Synchrotron-radiation vacuum-ultraviolet circular dichroism spectroscopy in structural biology: an overview

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Circular dichroism spectroscopy is widely used for analyzing the structures of chiral molecules, including biomolecules. Vacuum-ultraviolet circular dichroism (VUVCD) spectroscopy using synchrotron radiation can extend the short-wavelength limit into the vacuum-ultraviolet region (down to ~160 nm) to provide detailed and new information about the structures of biomolecules in combination with theoretical analysis and bioinformatics. The VUVCD spectra of saccharides can detect the high-energy transitions of chromophores such as hydroxy and acetal groups, disclosing the contributions of inter- or intramolecular hydrogen bonds to the equilibrium configuration of monosaccharides in aqueous solution. The roles of hydration in the fluctuation of the dihedral angles of carboxyl and amino groups of amino acids can be clarified by comparing the observed VUVCD spectra with those calculated theoretically. The VUVCD spectra of proteins markedly improves the accuracy of predicting the contents and number of segments of the secondary structures, and their amino acid sequences when combined with bioinformatics, for not only native but also nonnative and membrane-bound proteins. The VUVCD spectra of nucleic acids confirm the contributions of the base composition and sequence to the conformation in comparative analyses of synthetic polynucleotides composed of selected bases. This review surveys these recent applications of synchrotron-radiation VUVCD spectroscopy in structural biology, covering saccharides, amino acids, proteins, and nucleic acids.

Key words: amino acids, nucleic acids, proteins, saccharides, structural analysis

Vacuum-ultraviolet circular dichroism (VUVCD) spectroscopy (down to ~160 nm) using synchrotron radiation provides detailed and new information unobtainable in the far-UV region down to 190 nm. This spectroscopy discloses the equilibrium conformation of saccharides and amino acids associated with hydration. VUVCD spectroscopy can markedly improve estimations of the contents and number of segments of protein secondary structures, and their amino acid sequences when combined with bioinformatics. This spectroscopy also makes more-detailed conformational analysis of nucleic acids possible through comparative studies of synthetic polynucleotides. This review surveys these recent applications of synchrotron-radiation VUVCD spectroscopy that is now becoming an important tool in structural biology.
available for the high-energy transitions of chromophores such as hydroxy and acetal groups that involve absorption in the vacuum-ultraviolet (VUV) region below 190 nm [4]. This situation has prompted considerable efforts in developing vacuum-ultraviolet circular dichroism (VUVCD) spectrometers for obtaining more-detailed and new information about the structures of biomolecules.

The first VUVCD spectrometer was constructed based on a hydrogen Hinteregger discharge lamp in the latter half of the 1960s, and the device was applied to the structural analysis of biomaterials (mainly saccharides) from the beginning of the 1970s [5–10]. These pioneering studies demonstrated that the CD spectra in the VUV region can provide important information that is unobtainable in the far-UV region, but also that VUVCD measurement in solution was limited by the insufficient luminance of the light source. VUVCD spectrometers constructed since the 1980s have used synchrotron radiation (SR) because this provides an excellent high-flux source of photons that is three to six orders of magnitude more intense than that available from xenon lamps in the VUV region around 180 nm [2,11–15]. More than 15 VUVCD beam lines were constructed worldwide by 2017, and at present 9 of these beam lines are operational at the Aarhus Storage Ring (Denmark), Diamond Light Source (UK), Hiroshima Synchrotron Radiation Center (HiSOR) (Japan), Beijing Synchrotron Radiation Facility (China), National Synchrotron Radiation Research Center (Taiwan), Synchrotron SOLEIL (France), BESSYII (Germany), and ANKA (Germany). Most of the instruments use nitrogen purge and the technique is widely called synchrotron-radiation circular dichroism (SRCD) spectroscopy, which is equivalent to SR-VUVCD spectroscopy used in this review. The VUVCD spectrometer at HiSOR was established in 2000 and has been open to public use since 2004 [16].

SR-VUVCD spectroscopy is now becoming an important tool in structural biology (mainly protein structural analysis) in combination with advanced CD theory, computational calculation, and bioinformatics, which has been comprehensively reviewed for saccharides [16–18], proteins [16,19,20], and nucleic acids [21]. The accuracy of predicting the protein secondary structures (contents, numbers of segments, and amino acid sequences) has been markedly improved for native states but also unfolded and membrane-bound states. The relationships between the conformation and the base composition or sequence of nucleic acids have been clarified based on the SR-VUVCD spectra of synthetic polynucleotides. The SR-VUVCD spectra of monosaccharides have confirmed the contributions of configurations of anomeric hydroxy group, staggered configurations of hydroxymethyl group, and hydration to the equilibrium conformation in solution. The VUVCD spectra of amino acids indicate the important contribution of hydration to the fluctuation of dihedral angles of carboxyl and amino groups.

This review first provides a brief description of the theoretical basis of CD and its calculation, and then surveys the recent applications of SR-VUVCD spectroscopy in structural analyses of saccharides, amino acids, proteins, and nucleic acids, primarily based on our own work [16,18,20]. We also provide a perspective on the application of SR-VUVCD spectroscopy in structural biology.

### Basic Theory and Calculation of Circular Dichroism

CD is defined as the difference (ΔA) between the absorbance of left- and right-handed circularly polarized light \( A_\lambda = \varepsilon_\lambda LC \) and \( A_\lambda = \varepsilon_\lambda LC \), respectively) at a given wavelength:

\[
\Delta A = A_L - A_R = (\varepsilon_L - \varepsilon_R)L = \Delta\varepsilon LC
\]

where \( \varepsilon_L \) and \( \varepsilon_R \) are the molar absorptions of the sample for left- and right-handed circularly polarized light, respectively, \( C \) is the molar concentration of the sample, and \( L \) is the path length of the optical cell (in cm). The CD intensity is usually expressed as \( \Delta\varepsilon \) (in M\(^{-1}\) cm\(^{-1}\)) or the molar ellipticity \( [\theta] \) (in degrees cm\(^2\) dmol\(^{-1}\)); these two units are related as follows:

\[
[\theta] = 3298 \Delta\varepsilon
\]

SR-VUVCD measurements are basically the same as conventional CD measurements, but they require a higher concentration of sample because a short-path-length cell (<50 \( \mu \)m) is used in order to reduce the absorption of the solvent in the VUV region [20].

CD is induced by the interaction between electric and magnetic dipole transition moments of chromophores, and its intensity is related to the rotational strength that is theoretically defined by

\[
R_{\theta \lambda} = \text{Im}\{\langle \Psi_{\theta} | \hat{m} | \Psi_{\lambda} \rangle \cdot \langle \Psi_{\lambda} | \hat{m} | \Psi_{\theta} \rangle \}
\]

where \( R_{\theta \lambda} \) is the rotational strength of the electric transition from the “0” to “a” states, \( \hat{m} \) and \( \hat{m} \) are the electric and magnetic dipole moments, respectively, and \( \text{Im}\{\} \) is the imaginary part of a complex number. The final CD spectrum can be calculated using the following equations:

\[
R_i = 1.23 \times 10^{-42} \frac{[\theta]_i \Delta\lambda_i}{\lambda_i^3}
\]

\[
[\theta](\lambda) = \sum_i [\theta]_i \exp \left[ -\left( \frac{\lambda - \lambda_i}{\Delta\lambda_i} \right)^2 \right]
\]

where \( R_i \), \( [\theta]_i \), and \( \lambda_i \) are the rotational strength, molar ellipticity, and wavelength of the \( i \)th transition, respectively, and \( \Delta\lambda_i \) is the half bandwidth of a spectrum calculated assuming that it conforms to a Gaussian distribution.

