The abnormal fibrillation of human islet amyloid polypeptide (hIAPP) is associated with development of type II diabetes mellitus (T2DM). Epigallocatechin gallate (EGCG) can bind amyloid proteins to inhibit the fibrillation of these proteins. However, the mechanistic detail of EGCG inhibiting amyloid formation is still unclear at the molecular level. In the present work, we sought to investigate the effect of EGCG on amidated hIAPP (hIAPP-NH₂) fibrillation and aggregation by using spectroscopic and microscopic techniques, and also sought to gain insights into the interaction of EGCG and hIAPP₂₂₋₂₇ by using spectroscopic experiments and quantum chemical calculations. ThT fluorescence, real-time NMR, and TEM studies demonstrated that EGCG inhibits the formation of hIAPP-NH₂ fibrils, while promoting the formation of hIAPP-NH₂ amorphous aggregates. Phenylalanine intrinsic fluorescence and NMR studies of the EGCG/hIAPP₂₂₋₂₇ complex revealed three important binding sites including the A ring of EGCG, residue Phe23, and residue Ile26. DFT calculations identified the dominant binding structures of EGCG/Phe23 and EGCG/Ile26 complexes, named structure I and structure II, respectively. Our study demonstrates the inhibitory mechanism of EGCG on fibrillation and aggregation of hIAPP-NH₂, in which EGCG interacts with hIAPP-NH₂ through hydrogen bonding and π-π interactions between the A ring and residue Phe23 as well as hydrophobic interactions between the A ring and residue Ile26, which can thus inhibit the interpeptide interaction between hIAPP-NH₂ monomers and finally inhibit fibrillation of hIAPP-NH₂. This study agrees with and reinforces previous studies and offers an intuitive explanation at both the atomic and molecular levels. Our findings may provide an invaluable reference for the future development of new drugs in the management of diabetes.

1. Introduction

Amyloid fibrils are involved with more than 30 human diseases,[1] including neurodegenerative disorders such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), and amyotrophic lateral sclerosis (ALS), as well as systemic disorders such as secondary amyloidosis and type II diabetes mellitus (T2DM). Despite many differences in primary sequence and natively unfolded structure of the precursor proteins, the amyloid deposits share common structural, chemical, and biological features, for instance, an extensive cross-β-sheet quaternary structure with 80–150 nm in diameter,[2] the appearance of apple green birefringence after staining with Congo red, and the increased fluorescence intensity of thioflavin T (ThT).[3]

A number of experimental methods, such as circular dichroism spectroscopy, NMR spectroscopy, infrared spectroscopy, fluorescence spectroscopy, and light scattering techniques, can be used to monitor the kinetics of amyloid fibrillation in situ and to determine the kinetic parameters. ThT fluorescence is one of the most widely used experimental approaches to probe the kinetics of β-sheet enriched fibrillation with high sensitivity, and increases markedly upon binding to the stacked β-sheets of amyloid fibers. The observed increasing ThT signal corresponds to the content of hIAPP-NH₂ fibrils.[4] NMR spectroscopy can monitor the aggregation of protein on the fact that the solidification of protein makes the resonance peaks broader or even cover in the noise background for the specific amino acid residues on the atomic level.[5]

