1})	HOS Structural Element
1618	Intermolecular β-sheet
1624	Intermolecular β-sheet
1627	β-sheet
1638	β-sheet
1642	β-sheet
1650	Unordered
1656	α-helix
1666	Turn
1672	Turn
1680	Turn
1688	Turn
1695	Intermolecular β-sheet

Results

Effect of DMSO on glucagon structure

MMS analysis of glucagon in varying concentrations of DMSO revealed a clear transition in the peptide's secondary structure (Figure 1). In pure water, glucagon displayed predominantly beta-sheet and intermolecular beta-sheet features (notably around 1630 cm^{-1}), indicative of structural aggregation. With increasing DMSO content, there was a progressive decrease in beta-sheet content (1630 cm^{-1}, native and intermolecular) and a corresponding increase in alpha-helical structure (around 1658 cm^{-1}) and turn structure (1664 – 1680 cm^{-1}) (Figure 1B). At 50% DMSO, turn structure became the major conformation with moderate amount of beta-sheet and alpha-helix structures. At 100% DMSO, the turn structure became dominant at over 60%, while maintaining the level of alpha-helix structure with less than 10% native beta-sheet and intermolecular beta-sheet structures (Figure 1C). Note that these peak assignments are based on the IR absorption of protein secondary structure in aqueous solutions (Table 1).[3,4] Previous studies in the literature have suggested that the IR absorption of protein secondary structure in DMSO remains unchanged compared to water.[5]

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Results, continued

These observations are consistent with DMSO's known property of stabilizing alpha-helical structures and disrupting intermolecular interactions that promote aggregation.[6] Overall, at 0% to 50% concentration in water, DMSO effectively reduces glucagon aggregation by shifting the conformation toward a monomeric, moderately helical structure. At 100% DMSO, almost all intermolecular interactions disappeared. The glucagon conformation becomes mostly disordered (i.e. loose turn structure) with moderate amounts of stable helical structure.

This transition is in line with a previous study on DMSO-dissolved insulin, where the amyloid fibril of insulin converts to a monomeric disordered structure from aqueous to DMSO.[7]

Effect of pH on glucagon structure

When comparing glucagon's structure at different pH conditions, we observed that in both 0% and 50% DMSO, glucagon at pH 2 exhibited a higher alpha-helical content and reduced beta-sheet formation (Figure 2).

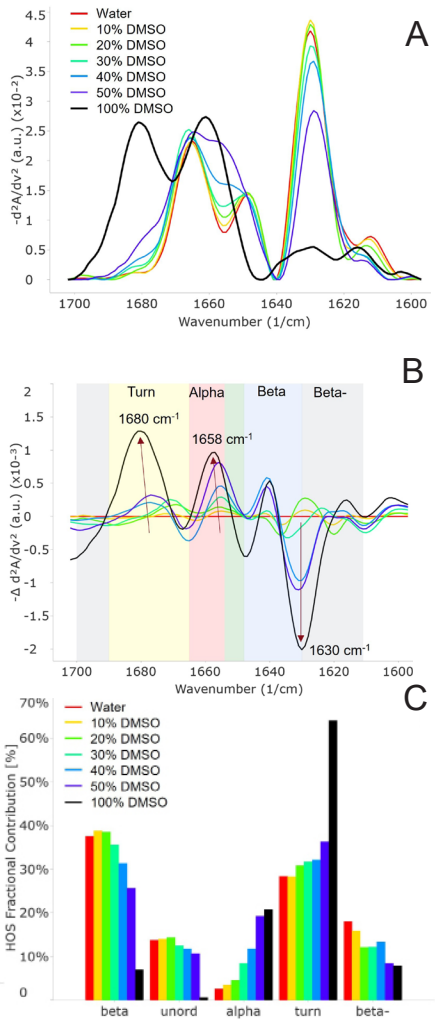

Figure 1. Structural transition of glucagon from 0% to 100% DMSO. (A) Similarity spectra, or inversed and baselined second derivative spectra. (B) Delta of similarity spectra showing clear changes of turn (1680 cm^{-1}), alpha (1658 cm^{-1}), and beta (1630 cm^{-1}) as the concentration of DMSO increases. (C) HOS bar graph reveals loss of beta-sheet and intermolecular beta-sheet and gain in alpha-helix and turn structures with increasing DMSO concentration.