For small molecules such as monosaccharides and amino acids, the initial structure of a target molecule is obtained using X-ray crystallography or NMR spectroscopy, or it is modeled using the standard molecular parameters. This ini-
Structural Analysis of Saccharides

Many VUVCD data were obtained for saccharides during the 1970s and 1980s without using an SR source, which revealed or predicted the relationships with structure and conformation, as comprehensively reviewed by Johnson and Stevens [4,17]. CD spectra of saccharides can be roughly divided into three wavelength regions: the two most common substituents, acetamido and carboxyl groups, display CD bands associated with the n–π* transitions at 200–240 nm and the π–π* transitions at 180–200 nm, whereas the n–σ* transitions of acetal and hydroxyl groups produce bands at 140–180 nm. VUVCD spectroscopy is especially advantageous for the structural analysis of unsubstituted saccharides because their chromophores exhibit absorbance only in the VUV region.

Unsubstituted saccharides

Monosaccharides

The VUVCD spectra of many monosaccharides and methyl aldopyranosides have been measured down to 165 nm in H₂O and D₂O, and to 140 nm using dried film samples [4,17]. Monosaccharides have very similar structures, but they exhibit markedly different VUVCD spectra in terms of peak positions and intensities: most monosaccharides show positive bands, but galactose shows negative bands around 160–180 nm. The CD bands around 160–180 nm predominantly arise from the electronic transitions (n–σ*) of the ring oxygen atom [24], which would be affected by the nearby hydroxy group at C-1 and the hydroxymethyl group at C-5. Film CD spectra provide important information about the originating orbital and energy levels (state assignments), but the relationships between CD spectra and structure in aqueous solution have not been determined explicitly due to the complexity of the equilibrium conformations, which include two anomic forms (α and β) of the hydroxy group at C-1, three staggered configurations—gauche–gauche (GG), gauche–trans (GT), and trans–gauche (TG)—of the hydroxymethyl group at C-5, and two chair conformations (C₁ and C₂). The contributions of these equilibrium conformers including solvent effects (hydration) have been investigated by deconvolution analysis and theoretical calculations of the VUVCD spectra down to 160 nm [18,23,25,26].

Disaccharides

The VUVCD spectra of disaccharides qualitatively resemble those of their monomer components [4,17,25]. For example, lactose, which is a disaccharide consisting of d-galactose and d-glucose joined by a β-1,4-glycosidic linkage, exhibits two successive negative CD peaks around 168 and 177 nm, which would arise from the constituent galactose unit. However, there is no additivity between the spectra of disaccharide and the constituent monomers since the anomic hydroxy group that links two monomers is fixed to an α or β form and the resulting ether linkages (e.g., 1→4 and 1→6) produce bands at 140–180 nm. VUVCD spectroscopy is especially advantageous for the structural analysis of unsubstituted saccharides because their chromophores exhibit absorbance only in the VUV region.

Matsuo and Gekko deconvolved the VUVCD spectra of d-glucose, d-mannose, and d-galactose into six independent Gaussian components (α-GG, α-GT, α-TG, β-GG, β-GT, and β-TG) based on their compositions as determined by NMR spectroscopy [25]. Those authors suggested that the GG and GT conformers exhibit positive and negative peaks, respectively, and that the negative peaks around 165 and 177 nm for d-galactose are attributable to the GT and β-TG conformers, respectively. The component spectra for three rotamers (α-GG, α-GT, and α-TG) of methyl α-1,6-glucopyranoside were calculated by Matsuo, K. et al., using the TDDFT combined with MD simulations [23,27]. The obtained spectra were similar to the corresponding component spectra revealed by deconvolution analysis, and a linear combination of these three spectra, which differ markedly from each other, essentially reproduced the experimentally observed spectrum of methyl α-1,6-glucopyranoside. These results indicate that the VUVCD spectra of monosaccharides are dominantly influenced by the configurations of the hydroxy group (α/β) at C-1 and the hydroxymethyl group (GG/GT/TG) at C-5. Furthermore, the differences in α-GT, α-GG, and α-TG spectra were found to be due to fluctuations of the hydroxymethyl group at C-5 and the hydroxy group at C-4, which strongly affected the orientations of intramolecular hydrogen bonds around the ring oxygen atom [18,23].

Hydration (intermolecular hydrogen bond) produces different VUVCD spectra of methyl α-1,6-glucopyranoside in H₂O and D₂O, with all hydrogen atoms of the hydroxyl groups being replaced by deuterium in the latter. The observed isotope differences in the peak position (blue shift) and the intensity of spectra were compared with those theoretically calculated using multicomponent quantum mechanics, which utilizes the quantum deviation of hydrogen nuclei from an equilibrium geometry to provide information about the conformation of the hydroxy groups and water molecules along the solvation surface [26]. That study revealed that modification of the solvation surface is essential for reproducing the observed isotope effect on the spectrum; that is, the isotope differences in the VUVCD spectra are strongly dependent on solute–solvent interactions. These observations demonstrate that the VUVCD spectrum can offer new insight into not only equilibrium conformations but also the hydration of saccharides.
Although the positive CD band around 167 nm is observed for a molecular weight ranging from 410 to 303,000 [29]. The different CD features of α-glycosidic linkage than for the β-glycosidic linkage in cellobiose are red shifted relative to those of isomaltose and 1,4-glycosidic linkage. The CD peaks of gentiobiose and mannohexaose, which is a nonreducing disaccharide formed by a 1,1-glycosidic linkage between any of two α-glucose and β-glucose molecules are joined by β-1,6- and β-1,4-glycosidic linkages, respectively, also both show a positive CD peak around 170 nm, whose intensity is larger for gentiobiose. These results suggest that the 1,6-glycosidic linkage has a greater CD-increasing potential than the 1,4-glycosidic linkage. The CD peaks of gentiobiose and cellobiose are red shifted relative to those of isomaltose and maltose, suggesting that the bond energy is higher for the α-glycosidic linkage than for the β-glycosidic linkage in both 1,4- and 1,6-linkages. The different CD features of the two glycosidic linkages may be more clearly reflected in the VUVCD spectra of α/α, α/β, and β/β isomers of trehalose, which is a nonreducing disaccharide formed by a 1,1-glycosidic linkage between any of two α-D-glucose and β-D-glucose units. In any case, these predictions must be confirmed by theoretical calculations of the VUVCD spectra of disaccharides, which may require a large amount of computation.

**Oligosaccharides and polysaccharides**

While the VUVCD spectra of oligosaccharides and polysaccharides have interesting features, few SR-VUVCD spectra have been measured. Three malto-oligosaccharides (maltose, maltotriose, and maltotetraose) with an α-1,4-glycoside linkage between glucose units exhibited negative CD around 190 nm and positive CD around 170 nm, which markedly changed as the chain length increased [28]. However, there were no indications of a chain-length dependence of CD attributable to the formation of a helical structure of amylose, which is a polysaccharide composed of maltose units. Stipanovic, A. J. et al. measured the VUVCD spectra of an isomalt-oligosaccharide series with an α-1,6-glycoside linkage between glucose units (dextran) in aqueous solution down to 175 nm and in films down to 150 nm over average molecular weights ranging from 410 to 303,000 [29]. Although the positive CD band around 167 nm in film spectra was not detectable in solution spectra, the ellipticity at 177 nm increased slightly (by less than 10%) with the molecular weight, but without showing the critical point around a molecular weight of 2,000 (about 12 degrees of polymerization) that was observed for various solution properties, including the amount of hydration [30]. Knowledge of the VUVCD spectra of oligosaccharide series down to 160 nm in solution would be useful for understanding the intra- and intermolecular interactions in the conformational transition from an oligomer (rod-like form) to a polymer (coiled form), which is closely related to the chain flexibility and properties of polysaccharides.