Deposition of human islet amyloid polypeptide (hIAPP, Figure 1A) is found in most of type II diabetes mellitus (T2DM) patients with loss of at least 50% β-cell mass.[6] hIAPP is a 37-amino acid polypeptide derived from an 89-amino acid preproprotein, which is coproduced and costored with insulin in pancreatic islet β-cells.[7] The amidated hIAPP (hIAPP-NH₂) is the mature form of soluble hIAPP. The functions of hIAPP include
However, hIAPP tends to form fibrils. solutions for the ThT fluorescence assay and NMR spectroscopy. Additionally, EGCG has shown the ability to scavenge free radicals including superoxide, hydroxyl radicals, hydrogen peroxide, and nitric oxide, which are involved in the progression of β-cell apoptosis as well as the development of diabetic complications.\\n
The goal of this paper is to investigate the mechanism of the molecular recognition between EGCG and hIAPP-NH2. We first focus our attention on the influence of EGCG on the fibrillation of hIAPP-NH2 by using thioflavin T (ThT) fluorescence, nuclear magnetic resonance (NMR) spectroscopy, and transmission electron microscopy (TEM). We then sought to investigate the interaction between hIAPP-NH2 and EGCG by using experimental methods including phenylalanine intrinsic fluorescence assay and NMR spectroscopy. Finally, we performed quantum chemical calculations for residues Phe23 and Ile26 of hIAPP with or without EGCG at the density functional theory level and identified the possible binding modes of hIAPP-NH2 to EGCG. The obtained results will provide valuable information for the design and discovery of amyloid inhibitors.

**Materials and Methods**

**Materials**

hIAPP22–27 peptide (NL6) and amidated hIAPP1–37 peptide (hIAPP-NH2) were synthesized by Chinese Peptide, China. EGCG was purchased from Aladdin, China. Deuterated water (D2O) was purchased from J&K, China. Purities of hIAPP-NH2 and NL6 were higher than 95% and their molecular weights were checked by ESI-MS.

**Preparation of samples**

**Preparation of Peptide Stock Solutions**

hIAPP-NH2 was dissolved in HFIP and sonicated at room temperature for 3 min before use.

**Preparation of Samples for Kinetic Study of hIAPP-NH2**

For the fibrillation kinetic studies, the hIAPP-NH2 stock solution (800 μM) was mixed with an equivalent amount of ThT aqueous solution, as well as various volumes of 15 mM EGCG fresh aqueous solution, and then diluted with phosphate buffered saline (PBS) aqueous solution (20 mM, pH 7.4). The final concentrations of hIAPP-NH2 and ThT were both 12 μM for the ThT fluorescence study. Three replicates of each sample were prepared in a 96-well plate (NUNC no. 237108) with volume of 200 μL per well.

For aggregation kinetic studies, the hIAPP-NH2 stock solution (800 μM) was added into 0.4 mM PBS D2O solutions with 0, 6.25, 25, and 100 μM EGCG to give a 100 μM hIAPP-NH2 solution for the 1H NMR study.
Preparation of Samples for TEM Observations

The incubated solutions used for the real-time NMR assay were diluted to 16 μM hIAPP-NH₃ solution for TEM observations. Aliquots (10 μL) of the diluted solutions were dropped on the 300 mesh formvar-coated copper grids for 10 min, and dried at room temperature for 30 min. Then, the samples were gently rinsed with water to remove the PBS salt, and stained with 2% fresh uranyl acetate for another 2 min, and finally dried under vacuum overnight.

Preparation of EGCG/hIAPP-NH₃ Samples for ¹H NMR Study

hIAPP-NH₃ stock solution (800 μM) was added into D₂O with 0, 6.25, 25, and 100 μM EGCG to give a 100 μM hIAPP-NH₃ solution for the ¹H NMR study of hIAPP-NH₃ characteristic resonances.

Preparation of EGCG/NL₈ Samples for Fluorescence Spectroscopy and NMR Studies

NL₈ aqueous solution (320 μM) was mixed with 0, 10, 20, 40, and 80 μM EGCG for the intrinsic phenylalanine fluorescence spectroscopy study. NL₈ deuterium aqueous solution (1 mM) was mixed with 0, 1, 5, and 10 mM EGCG in D₂O for the ¹H NMR study of NL₈. EGCG (10 mM) was mixed with 0, 1, 2, and 10 mM NL₈ in D₂O for the ¹H NMR study of EGCG. EGCG (10 mM) was mixed with 0, 1, 2, and 10 mM NL₈ in D₂O. EGCG (15 mM) in D₂O, NL₈ in D₂O (10 mM), EGCG (6 mM) and NL₈ (6 mM) in D₂O were prepared for ¹³C NMR spectroscopy study.