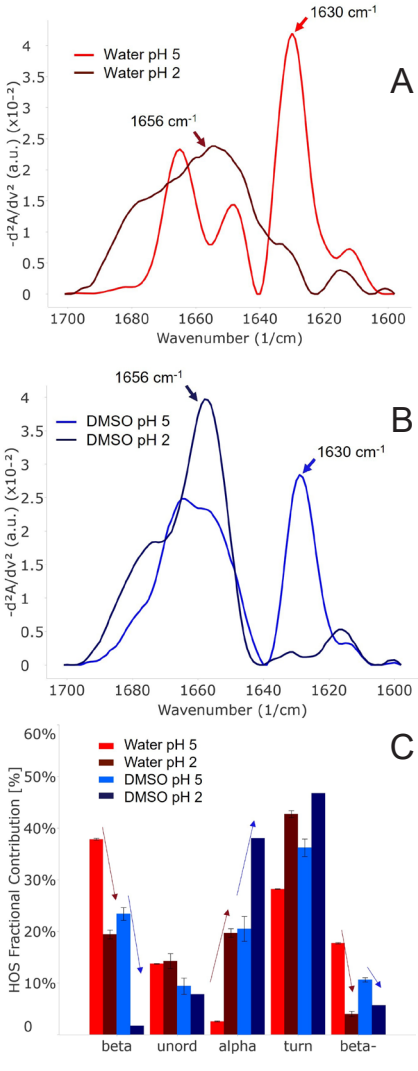

Figure 2. Similarity spectra of pH 2 (dark colors) and pH 5 (light colors) samples in water (A) and in 50% DMSO (B) show that the pH 2 samples consistently exhibit higher alpha-helix content and significantly reduced beta-sheet structures compared to pH 5. HOS bar graph (C) shows an overall reduction of 20% beta-sheet structure, 10-15% of aggregated beta-sheet, and nearly 20% increase of alpha-helix when the pH changes from 5 to 2.

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Results, continued

The effect was more pronounced in aqueous conditions, suggesting enhanced stabilization under acidic environments. As expected, low pH conditions stabilize glucagon's alpha-helical form, minimizing aggregation-prone beta structures. This behavior aligns with studies in literature and highlights low pH as a favorable condition for the solubilization of glucagon.[1,8]

Conclusion

This study confirms that both DMSO concentration and acidic pH significantly influence glucagon's conformation. Increasing DMSO shifts glucagon toward a more helical and monomeric structure, while low pH enhances helix formation and reduces intermolecular beta-sheet aggregation. These findings provide actionable insights for the rational design of glucagon formulations, especially for liquid or injectable products requiring enhanced shelf life and stability.

Moreover, this work highlights the value of having a robust biophysical tool capable of analyzing peptides in the presence of organic solvents. MMS's solvent-referencing capability overcomes the spectral interference challenges that limit many conventional techniques, enabling accurate secondary structure characterization under formulation-relevant conditions. Many therapeutic peptide candidates in early discovery require dissolution in organic solvents like DMSO for solubility or assay compatibility, making MMS essential for accurate structural characterization and high-throughput screening under these conditions.

Contributors

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References

1. Pisano, R. et al. Understanding Glucagon Aggregation: In Silico Insights and Experimental Validation. *Mol Pharm* 21, 3815–3823 (2024).

2. Lucas, L., Tsoi, P. S., Nair, A., Ferreon, A. C. M. & Ferreon, J. C. Sensing Protein Structural Transitions with Microfluidic Modulation Infrared Spectroscopy. *Biosensors* (Basel) 15, (2025).

3. Dong, A., Huang, P. & Caughey, W. S. Protein Secondary Structures in Water from Second-Derivative Amide I Infrared Spectra. *Biochemistry* 29, 3303–3308 (1990).

4. Dong, A., Meyer, J. D., Brown, J. L., Manning, M. C. & Carpenter, J. F. Comparative fourier transform infrared and circular dichroism spectroscopic analysis of α1-proteinase inhibitor and ovalbumin in aqueous solution. *Arch Biochem Biophys* 383, 148–155 (2000).

5. Liu, C., Bo, A., Cheng, G., Lin, X. & Dong, S. Characterization of the structural and functional changes of hemoglobin in dimethyl sulfoxide by spectroscopic techniques. *Biochimica et Biophysica Acta* (BBA) - Protein Structure and Molecular Enzymology 1385, 53–60 (1998).

6. Duarte, A. M. S., Van Mierlo, C. P. M. & Hemminga, M. A. Molecular dynamics study of the solvation of an α-helical transmembrane peptide by DMSO. *Journal of Physical Chemistry B* 112, 8664–8671 (2008).

7. Dzwolak, W. et al. On the DMSO-Dissolved State of Insulin: A Vibrational Spectroscopic Study of Structural Disorder. *J Phys Chem B* 116, 11863–11871 (2012).

8. Joshi, A. B., Rus, E. & Kirsch, L. E. The degradation pathways of glucagon in acidic solutions. *Int J Pharm* 203, 115–125 (2000).