Many VUVCD data for polysaccharides are available, most of which have been reported by Stevens and coworkers [17,29,31,32]. All of the studied α-glucans consisting of 1,3- and 1,4-glycosidic linkages exhibit positive bands for the α-linkage (e.g., amyllose) and negative bands for the β-linkage (e.g., cellulose) around 164–172 nm, but no such correlation with anomeric configuration is observed for 1,6 glucans: both dextran (α-linkage) and pullulan (β-linkage) show a positive band around 167 nm. Some of the gelling polysaccharides display a small negative band around 180–190 nm. However, the gel of agarose, which is a polymer of repeating disaccharide units of d-galactose and 3,6-anhydro-L-galactopyranose, shows a positive band at 180 nm with a lower intensity and red shift upon melting [17]. This band around 180–190 nm is assigned to the ether oxygen atom of the linkage, and hence it may reflect the local flexibility of the polysaccharide chain. Comparing the VUVCD spectra of gels with theoretically calculated spectra would provide new insight into the cross-linking structures. Such studies may also be useful for confirming the modified hydration (volume change) due to the solvent–gel transition of polysaccharides [33].

**Substituted saccharides**

Many biologically important saccharides include various substituents such as carboxyl, acetamido, and sulfate groups, some of which exhibit absorption in the far-UV to VUV region. These substituents exert greater effects in the VUV region (160–190 nm) than in the far-UV region (190–240 nm) [34], and so SR-VUVCD spectroscopy provides much more information for characterizing the structures of substituted saccharides in aqueous solution.

Typical substituted polysaccharides are glycosaminoglycans such as heparin, chondroitin, chondroitin sulfates, and hyaluronic acid, which are linear polymers composed of repeating disaccharide units of hexosamine (glucosamine or galactosamine) and uronic acid (glucuronic or iduronic acid). These disaccharide units have similar structures, but the substituents such as carboxyl, acetamido, and sulfate groups, which are linear polymers composed of repeating disaccharide units of hexosamine (glucosamine or galactosamine) and uronic acid (glucuronic or iduronic acid). These disaccharide units exhibit different VUVCD spectra depending on the configuration, number, and position of the substituents [34–36]. Chondroitin sulfates A and B show markedly different spectra mainly due to differences in the configurations of the carboxyl group (equatorial or axial) and in the ring conformations (C1 or C1) [34]. Although theoretically assigning the bands in the VUV region is difficult due to the large number of overlapping electronic transitions (n–π*, π–π*, and n–σ*) and the modified ring conformation, the contributions of the substituents may be estimated by comparing the VUVCD spectra of glycosaminoglycans with those of the constituent monomers such as N-acetylgalactosamine and d-glucuronic acid.

Matsuo, K. et al. measured the SR-VUVCD spectra
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of four N-acetylaminosugars (N-acetylglucosamine, N-acetylgalactosamine, N-acetylgalactosamine 4-sulfate, and N-acetyllactosamine) and one uronic acid (p-glucuronic acid) down to 160 nm in aqueous solution [34]. N-acetylglucosamine has a negative band around 210 nm, a positive band around 180 nm, and a shoulder around 190 nm, while N-acetylgalactosamine shows two negative bands around 210 and 170 nm and a positive band around 190 nm [34,37]. The oligomers of chitin, which consists of β-1,4-glycoside linkages between N-acetylglucosamine units, all exhibit a negative CD band at about 210 nm and a positive band at about 192 nm, whose intensity increases with the chain length [38]. D-Glucuronic acid shows a positive band around 210 nm and a negative band around 185 nm, both of which are strongly pH dependent. The electronic transitions of the carboxyl group are influenced in complicated ways by the solvent conditions, the surrounding substituents, and the ring conformation: carboxymethylcellulose, which is a nonbiological polysaccharide but involves the biologically important carboxyl chromophore, shows very different CD spectra depending on the content (charge density), degree of neutralization, and counterions of the carboxyl group and added salts [4,39]. SR-VUVCD spectroscopy could be useful for studying not only the structures but also the electrostatic properties of substituted saccharides.

As described above, the VUVCD spectra of saccharides are complex due to their structural diversity. However, the high sensitivity of VUVCD spectra to the electronic transitions of chromophores can provide important and new information about the conformation, interaction, and hydration of saccharides in solution with the aid of theoretical calculations.

Structural Analysis of Amino Acids

Amino acids contain COOH and NH₂ groups as common chromophores that exhibit certain characteristic CD peaks based on their n–π* and π–π* transitions in the VUV region. In aqueous solution, these two groups exist in the form of zwitterions COO⁻ and NH₃⁺ whose ionization states or conformations are sensitively influenced by the solvent conditions (pH or hydration) and the side chains. This situation makes it difficult to assign the VUVCD spectra of amino acids. The first VUVCD spectra of amino acids were recorded to 160 nm in solution by Snyder, P.A. et al. [40], but those authors found it difficult to extract definitive conformational information due to the low accuracy of the spectra. Detailed theoretical analysis methods have been developed based on the VUVCD spectra of amino acids measured in the film and gas states [41–43]. Clear differences in the spectra between the solution and solid states indicate the presence of different molecular structures. The theoretical calculation of VUVCD spectra in solution can disclose the role of hydration in the equilibrium conformation of amino acids.

Contributions of side chains

The VUVCD spectra of various amino acids were measured from 260 to 140 nm in aqueous solution by Matsuo, K. et al. [44]. The spectra of l-alanine, l-valine, l-isoleucine, and l-leucine show two successive positive peaks around 200 and 180 nm at about pH 6, which are mainly attributable to the n–π* and π–π* transitions of COO⁻ and NH₃⁺ groups. These two peaks exhibit blue shifts and increased intensity when the alkyl side chains become more bulky. The [θ] values of both peaks are evidently smaller (two-thirds at around 200 nm) for l-isoleucine with an asymmetric β-carbon atom than for l-leucine, even though the side chains of the two amino acids have the same number of carbon atoms. This difference illustrates how the VUVCD spectra of amino acids are sensitively influenced by the size and asymmetry of the side chains.

The polar amino acids l-serine and l-threonine also show two positive peaks around 200 and 180 nm due to the COO⁻ and NH₃⁺ groups, and peaks or shoulders below 170 nm that are probably attributable to the side-chain OH group. The two successive positive peaks exhibit red shifts and large decreases in intensity for l-threonine with an asymmetric β-carbon atom. For l-lysine, the two peaks are more-clearly separated and red shifted to 210 and 185 nm accompanying shoulders below 180 nm, which may be associated with the transition of the side-chain NH₂ group. l-Proline shows a unique VUVCD spectrum, with a positive peak around 214 nm and two negative peaks around 194 and 166 nm, which is undoubtedly due to their characteristic cyclic five-member ring structure that differs from those of other amino acids [44]. Understanding these side-chain effects on the VUVCD spectra must await more-detailed theoretical analysis of the n–π* and π–π* transitions of COO⁻ and NH₃⁺ groups.

Contributions of side chains are also observed in hydroxy acids, in which an amino group (NH₂⁺) in amino acids is replaced by a hydroxy group (OH). Lactic acid, (S)-(+) 2-hydroxy-3-methylbutyric acid, and (S)-(−) 2-hydroxyisocaproic acid, which have the same side chains as alanine, valine, and leucine, respectively, show more-complicated VUVCD spectra in aqueous solution compared to the corresponding l-amino acids: no systematic size dependence of alkyl side chains is observed in hydroxy acids [45]. This suggests that the OH group in hydroxy acids exerts weaker hydration effects in fixing the equilibrium conformation than the NH₂⁺ group in amino acids.