Experimental Methods

Fluorescence Spectroscopy

ThT fluorescence of the hIAPP-NH₃ samples was detected by using a fluorescence plate reader (ThermoFisher, USA) with the excitation wavelength of 450 nm and emission wavelength of 480 nm and a 40 s interval for the kinetic study at 37 °C. The fluorescence intensity was referenced to that of ThT free solution. Experiments were replicated three times and averaged.

The intrinsic phenylalanine fluorescence of NL₈ interacting with EGCG was studied by using a QM 40 fluorescence spectrometer (PTI, USA) with a 1.0 cm path length quartz cell at the excitation wavelength of 218 nm and emission wavelengths from 258 to 318 nm and resolution of 1 nm at 25 °C. The fluorescence spectrum of the buffer solution was subtracted from that of the samples.

NMR Spectroscopy

All NMR spectra were recorded by using a Bruker Avance III HD 400 MHz spectrometer (Bruker BioSpin International, Germany) at 25 °C. ¹H NMR spectra were recorded for 48 transients and with 3.0 s pulse delays between each transient. For the aggregation study of hIAPP-NH₃, the kinetic measurements were performed with a 6 min interval. ¹³C NMR spectra were recorded for 1024 transients with 3.0 s pulse delays between each transient.

Transmission Electron Microscopy

The images of hIAPP-NH₃ aggregates were recorded by using a Tecnai G2 20 TWIN transmission electron microscope (FEI, USA) at 200 kV under vacuum. The point resolution was 0.27 nm, and the tracer resolution was 0.14 nm.

Logistic Equation

The ThT fluorescence and ¹H NMR integration intensity of hIAPP-NH₃ on the incubation time were fitted with the logistic equation, Equation (1).[27]

\[
I = \frac{I_{\text{max}}}{1 + e^{(t - t_{1/2})/k}}
\]

where \(I_{\text{max}}\) is the maximum fluorescence values of ThT or the maximum integration values of ¹H NMR resonances; \(t_{1/2}\) is the time required to reach half \(I_{\text{max}}\); and \(k\) is an apparent first-order rate constant for the hIAPP-NH₃ fibrillation. The symbol “-” of “±” was selected for the ThT fluorescence assay with the increased signal intensity, and “±” for the ¹H NMR assay with the decreased signal intensity. As reported,[27] the lag-time \(t_{0}\), the time before detectable amyloid aggregation occurred, was described by \(t_{0} = t_{1/2} - 2/k\).

Computational Methods

To get the geometries of possible configurations of EGCG/peptide complexes, quantum chemical calculations were performed at the density functional theory level by using the B97-D3[28] method with 6–31+G(d,p) basis set.[19] The Gaussian 09 program package[20] was used for all calculations. hIAPP-NH₃ has two residues, Phe23 and Ile26. To avoid large computational loading when studying the interaction between hIAPP-NH₃ and EGCG, as shown in Figure S1 (in the Supporting Information), 2-acetamido-N-methyl-3-phenylpropanamide (fragment A), chroman-5,7-diol (fragment B), and 2-acetamido-3-methylpentanamide (fragment C) were employed as models for Phe23, EGCG, and Ile26, respectively. Such an approach allows for the study of large molecules by rational fragmentation of their interaction sites.[21]

The B97 functional in combination with the D3 dispersion correction of Grimme et al. (B97-D3) has been proven to give reliable results to the structural and energetic properties of non-covalent systems.[22] Full geometry optimizations were carried out in the water solution, which was modelled by the polarizable continuum solvation model (IEFPCM)[23] with radii and non-electrostatic terms for the Truhlar and co-workers’ SMD solvation model based on density.[24] This solvation model is by far the most reliable one in predicting solvation free energies. The dielectric constant used for water is 78.3553. The convergence criteria used for the geometry optimization are 4.50 × 10⁻⁶ a.u. for gradients, and 1.80 × 10⁻⁶ a.u. for displacements. Harmonic vibrational analyses were carried out to verify if the optimized structure is a local minimum and to provide zero-point vibrational energy corrections and thermal corrections to various thermodynamic properties.