Contributions of hydration

Theoretical calculations of VUVCD spectra are useful not only for assigning the CD peaks but also for analyzing the solution structure of amino acids, including the hydration state. Sebek, J. et al. compared the VUVCD spectra of diolamine measured down to 170 nm at three pH values (1.43, 6.17, and 12.1) with those theoretically calculated using the TDDFT method, which revealed that the anionic, cationic,
and zwitterionic forms of dialanine were hydrated with ten water molecules [46]. We compared the VUVCD spectra of \(l\)-alanine and \(l\)-lactic acid with those theoretically calculated using the DFT and TDDFT methods to clarify their hydrated structures [22,45]. Among 36 initial structures with various values for the pairs \((\phi, \psi)\) of dihedral angles of \(\text{COO}^- (\phi)\) and \(\text{NH}_3^+ (\psi)\) groups of alanine, the \((90^\circ, 60^\circ)\) structure was most-accurately optimized using a continuum model. In this optimized structure, six water molecules together formed a hydrogen-bond network around the \(\text{COO}^-\) group, and two of three hydrated water molecules around the \(\text{NH}_3^+\) group were incorporated in the hydrogen-bond network to restrict the rotation of the two groups. The VUVCD spectrum for this optimized structure showed positive peaks around 203 and 185 nm, negative peaks around 225 and 160 nm, and a small shoulder around 170 nm. These peaks and shoulder were comparable with the experimentally observed ones with the exception of the negative peak around 225 nm, which was experimentally observed as a small and broad band around 252 nm in a high-concentration sample [22].

While the observed VUVCD spectrum of \(l\)-alanine could be reproduced with only one optimized structure and nine hydrated water molecules, that of \(l\)-lactic acid required four optimized structures with seven hydrated water molecules (one around the \(\text{OH}\) group and six around the \(\text{COO}^-\) group) for theoretically reproducing [45]. Such diverse structures of \(l\)-lactic acid relative to \(l\)-alanine might originate from the hydration effects being weaker for the \(\text{OH}\) group than for the \(\text{NH}_3^+\) group: the hydrogen-bond networks of \(l\)-lactic acid were formed separately around the \(\text{OH}\) and \(\text{COO}^-\) groups but did not extend throughout the molecule, unlike those of \(l\)-alanine. The network with loose hydrogen bonding would allow easy rotation of the dihedral angle of the \(\text{COO}^-\) group of \(l\)-lactic acid. Since the four optimized structures showed very different VUVCD spectra, the modified population of such flexible conformations in equilibrium should be responsible for the complicated side-chain dependence of the VUVCD spectra for hydroxy acids.

This comparison of the VUVCD spectra between \(l\)-alanine and \(l\)-lactic acid clearly demonstrates that hydrated water molecules play a crucial role in stabilizing the conformation of amino acids and hydroxyl acids in aqueous solution. Future comparative studies of the experimental and theoretical VUVCD spectra for small molecules could yield important information not only for assigning the electronic transitions of chromophores but also for characterizing the equilibrium conformation, including the hydration states.

**Structural Analysis of Proteins**

The tertiary structures of numerous proteins remain unknown due to the experimental restrictions (crystallization or size limit of proteins) of X-ray crystallography and NMR spectroscopy. Although CD spectroscopy does not provide atomic-level resolution (unlike these other two techniques), it can provide valuable information about the secondary structures of both native and nonnative proteins because it can be applied in a wide range of solution conditions (e.g., pH, salts, organic solvents, lipids, and temperature). CD spectroscopy might be useful even when we have the atomic-resolution structure or even before getting it to verify the right conformation/proper fold or the solution conditions for NMR measurements. Combining the SR-VUVCD technique with bioinformatics improves the predictive accuracy and the amount of information about the secondary structures, and hence it is becoming increasingly important in protein structural biology [19].

The VUVCD spectra of various globular proteins have been measured down to 160 nm [19, 47–50]. Helix-rich proteins (\(\alpha\)-proteins) such as myoglobin (76% \(\alpha\)-helices) and horse serum albumin (70% \(\alpha\)-helices) exhibit three negative peaks around 222, 208, and 170 nm, a positive peak around 190 nm, and a shoulder around 180 nm. Strand-rich proteins (\(\beta\)-proteins) exhibit large variations in their spectra compared with \(\alpha\)-proteins: lysozyme (42% \(\alpha\)-helices and 6% \(\beta\)-strands) shows a similar spectrum to that of myoglobin, but concanavaline A (4% \(\alpha\)-helices and 46% \(\beta\)-strands) exhibits two negative peaks around 220 and 175 nm, and two positive peaks around 200 and 165 nm, while the spectrum of soybean trypsin inhibitor (1.7% \(\alpha\)-helices and 37% \(\beta\)-strands) oppositely exhibits a positive peak around 220 nm and a large negative peak around 200 nm that resemble those of poly-L-proline II-type helix (PPI) [51]. It is evident that these characteristic variations of VUVCD spectra over wide wavelength ranges provide much more information about the secondary structures of proteins compared with that available from far-UV CD spectroscopy.

The CD spectra of proteins have been calculated theoretically, but at present it seems difficult to reproduce the observed spectra because of various electronic excitations affecting the backbone amide chromophore [3,52–55]. The current situation of theoretical methods for CD calculations is reviewed by Woody [56]. One of the interfering excitations is from aromatic side chains. In particular, exciton coupling between aromatic side chains makes significant contributions to CD spectra in some proteins, as typically observed for exciton coupling between Try47 and Try74 of dihydrofolate reductase [57,58], although the contributions may be theoretically inferred by comparing the CD spectra of mutants replaced with a nonaromatic amino acid [54]. The secondary structures are affected by these various interferences, and hence have been deductively estimated by comparing the CD spectrum of an unknown protein with a VUVCD data set of the reference proteins of known atomic coordinates. There are several VUVCD data sets with different numbers and special categories of proteins [19]. The Protein Circular Dichroism Data Bank (PCDDB) established in 2011 by Whitmore, L. et al. is in operation as a public repository that archives and freely distributes CD and SRCD.
spectral data and their associated experimental metadata (http://pcedb.cyst.bbk.ac.uk) [59]. The predictive accuracy for the secondary structures has been improved in combination with the accumulation of protein structures in the Protein Data Bank, the development of programs (e.g., DSSP and STRIDE) for determining the secondary structures from atomic coordinates [60,61], and advancements in software (e.g., CONTIN, SELCON3, and CDSSTR) for analyzing CD spectra [62,63]. The secondary structures of proteins can now be analyzed online using various software packages, including DichroWeb (http://dichroweb.cyst.bbk.ac.uk/html/home.shtml), CTool (http://cdtools.cyst.bbk.ac.uk), CDPro (https://sites.bmb.colostate.edu/sreeram/CDPro), and DichroCalc (http://comp.chem.nottinham.ac.uk/dichrocac/) [19]. Recently, a new web server BeStSel (http://bestsel.elte.hu) is developed for accurately estimating the secondary structures for a broad range of protein folds, particularly for β-sheet-rich proteins and amyloid fibrils [64,65].

**Improved secondary-structure prediction**

The secondary-structure contents of an unknown protein are estimated by assuming additivity of the component spectra that are extracted from a CD data set of reference proteins with known atomic coordinates. In general, either three components (α-helices, β-strands, and unordered structures) or four components (with the addition of β-turns) are used for such calculations depending on the quality of the available CD spectra [1,3,20,48,63]. The ability to improve estimations of the secondary-structure contents by extending the short-wavelength limit has been demonstrated using conventional CD spectroscopy down to 170 nm [66,67]. As the short-wavelength limit was further decreased to 160 nm using SR, the root-mean-square deviation (δ) between X-ray and CD estimates for the contents of α-helices and β-strands decreased, with the correlation coefficients (r) increasing to 0.937 and 0.826, respectively [47,48]. High performance of VUVCD spectroscopy can increase the number of secondary-structure components to a maximum of eight, which makes it possible to improve the accuracy of predicting the numbers of α-helix and β-strand segments. The recently developed BeStSel method takes into account eight secondary-structure components including the twist of β-sheets and can distinguish parallel and antiparallel β-sheets, which was not available previously and might be useful to predict the protein fold [64,65].

The numbers of α-helix and β-strand segments have been estimated from CD spectra using two methods. Pancoska, P. et al. used a matrix descriptor of secondary-structure segments for the neural network (NN)-based analysis of proteins [68], while Sreerama, N. et al. estimated the number of segments from the distorted residues in α-helices and β-strands (on average four and two, respectively), classifying the secondary structures into six components: regular α-helix, distorted α-helix, regular β-strand, distorted β-strand, turn, and unordered structures [62]. The numbers of α-helix and β-strand segments calculated by this method from the VUVCD spectra of 31 reference proteins down to 160 nm agree well with the structures revealed by X-ray crystallography: r values between the VUVCD and X-ray estimates for the numbers of α-helix and β-strand segments are 0.954 and 0.849, respectively, corresponding to root-mean-square differences of 2.6 and 4.0 [47,48]. Thus, VUVCD spectroscopy is superior to conventional CD spectroscopy for estimating both the contents and the numbers of segments of the secondary structures of proteins.

The sequence-based prediction of secondary structures requires computational algorithms for the correlations between the X-ray crystal structures and amino acid sequences of reference proteins, because CD spectroscopy in principle yields no information about the amino acid sequences. Various types of algorithms have been developed for this purpose [69–71], and their predictive accuracy has gradually improved with the application of various computational techniques, including NNs [72,73], the Profile Network from HeiDelberg [74], and discrimination of the secondary-structure class [75]. However, the accuracy of these computational techniques in predicting the secondary-structure contents and numbers of segments is lower than that of VUVCD spectroscopy, and hence it can be improved by combining with the VUVCD data. We incorporated VUVCD data into an NN algorithm (VUVCD-NN method) [76]. In this method, the α-helix and β-strand weights of 20 amino acids first calculated by the NN method were revised until the determined contents and numbers of α-helix and β-strand residues converged to those estimated by VUVCD analysis, which resulted in the per-residue assignment (Qr value) of the NN algorithm increasing from 70.9% to 74.9%. When combined with the currently best sequence-prediction algorithms, a predictive accuracy exceeding 80% could be expected. A similar approach was adopted by Lees and Janes using information from both VUVCD and FTIR measurements [77].

The secondary-structure information obtained by VUVCD spectroscopy and VUVCD-NN method can be used to test the tertiary-structure models predicted by homology modeling or ab initio calculations such as the Modeler package [78–80]. Such information cannot prove which model is correct, but it does represent a novel criterion for eliminating uncertain models. Thus, VUVCD spectroscopy and VUVCD-NN method are important tools for predicting the structures of proteins that are difficult to determine using X-ray crystallography and NMR spectroscopy.

**Application to native proteins**

Human α₁-acid glycoprotein (AGP), which is a major acute-phase protein that can bind to neutral drugs and steroid hormones, includes 183 amino acid residues and 5 glycan chains that constitute about 40% of its total mass (36 kDa). Its tertiary structure was unknown until Schönfeld, D. L.
et al. succeeded in applying X-ray crystallography with the recombinant unglycosylated protein [81]. Although carbohydrate components are generally thought to have little effect on the structure and function of glycoproteins, as was found to be the case for sodium channels [82], the tertiary structure of intact AGP (glycosylated form) and the roles of carbohydrate components are still unknown. VUVCD spectroscopy is highly advantageous for such glycoproteins because it can detect the CD spectra of carbohydrate moieties in the VUV region. The VUVCD spectrum (down to ~160 nm) of the protein moiety of AGP was obtained by subtracting the contributions of the constituent carbohydrates (l-fucose, d-mannose, d-galactose, N-acetylglucosamine, and N-acetylmuramic acid) [83]. The secondary-structure analysis classifying 3.10-helices into unordered structures revealed 37.7% β-strands (10 segments) and 14.4% α-helices (3 segments), which were almost consistent with those predicted by homology modeling (Modeler package). However, this α-helix content was considerably smaller than that indicated by the X-ray structure of unglycosylated protein (18.8%, 4 segments), although the β-strand content was highly consistent with the X-ray structure (37.7%, 9 segments). The positions of β-strands and α-helices predicted by the VUVCD-NN method were roughly comparable with those of the X-ray structure. Although the effects of crystal packing may not be disregarded [81], the observed difference between the VUVCD estimate and X-ray structure predicts that the constituent glycan chains—if they have any structural effects—may affect the secondary structures dominantly in the N- and C-terminal regions. Thus, VUVCD spectroscopy in combination with the VUVCD-NN method is useful for predicting the intact structure of glycoproteins relevant to their functions (the conformational changes of AGP bound to membrane are described below).

Another interesting target protein for VUVCD spectroscopy is a uracil-DNA degrading factor (UDE) of D. melanogaster, which recognizes and removes uracil from DNA at the end of the third larval stage [84]. This protein consists of 355 amino acid residues, beyond the upper limit of NMR measurements, and it has not been successfully crystallized, probably due to the predicted high conformational freedom of several segments. The VUVCD measurements were performed down to 170 nm for full-length UDE and its nine truncated fragments covering the full-length region, which were constructed using a random-screening method [85]. The VUVCD-NN analysis predicted a large α-helix content (62%, 13 segments) and a small amount of β-strands (8%, 7 segments) for the full-length protein, with most of these segments being preserved in the truncated fragments. The arrangement of the α-helix bundles within the truncated fragments suggested new domain boundaries that differ from the conserved motifs determined by sequence-based alignment of UDE homologs. This new structural description of UDE forms a basis for further detailed functional studies [85].

These two examples demonstrate the validity of applying VUVCD spectroscopy in combination with the VUVCD-NN method for estimating the secondary structures of native proteins whose tertiary structures are unknown. The predictive accuracy of this method would be improved by taking into account additional information such as the NMR spectroscopy or the X-ray crystal structures of any constituent fragments, and small-angle X-ray scattering data for the whole molecule.

**Application to nonnative proteins**

While proteins generally exert their functions within a unique stable three-dimensional structure associated with conformational fluctuations, recent studies have revealed that there are many functional proteins and functional protein regions without such unique structures under physiological conditions, which are called intrinsically disordered proteins (IDPs) [86]. The stability of the structure of a protein is generally marginal due to only a small difference in free energy between its native and denatured states, and most such proteins are easily denatured in certain solvent conditions or chemical modifications. The structures of denatured proteins are very important for understanding the mechanisms of protein folding and stability. VUVCD spectroscopy is particularly useful for the structural analysis of such nonnative proteins that are difficult targets for X-ray crystallography or NMR spectroscopy.

**Intrinsically disordered proteins**

CD spectroscopy has been widely used for the structural analysis of IDPs [87], but the small amount of VUVCD data has also provided valuable information about the structure of IDPs [88–91]. Kumagai, P. S. et al. found that the secondary-structure analysis of IDPs was greatly improved by comparing VUVCD spectra (down to ~170 nm) with conventional CD spectra (down to ~184 nm) of the two proteins MEG-14 (micro-exon gene protein 14) and soybean trypsin inhibitor [89]. Lopes, J. L. et al. measured VUVCD spectra of collagen and polyproline down to ~170 nm to investigate the spectral properties of PPII-type structures abundant in IDPs that are classified as an “other” category in a CD analysis [92]. Their study revealed the characteristic spectral feature distinguishing a PPII structure from a disordered structure, which improves the results of secondary-structure analyses based on CD spectroscopy.