We applied the continuous set of gauge transformation (CSGT) method using the B97-D3/6–31+G(d,p) level to calculate the nucleus-independent shift (NICS) of side chain of Ile26.[25]

2. Results and Discussion

2.1. Influence of EGCG on the Fibrillation of hIAPP-NH₃ Revealed by ThT Fluorescence

To investigate the influence of EGCG on the fibrillation of hIAPP-NH₃, 12 μM hIAPP-NH₃ was incubated with various concentrations of EGCG from 0 to 48 μM, the fibrillation kinetics of the samples was monitored by ThT fluorescence (Figure 2).
The time courses of ThT fluorescence intensity shown in Figure 2A has a typical sigmoidal shape. The fibrillation kinetic parameters were determined by fitting the curves in Figure 2 by using Equation (1). Table S1 (in the Supporting Information) summarizes the $I_{\text{max}}$, $t_{1/2}$, $k$, and $t_0$ values. EGCG significantly affected the fibrillation of hIAPP-NH$_2$, the maximum ThT fluorescence $I_{\text{max}}$ sharply dropped from 3204 ± 19 to 757 ± 6 with the increased concentration of EGCG from 0 to 48 μM, whereas the lag time $t_0$ sharply increased from 13 ± 11 to 569 ± 5 s with the increased concentration of EGCG from 0 to 3 μM (Figure 2B and C). These findings demonstrated that EGCG can significantly inhibit the fibrillation and nucleation of hIAPP-NH$_2$.

2.2. Influence of EGCG on the Aggregation of hIAPP-NH$_2$ Revealed by Real-Time NMR Spectroscopy

NMR spectroscopy is a sensitive tool to detect monomers and oligomers, and can yield rapid atomic-level structural details. To evaluate the influence of EGCG on the aggregation of hIAPP-NH$_2$, we measured the $^1$H NMR spectrum of hIAPP-NH$_2$ in D$_2$O solution first (Figure S2 in the Supporting Information). Figure S2B shows the $^1$H NMR spectrum of the aromatic residues of hIAPP-NH$_2$. The assignments of the $^1$H NMR spectrum of the aromatic resonances of hIAPP-NH$_2$ are listed in Table S2 (in the Supporting Information).

Furthermore, the aggregation process of hIAPP-NH$_2$ with or without EGCG was investigated by real-time NMR spectroscopy (Figure 3). hIAPP-NH$_2$ (100 μM) was incubated with 0, 6.25, 25, and 100 μM EGCG in 0.4 mM PBS/D$_2$O solution at pH 7.4 and 25 °C. Figure 4 shows the normalized $^1$H NMR integrated intensities of the residues in hIAPP-NH$_2$ within the chemical shift range from 7.00 to 7.20 ppm as a function of incubation time for those samples. The integrated intensities were decreased in
a typical sigmoidal manner and no signals were observed after 2 h of incubation as large aggregates were formed, resulting in the short transverse relaxation time of the resonance and the broadened linewidth. Previous studies have also observed this phenomenon in amyloid fibrillation\(^\text{[5]}\) and amorphous aggregation.\(^\text{[26]}\) The fibrillation kinetic parameters were determined by fitting the curves in Figure 4 by using Equation (1) and the parameters are summarized in Table S3 (in the Supporting Information). The elongation rate \(k\) was sharply increased when the EGCG concentration increased to 25 and 100 \(\mu\text{M}\). The lag time \(t_0\), that is, nucleation time, was increased from 11 ± 3 to 22 ± 1 min when the concentration of EGCG was increased from 0 to 6.25 \(\mu\text{M}\), indicating that low concentrations of EGCG inhibit the formation of hIAPP-NH\(_2\) aggregation. However, \(t_0\) started to reduce to 7 ± 1 min and even shorter when the concentration of EGCG was higher than 6.25 \(\mu\text{M}\), which indicated that high concentrations of EGCG promoted the formation of hIAPP-NH\(_2\) aggregates.

2.3. Influence of EGCG on the Aggregation of hIAPP-NH\(_2\) Revealed by TEM

The aggregate morphologies of the real-time NMR samples were observed by TEM (Figure 5). Distinct and well-defined fibrils with diameters of about 35 nm were observed in the hIAPP-NH\(_2\) sample, which demonstrated that hIAPP-NH\(_2\) dominantly self-aggregated into fibrils (Figure 5 A). In contrast, only rare and morphological altered fibrils with diameters of about 20 nm were visible in the hIAPP-NH\(_2\) sample treated with 6.25 \(\mu\text{M}\) EGCG; fewer and thinner than the hIAPP-NH\(_2\) alone, indicating that a low concentration of EGCG could inhibit the hIAPP-NH\(_2\) fibrillation significantly (Figure 5 B). It is also found that hIAPP-NH\(_2\) aggregates incubated with 100 \(\mu\text{M}\) EGCG were twisted and ribbon-like in shape with average diameters of about 50 nm (Figure 5 C and D), suggesting that high concentrations of EGCG could promote hIAPP-NH\(_2\) forming amorphous aggregates and thus inhibit the formation of typical fibrils. These results demonstrate that EGCG could inhibit the formation of hIAPP-NH\(_2\) fibrils, while promoting the formation of hIAPP-NH\(_2\) amorphous aggregates.

2.4. Sites of Interaction of EGCG with NL\(_6\) Revealed by Phenylalanine Intrinsic Fluorescence

Intrinsic fluorescence can clearly reflect the internal changes in the protein upon ligand complexation. Experimental and theoretical studies suggest that aromatic interactions play an important role by affecting the orientation needed for the \(\beta\)-sheet fibrillation.\(^{[13b, 27]}\) As residue Phe23 plays a key role in the self-assembly fibrillation of hIAPP through interpeptide aromatic interactions\(^{[13b]}\), we investigated the interaction of Phe23 of NL\(_6\) with EGCG through phenylalanine intrinsic fluorescence. Fluorescence spectra of 320 \(\mu\text{M}\) NL\(_6\) with various concentrations of EGCG from 0 to 80 \(\mu\text{M}\) were recorded. The significantly decreased fluorescence intensity upon the addition of EGCG (Figure 6) demonstrated that EGCG binds to NL\(_6\). The slight blueshifts in the emission peak were due to restricted motion of the Phe23 residue\(^{[28]}\) as well as the movement of the Phe23 residue to more hydrophobic environment upon EGCG binding.\(^{[29]}\)

2.5. Sites of Interaction of EGCG with hIAPP-NH\(_2\) and NL\(_6\) Revealed by NMR Spectroscopy

NMR spectra were used to indicate the atomic-level detail of the interactions between EGCG and peptides. The \(^1\text{H}\) NMR spectra of hIAPP-NH\(_2\), NL\(_6\), and EGCG are shown in Figures S2, S3A, and S3B (in the Supporting Information), respectively. The assignments of the \(^1\text{H}\) NMR spectra of hIAPP-NH\(_2\) (aromatic
residues), NL₆, and EGCG are listed in Tables S2, S4, and S5 (in the Supporting Information), respectively.