Yoneda, J. S. et al. measured the VUVCD spectra of the four IDPs MEG-14, α-synuclein, β-synuclein, and small hydrophilic endoplasmic reticulum-associated protein down to ~170 nm [91]. All of these IDPs, which are almost entirely unstructured (nonregular secondary structure) in solution, exhibited increased amounts of regular (mostly helical) secondary structures in films, although such a significant conformational change was not observed for globular soluble proteins. That study indicates that IDPs and globular proteins are very different in secondary-structure formation.
upon dehydration, and that film spectra are useful for assessing the structure of IDPs.

VUVCD spectroscopy was also applied to study the structure, dynamics, and function of the C-terminus of a hexameric E. coli RNA chaperone Hfq, which is involved in the riboregulation of target mRNAs by small trans-encoded RNAs [88]. Although the structure of the conserved core was elucidated for several Hfq proteins, no structural information has yet been obtained for the C-terminus. In combination with bioinformatics, NMR spectroscopy, and small-angle X-ray scattering, VUVCD spectroscopy revealed that the C-terminals have features of typical IDPs that facilitate intermolecular interactions with RNA fragments, supporting the hypothesis that RNA fragments exceeding a certain length interact with the C-terminals of Hfq. VUVCD spectroscopy would be of considerable help for the structural and functional characterization of IDPs.

**Denatured proteins**

CD spectroscopy is the most widely used technique for the structural analysis of denatured proteins, since unlike for other methods (e.g., NMR spectroscopy, small-angle X-ray scattering, and FTIR), CD spectra are measurable for any protein at a low concentration under various solvent conditions although the VUVCD measurement is often difficult for the solvents containing a high concentration of denaturant or additive due to their high absorption.

Matsuo, K. et al. measured the VUVCD spectra of metmyoglobin (metMb), staphylococcal nuclease, and thioredoxin in four denatured states: acid-, cold-, and heat-denatured states were compared with that of the fully unfolded state induced by guanidine hydrochloride (GdnHCl) [93]. Expansion of the short-wavelength limit of the spectra made it possible to clearly identify characteristic differences in the secondary structures of these denatured states. Although the extent of the residual secondary structures depends on the denaturing conditions and the types of proteins, these VUVCD data reveal the following general structural features associated with protein denaturing: (1) α-helices are broken, (2) β-strands increase, (3) turns are almost unchanged, and (4) PPII and unordered structures increase. A loss of α-helices is the most common feature of denatured proteins with the exception of alcohol denaturation (as described below), as also observed when using other techniques [94–96]. An important finding from VUVCD spectroscopy is that many β-strands are formed in all denatured proteins, even in the fully denatured state induced by GdnHCl, which is clearly inconsistent with the assumption that the starting conformation for the folding process is a completely unfolded random coil. Since NMR measurements have provided no direct evidence of β-sheets, most of these β-strands would not exist as β-sheets, but rather as an ensemble of many short peptide segments with the dihedral angles of β-strands. Excess β-strands in the denatured proteins could form intermolecular β-sheets to initiate amyloid formation under appropriate conditions, such as high concentrations of protein and added salts.

Alcohol denaturation was investigated by measuring the VUVCD spectra of six proteins (metMb, human serum albumin, α-lactalbumin, thioredoxin, β-lactoglobulin, and α-chymotrypsinogen A) down to 170 nm in trifluoroethanol (TFE, 0–50%) and 175 nm in methanol (MeOH, 0–70%) solutions [97]. All of these proteins showed very high α-helix contents (up to ~90%) with several long helical segments that differed from the α-helices in the native structure. The helix-forming ability was higher in TFE than in MeOH, while small amounts of β-strands without sheets were formed in the MeOH solution. The content and mean length of α-helix segments decreased as the number of included disulfide (S–S) bonds increased, suggesting that such bonds hinder helix formation in alcohol denaturation. The VUVCD-NN analysis predicted that the produced α-helices were transformed dominantly from the β-strands and unordered structures, and less so from the turns in the native state. These results clearly indicate that alcohol-denatured structures distinctly differ from other types of solvent denaturation.

There are abundant thermodynamics and kinetics data indicating that S–S bonds play an important role in stabilizing the tertiary structure of proteins [98,99]. Most proteins containing S–S bonds unfold when these bonds are completely broken by reduction or mutation, even in the absence of a denaturant. However, the resulting unfolded structures and the effects of individual S–S bonds remain controversial. To address these problems, Matsuo, K. et al. measured the VUVCD spectra of hen lysozyme down to 170 nm for 13 disulfide-deficient variants in which cysteine residues of 4 S–S bonds (Cys6–Cys127, Cys30–Cys115, Cys64–Cys80, and Cys76–Cys94) were alternatively replaced with alanine or serine residues [100]. Each variant exhibited a characteristic VUVCD spectrum that depended on the positions and numbers of deleted S–S bonds. The three-disulfide variants lacking one of the four S–S bonds had native-like secondary structures, but other variants exhibited fewer α-helices with a border between the ordered and disordered structures around the two-disulfide variants. No additive effect of S–S bonds on secondary-structure formation was found, and even the all-disulfide-deleted variant contained a considerable amount of secondary structures, as was the case for the fully denatured state induced by GdnHCl. Deleting the Cys6–Cys127 bond, which is located at the longest distance on the primary structure, exerted the largest effects of disrupting α-helices among three-disulfide variants and of forming α-helices among the one-disulfide variants. The contents of α-helix and β-strand were correlated negatively ($r_m=-0.88$) and positively ($r=0.87$), respectively, with the conformational entropy of disulfide variants, suggesting that the reduction of chain entropy (flexibility) due to cross-linking is favorable for the formation of α-helices, while it is unfavorable for the formation of β-strands. This is supported by a strong correlation between the adiabatic compressibil-
Amyloid fibrils

Amyloid fibrils are highly ordered aggregates of proteins and peptide fragments that cause many amyloidosis diseases. These fibrils are known to be composed of some protofilaments, with β-sheet layers identified in electron microscopy and X-ray diffraction studies [102,103], but the structures and formation mechanisms of the fibrils vary in a complicated manner depending on the solvent conditions (e.g., pH and cosolvents) in addition to the involved amino acid sequences (e.g., S–S bonds and hydrophobicity) [104–106].

β₂-Microglobulin (β₂m) is one of the most typical proteins that form amyloid fibrils, and the deposition of β₂m amyloid fibrils in vivo causes dialysis-related amyloidosis [106]. It consists of 99 amino acid residues and contains 2 β-sheets bound with an S–S bond (immunoglobulin fold). β₂m forms amyloid fibrils with two morphologically different forms depending on the NaCl concentrations: needlelike long-straight (LS) and flexible wormlike (WL). These two forms exhibit distinct VUVCD spectra, although the measurable short-wavelength limit has been about 190 nm due to the high concentrations of NaCl: the WL fibril (in 200 mM NaCl) showed a largely blue-shifted spectrum compared with the LS fibril (in 100 mM NaCl) [107]. The two spectra predicted that the LS fibril consists of 1.4% α-helices (0.2 segments), 54.2% β-strands (7.6 segments), and 46.6% nonregular structures (23.6% turns and 23.0% unordered structures), whereas the WL fibril consists of 20.5% α-helices (2.8 segments), 41.9% β-strands (6.1 segments), and 40.3% nonregular structures (20.1% turns and 20.2% unordered structures) [107]. Micsonai, A. et al. obtained the similar values for the contents of α-helices, β-strands, and nonregular structures of the two fibrils using the BeStSel method, and showed that the structure of WL fibril is dominantly antiparallel β-sheet, whereas mature SL fibril contains parallel β-sheets [65]. These spectral and morphological differences of the two fibrils might be ascribed to the different protonation states (pKₐ) of the carboxyl side chains in the two fibrils relevant to the spatial arrangement of β-strands and β-sheets [107].