As shown in Figure S4 (in the Supporting Information), the ¹H NMR spectra of 100 μM hIAPP-NH₂ with 0, 6.25, 25, and 100 μM EGCG were recorded. The peak features of hIAPP-NH₂ significantly changed after the addition of EGCG. The Hε resonances (symbol *) of phenylalanine 15 and 23 (Phe15/23) residues were stepwise up-field shifted from 7.15–7.18 ppm to 7.12–7.14 ppm.[30] Whereas the chemical shifts and line widths of the Hδ resonances of the Phe15/23 residues (7.08–7.10 ppm),[30] the Hδ resonances of His18 residue (7.05–7.08 ppm), as well as the Hε resonances (7.00–7.05 ppm) and Hδ resonances (6.69–6.75 ppm) of the tyrosine 37 (Tyr37) residue[31] were not significantly changed. The results indicate the presence of interactions between residues of Phe15/23 of hIAPP-NH₂ and EGCG.

¹H NMR spectra of 1 mM NL₆ with 0, 1, 5, and 10 mM EGCG were also recorded (Figure 7). EGCG sufficiently influenced the resonance intensities and chemical shifts of NL₆. Figure 7A shows the ¹H NMR signals of α-protons of Phe23, Ala25, and Leu27. The signals of the α-proton of Phe23 broadened stepwise and finally disappeared on titration with EGCG, whereas the signals of the α-protons of Ala25 and Leu27 were nearly unchanged. It was thought that NL₆ formed complexes with EGCG, and that the motion of the α-proton of Phe23 was restricted, leading to its signal being broadened.[32] The deviations in the chemical shift of methyl protons of NL₆ are shown in Figure 7B, where the strong deviation of Ile26 upon binding EGCG is evident. A marked stepwise down-field shift in the H-22 signals of Ile26 on the scale of about 0.008 ppm and a marked stepwise up-field shift in the H-24 signals of Ile26 on the scale of about 0.008 ppm upon the addition of EGCG were observed, whereas the H-29 and H-30 signals of Leu27 were nearly unchanged. A similar phenomenon was also observed for methylene protons and methane protons of NL₆ (Figure 7C). The H-21 signals of Ile26 stepwise shifted downfield on the scale of about 0.010 ppm and the H-23 signals of Ile26 stepwise shifted upfield on the scale of about 0.018 ppm upon interaction with EGCG, whereas the H-27 signals of Leu27 were nearly unchanged. These chemical shifts were considered to be ring-current effects and resulted mainly from the magnetic anisotropy of the aromatic ring of EGCG.[33] These results revealed that the residues Phe23 and Ile26 of NL₆ played a key role for the fibrillation and aggregation of hIAPP through binding to EGCG.

Meanwhile, ¹H NMR spectra of 10 mM EGCG in the absence and presence of various concentrations of NL₆ were observed (Figure 7D and Table S6 in the Supporting Information). The

![Figure 7](https://www.chemphyschem.org/article-pdf/2017/18/1611/1611-1619/1611-1619.pdf)

**Figure 7.** Interaction between EGCG and NL₆. ¹H NMR spectra of 1 mM NL₆ with 0 (black), 1 (red), 5 (blue), and 10 mM (dark cyan) EGCG within chemical shift ranges A) from 4.185 to 4.31 ppm, B) from 0.75 to 0.88 ppm, or C) from 1.37 to 1.85 ppm in D₂O at 25 °C. D) ¹H NMR spectra of 10 mM EGCG with 0 (black), 1 (red), 2 (blue), and 10 mM (dark cyan) NL₆ within the chemical shift range from 4.875 to 6.935 ppm in D₂O at 25 °C and the C-9 signals of EGCG, indicating the motions of C-7 and C-9 were restricted by addition of NL₆.[32] These results suggest the A ring is the main interaction site of EGCG to NL₆.
signals of protons in the A, B, and D rings were stepwise broadened on titration with NL₆, whereas the signals of methylene protons in the C ring were nearly unchanged. What’s more, ¹³C NMR spectra of EGCG, NL₆, and an EGCG/NL₆ mixture in a molar ratio of 1:1 were recorded (Figure S5 in the Supporting Information). All resonances of EGCG and NL₆ were observed in the spectrum of the mixture except for peaks at δ = 95.1 and 95.9 ppm, which are the C-7 and C-9 signals of EGCG, indicating the motions of C-7 and C-9 were restricted by addition of NL₆.[32] These results suggest the A ring is the main interaction site of EGCG with NL₆.