The three peptide fragments of β₂m, β₂m20–41 (SNFLNCYVSGFHPSDIEVDLLK), β₂m21–31 (NFLNCYVSGFH), and β₂m21–29 (NFLNCYVSG), are considered to be candidates for the core regions of the fibrils because these peptide fragments successfully form amyloid fibrils under physiological conditions [106,108]. The three-dimensional structure of β₂m20–41 fibril was determined by a solid-state NMR analysis [109], but the detailed structures of β₂m21–31 and β₂m21–29 fibrils remain unclear although FTIR and Raman spectroscopy have suggested that both fragments form parallel β-sheets containing 55–65% β-stands [110,111]. Matsuo, K. et al. measured the VUVCD spectra of the three peptide fibrils from 260 to 178 nm to characterize, in combination with MD simulations, the intermolecular configurations of β₂m21–29 fibril, not only of the backbones but also of aromatic side chains (phenylalanine and tyrosine) in the fragment [112]. The VUVCD spectrum in the 178–260 nm region and the CD spectrum in the 240–300 nm region were compared with those theoretically calculated from the simulated structures of β₂m21–29 amyloid fibrils with various types of β-sheet stacking (parallel or antiparallel). The experimental spectrum of β₂m21–29 fibrils was greatly affected by the couplings between transitions within the aromatic and backbone chromophores, sensitively depending on the type of stacking among the β-sheets of the fibrils. Theoretical analyses of simulated structures incorporating mutated aromatic residues suggested that the β₂m21–29 fibrils are composed of parallel β-sheets stacked in an antiparallel manner and that three phenylalanine–tyrosine interaction modes among the β-sheet stacks affect the aromatic–backbone coupling. These models resemble the parallel orientation of β₂m20–41 fibril with two aromatic residues buried between the β-strands [109], and suggest that the spectral differences among β₂m20–41, β₂m21–31, and β₂m21–29 fibrils from the near-UV to VUV regions might be ascribed to the conformations of the aromatic side chains [112].

Gobeaux and Wien recently applied VUVCD spectroscopy to investigate the self-assembly properties of atosiban, a nonapeptide drug, whose sequence is very close to those of the oxytocin and vasopressin hormones [113]. In combination with transmission electron microscopy, small-angle X-ray scattering, and infrared and fluorescence spectroscopy, those authors found that the peptide undergoes conformational changes during the association, but its monomers and dimers assemble into fibrils without passing through an oligomeric intermediate species, which contrasts with what is usually reported for pathogenic amyloids. It was also suggested that the native β-hairpin conformation of the monomer and the tyrosine stacking play important roles in the straightforward assembly.

These studies indicate that combining VUVCD analysis with various other techniques is effective for characterization of the intermolecular structures that are crucial for understanding the formation mechanisms and functions of amyloid fibrils.
Conformational changes of membrane proteins

The in vivo functions of many proteins manifest via interactions with biomembranes. Such so-called membrane proteins are difficult targets for CD spectroscopy due to various artifacts arising from absorption flattening, differential scattering, and wavelength shifts [19]. However, these problems have been obviated in VUVCD spectroscopy to yield high-quality data for detecting the conformational changes of membrane proteins in the presence of a membrane [20, 114–116]. There are two main types of membrane proteins: (1) integral proteins that penetrate through the membrane and (2) nonintegral proteins that bind to the lipid surface. Most VUVCD measurements have been made using liposomes with various lipids instead of biomembranes, because it is generally difficult to reconstruct biomembrane–protein systems. The PCDDDB provides an MP180 reference set of membrane proteins which might be useful for structural analysis of membrane proteins [117].

Integral membrane proteins

Equinatoxin II (EqtII) is a member of the actinoporin family of sea-anemone toxins that function by forming pores in cell membranes via a multistep mechanism. Miles, A. J. et al. measured the VUVCD spectra of EqtII down to 180 nm in the absence and presence of small unilamellar vesicles with different lipid compositions of dioleoylphosphatidylethanolamine (DOPC), cholesterol (Chol), and sphingomyelin (SM) [118]. All of the VUVCD spectra were similar in the far-UV region, but they exhibited large differences in peak positions and intensities in the VUV region depending on the lipid composition. The estimated secondary-structure contents suggested that EqtII is in a free state in the presence of DOPC or DOPC/Chol vesicles, and in a bound state of high lytic activity in the presence of DOPC/SM vesicles, indicating the lipid specificity for structural rearrangements of EqtII associated with toxicity and lysis functions.

Miles, A. J. et al. measured the VUVCD spectra of pig Na,K-ATPase down to 175 nm in the membrane-bound state at various temperatures, and showed that the loss of enzymatic activity is correlated with changes in the protein secondary structures [115]. Furthermore, McKibbin, C. et al. used VUVCD spectroscopy to monitor structural changes in rhodopsin and opsin aged for 1–6 hours in vesicles of 1,2-dimyristoylphosphatidylcholine and 1,2-dihexanoyl-sn-glycero-3-phosphocholine at room temperature [119].

Nonintegral membrane proteins

β-Amyloid peptide Aβ (residues 1–40) is a major component of Alzheimer’s amyloid deposits that presumably exerts its neurotoxic action via interactions with neuronal membranes. Yagi-Utsumi, M. et al. measured the VUVCD spectra of Aβ down to 175 nm in the absence and presence of monosialotetrahexosylganglioside (GM1) at Aβ:GM1 ratios of 1:15 and 1:30, and found that the secondary-structure contents vary with this ratio [120]. Combining these data with those from NMR spectroscopy, those authors suggested that GM1 clusters promote specific Aβ–Aβ interactions that vary with the sizes and curvatures of the clusters.

The drug-binding capacity of the nonintegral membrane protein AGP decreases upon interaction with biomembranes, which is associated with a large conformational change of AGP [121]. Matsuo, K. et al. measured the VUVCD spectra of AGP down to 160 nm in the absence and presence of liposomes of 1,2-dihexanoylphosphatidylglycerol at pH 4.5 [83]. Its VUVCD spectrum was close to that in the native state (pH 7.4) but differed markedly in a liposome environment, indicating a conformational transition from a β-strand-rich to α-helix-rich structure. Based on the spectra of the protein moiety, the secondary structure was estimated to comprise 11.4% α-helices (3 segments) and 39.9% β-strands (12 segments) in the absence of liposomes, and 47.5% α-helices (7 segments) and 2.7% β-strands (2 segments) in the presence of liposomes, but with no significant changes observed in the contents of turns and unordered structures. The VUVCD-NN analysis suggested that most progesterone-binding sites change to α-helices or unordered structures and that the stereostructures of the drug-binding sites collapse completely upon interacting with liposomes. Considering the net charge and hydrophobicity of each helix, and the evidence for Trp25 and Trp160 interacting with liposomes, the following membrane-binding mechanism of AGP was proposed: (1) positively charged AGP binding a ligand approaches the anionic membrane surface via an electrostatic interaction, and (2) Trp25 and Trp160 are inserted into liposomes via hydrophobic interactions, forming two helices around the N- and C-terminals in contact with the membrane surface and subsequently other helices, accompanied by the release of ligand into the membrane. This model provides sequence-level information about the membrane-binding mechanism and the structure–function relationship of AGP, which could be more generally extended to the protein-mediated uptake and membrane-transport mechanisms of hormones and drugs [83].