2.6. Interaction Mechanism between EGCG and NL₆ Revealed by Quantum Chemical Calculations

Density functional theory (DFT) calculations can yield valuable atomic-level information about the structural changes that are difficult to obtain experimentally.[34] On the basis of the results obtained from phenylalanine intrinsic fluorescence and NMR methods, we constructed three model systems to study the interaction between residue Phe23 (modeled by fragment A) and EGCG (modeled by fragment B) and one model system to study the interaction between residue Ile26 (modeled by fragment C) and EGCG (modeled by fragment B) in the EGCG/petide complexes at the DFT level, as described in the Materials and Methods section. For each model system, we considered many possible configurations. The three lowest-energy structures for the complexes between fragment A and fragment B are shown in Figure 8A (denoted as structure I) and Figure S6 (denoted as structures III and IV), respectively. One lowest-energy structure for the complexes between fragment C and fragment B is shown in Figure 8B (denoted as structure II). The Cartesian coordinates of those structures are shown in Figures S7–S10 (in the Supporting Information). In structure III, the benzene ring of Phe23 (denoted as the R ring) and the A ring of EGCG form a parallel, displaced π-stacked structure, whereas in structure I and structure IV, the R ring and A ring of EGCG form a parallel π-stacked structure.[35]

What’s more, networks consisting of two and three intermolecular hydrogen bonds were observed in structures I and IV, respectively.

To obtain a more quantitative understanding of the relative strength of the interactions between NL₆ and EGCG through Phe23 binding to EGCG in structures I, III, and IV, we calculated the relative binding free energies (ΔG = ΔH − TΔS) of these three structures. The relative binding free energies and their enthalpic and entropic components are shown in Table S7 (in the Supporting Information). It can be seen from Table S7 that the order of relative enthalpy change (ΔH) of these structures is structure IV (0.33 kcal mol⁻¹) > structure I (0.00 kcal mol⁻¹) > structure III (−1.39 kcal mol⁻¹), whereas the order of the entropy term (<M> > TΔS) of these structures at 298 K is structure III (1.65 kcal mol⁻¹) > structure IV (0.62 kcal mol⁻¹) > structure I (0.00 kcal mol⁻¹). It is generally agreed that π-stacking contributes modestly to the enthalpy change (ΔH)[36] and that the parallel, displaced π-stacked configuration is more stable than the parallel π-stacked configuration,[35a] whereas the entropy term (<M> > TΔS) correlates linearly with the number of intermolecular hydrogen bonds. Our calculation is consistent with previous reports.[35a,36] As a result, the order of the relative binding free energy (ΔG) of these structures at 298 K is structure IV (0.95 kcal mol⁻¹) > structure III (0.26 kcal mol⁻¹) > structure I (0.00 kcal mol⁻¹). Therefore, structure I with the lowest ΔG is the most energetically favorable configuration among these three structures.[37]

For structure I, the distance between the planes of the R ring and A ring is 3.547 Å, whereas the hydrogen-bond lengths r(H1–O1), r(H2–O2), and r(H3–O3) are 2.610, 2.151, and 2.935 Å, respectively. The formation of π-stacking and intermolecular hydrogen bonds limits the flexibility of the R ring and A ring, then reduces the degree of rotational freedom of the α-protons of Phe23 and the restriction of C-7 and C-9, which is consistent with our above results from NMR spectroscopy.

Based on the results from Figure 7B and C, we can conclude that the A ring and side chain of residue Ile26 should have CH–π interactions.[33] It is very likely that in the complex between hAPP-NH₂ and EGCG, the C-23 and C-24 of Ile26 located directly above the plane of the A ring of EGCG, whereas C-21 and C-22 do not. This was also verified by quantum chemical calculations of structure II (Figure 8B).

To inspect the ring-current effect of EGCG on the NMR chemical shifts of residue Ile26 qualitatively, the average isotropic shielding values as well as corresponding chemical shifts
of the protons at side chain of Ile26 without and with EGCG were calculated (shown in Table S8 in the Supporting Information). The order of the calculated average 1H chemical shifts on different carbon atoms of the residue was C-21 (~0.162 ppm) > C-22 (~0.356 ppm) > C-23 (~0.540 ppm) > C-24 (~0.837 ppm). This order is basically coincidental with the experimental 1H NMR data for the EGCG/NL2 mixture in a molar ratio of 10:1, which was C-21 (0.01 ppm) > C-22 (0.008 ppm) > C-24 (~0.008 ppm) > C-23 (~0.018 ppm). The significantly small experimental values were mainly attributed to the fast equilibrium between bound and free Ile26 on the NMR timescale.\(^{[38]}\)

The driving forces to form and stabilize the amyloid fibrils are the hydrogen bonding between the polar groups, and π–π interactions between the aromatic residues\(^{[37,39]}\) as well as hydrophobic interactions between the hydrophobic residues.\(^{[39,41]}\) What’s more, residues Phe23 and Ile26 play a key role in the fibrillation of hIAPP.\(^{[39,42]}\) Our calculation results show that EGCG can interact with hIAPP-NH\(_2\) through hydrogen bonding and π–π interactions between the A ring of EGCG and residue Phe23 and hydrophobic interactions between the A ring and residue Ile26 which can thus inhibit the interpeptide interaction between hIAPP-NH\(_2\) monomers and finally inhibit fibrillation of hIAPP-NH\(_2\). These computational results support the experimental ones well.

### 3. Conclusions

In this work, spectroscopic, microscopic, and computational methods were employed to gain insights into the interaction of EGCG with hIAPP-NH\(_2\). The fibrillation inhibition process, binding location, and binding mode were investigated. EGCG can significantly inhibit the formation of hIAPP-NH\(_2\) fibrils and promote the formation of hIAPP-NH2 amorphous aggregates, thus supporting the hypothesis that polyphenols could remarkably inhibit the formation of fibrils in vitro as summarized by Porat et al. in a recent review.\(^{[40]}\) The key interaction sites between EGCG and hIAPP-NH\(_2\) are the A ring, Phe23, and Ile26. Owing to the lowest binding free energy at structure I, this structure was considered as the dominant binding mode of the A ring of EGCG to Phe23. In structure I, EGCG forms parallel π-stacking and three intermolecular hydrogen bonds with Phe23. In addition, EGCG could interact with Ile26 through CH–π interactions between the A ring and side chain of Ile26, thus inducing a ring-current effect on Ile26. Structure II was considered the most probably structure with the A ring interacting with Ile26, in which the C-23 and C-24 of NL2 locate directly above the plane of the A ring. EGCG interacts with hIAPP-NH\(_2\) through hydrogen bonding and π–π interactions between the A ring and residue Phe23 as well as hydrophobic interactions between the A ring and residue Ile26 and thus can inhibit the interpeptide interaction between hIAPP-NH\(_2\) monomers and finally inhibit fibrillation of hIAPP-NH\(_2\).

It should be emphasized that the present work is, to our knowledge, the first focusing on the NMR spectroscopy of methyl and methylene protons of the protein whereas other NMR studies have mostly focused on the resonances of amide, α- and even β-protons.\(^{[39,42]}\)

This study agrees with and reinforces these previous studies and offers an intuitive explanation at the atomic level. Our findings may provide an valuable reference for the future development of new drugs for the management of diabetes.

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### Conflict of interest

The authors declare no conflict of interest.

### Keywords:

epigallocatechin gallate · human islet amyloid polypeptide · hydrophobic interactions · quantum chemical calculations · π–π interactions

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