Thioredoxin is widely distributed in tissues and organs, and it is often associated with membrane-adhesion sites relevant to cell–cell communication [122]. α-Lactalbumin and β-lactoglobulin are biologically important milk proteins that can interact with lipid molecules and liposomes [123,124]; the former is a secretory protein that exerts its function in a membrane environment, while the latter is related to the transport of certain small hydrophobic and amphiphilic compounds such as phospholipids [125,126]. Matsuo, K. et al. measured the VUVCD spectra of these three proteins down to 170 nm in the presence or absence of liposomes of 1,2-dihexanoylphosphatidylglycerol [127]. These three proteins showed characteristic helix-rich conformations consisting of several helical segments, of which two amphiphilic or hydrophobic segments participated in interactions with the liposome.

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Orientation of membrane-bound proteins and peptides

In addition to the secondary structures, the orientation of proteins and peptides in a membrane is also an important factor influencing their biological functions. The n-π* transition of the backbone amide bonds in helical polypeptides is polarized perpendicularly to the helix axis around 220 nm, and two π-π* transitions around 210 and 190 nm are polarized parallel and perpendicularly to the helix axis, respectively. This means that the relative magnitudes of the peaks for orientation under various external stresses (e.g., shear flow, stretched films, and electric fields) can be used to estimate the orientation and/or tilt of the helix with respect to the membrane.

Linear dichroism (LD) generated during shear flow has been widely used for estimating the orientation and/or tilt of the helix [127–129]. Hicks, M. R. et al. found that the LD spectra (down to ~183 nm) of the antibiotic peptide gramicidin D in lipid membranes exhibited dramatically different kinetics and equilibrium folding and orientation behaviors when interacting with lipid membranes with different charges [129]. The LD spectra (down to ~185 nm) measured using an LD spectrometer installed in a VUVCD spectrometer suggested that the average orientations of the two amphiphilic helices of α-lactalbumin are parallel to the liposome surface, two hydrophobic helices of thiodexin are perpendicular, and a hydrophobic helix and an amphiphilic helix of β-lactoglobulin are perpendicular and parallel to the liposome surface, respectively [127].

Another increasing popular method is oriented circular dichroism (OCD), which is typically performed by spreading a thin film of a concentrated sample on a quartz or calcium fluoride plate, followed by drying and then rehydrating under a controlled humidity condition [130,131]. This method has been applied to detect the orientation of various membrane-active proteins and peptides using an SR-VUVCD or conventional CD instrument, occasionally in combination with solid-state NMR analysis [132–136]. Bürck, J. et al. measured the OCD spectra of the antimicrobial peptides PGLa and MSI-103 and their structurally related cell-penetrating peptide MAP from 260 to 180 nm to characterize and compare the secondary structures and concentration-dependent realignment in phospholipid bilayers [132]. The observed changes in peptide conformation and membrane alignment were correlated with the membrane-perturbing activities of the three antimicrobial and cell penetrating sequences.

Muruganandam, G. et al. found that the OCD spectra (down to ~180 nm) of myelin proteins and peptides showed a highly tilted orientation of a helical peptide from the central immunodominant epitope with respect to the membrane surface, which provided important insights into the mechanisms of membrane binding and stacking of myelin proteins [133]. Perrone, B. et al. measured the OCD spectra (down to ~178 nm) of the cationic amphipathic designer peptide LAH4—which exhibits potent antimicrobial, nucleic acid transfection, and cell-penetration activities—as a function of pH, buffer type, phospholipid head group, and fatty acyl chain composition [134]. That study demonstrated that the membrane topology of this peptide is readily modulated by the environmental conditions. OCD has also been applied to investigations of the alignment of crown ether-modified peptides at variable peptide:lipid ratios [135]. Those authors found that the peptides were predominantly incorporated as a transmembrane helix into the membrane, and the preferred trans-bilayer alignment of the crown ether functional groups explains their ion conductivity. Windisch, D. et al. applied OCD analysis to the oncogenic E5 protein from bovine papillomavirus, which activates platelet-derived growth factor receptor by transmembrane helix–helix interactions [136]. They revealed that a very long transmembrane helix of ~26 amino acids is formed and that the alignment and stability of this unusually long segment depend critically on the membrane thickness, forming a closely packed bundle of mutually aligned transmembrane helices.

While the application of VUVCD to membrane proteins—especially integral membrane proteins—is still restricted by difficulties related to sample preparation [19,20,131], it will undoubtedly become a powerful technique in combination with LD and OCD spectroscopy for monitoring the conformational changes and orientations of proteins and peptides in a membrane environment.

Structural Analysis of Nucleic Acids

CD spectroscopy has also been used as a powerful technique for the structural analysis of nucleic acids (e.g., DNA and RNA), because it is sensitive to base composition/sequence, coupling between bases, and strand conformation [137]. Nucleic acids display positive and negative bands below 300 nm, while signal intensities in the VUV region are often significantly larger than those in the UV region, and so VUVCD spectroscopy provides more-detailed information than that available from the far-UV region. Theoretical and quantum-mechanical calculations of spectra have not yet been successfully applied to nucleic acids as well as polysaccharides and proteins due to various complex overlapping electronic excitations, but theoretical calculations of CD are in better agreement with experiments in the VUV region [138]. VUVCD spectroscopy of nucleic acids has been developed in parallel with comparative analyses of synthetic polynucleotides composed of selected bases [21,139–145].

Double-stranded DNA consists of three primary structures (A-, B-, and Z-forms), although various kinds of other structures such as hairpins, triplexes, and quadruplexes are also found [146]. The A- and B-forms are both right-handed helices, with the A-form having a shorter and more-compact structure, whereas the Z-form is a left-handed helix with the opposite base-pair orientation compared to the A- and B-forms. The VUVCD spectra of B- and A-forms of DNA from various species including calf thymus, C. perfringens,
sequences—not only for native proteins (including glyco-proteins) but also unfolded, associated, and membrane-bound states that are difficult targets for other spectroscopic techniques, thereby providing new insight into structure–function relationships and medical applications. VUVCD spectroscopy of nucleic acids also makes more-detailed conformational analysis possible through comparative studies of synthetic polynucleotides with certain base compositions and sequences. Therefore, SR-VUVCD spectroscopy is a useful technique in structural biology that complements NMR spectroscopy and X-ray diffraction.

Further developments of this technique could be promised by challenging the following issues:

1. For small molecules (e.g., saccharides, amino acids, and nucleosides), experimental and theoretical comparative studies of VUVCD spectra in film, gas, and solution states would lead to a better understanding of their equilibrium conformations and the intra- and intermolecular interactions in aqueous solutions.

2. Quantum mechanical or \textit{ab initio} calculations of VUVCD spectra of polysaccharides, proteins, and nucleic acids (which might be a hard task) could predict their conformations based on the compositions and sequences of the constituents.

3. Combining VUVCD spectroscopy with bioinformatics (e.g., VUVCD-NN method) would be practically useful for increasing the accuracy of predicting secondary structures. For proteins, the PCDDB will be a good resource for this purpose [19,59].

4. VUVCD spectroscopy has mostly been applied to static (or equilibrium) structures, and only rarely in kinetics studies. Time-resolved techniques such as stopped/continuous flows and temperature jumps are progressively being incorporated into VUVCD spectrometers [19,158]. These advances will allow VUVCD spectroscopy to provide new perspectives in kinetics studies of conformational changes and interaction mechanisms of biomolecules, such as protein folding/association, protein–DNA interactions, and ligand binding.

5. Novel applications of VUVCD spectroscopy would provide considerable potential in diverse areas of structural biology, including glyco-biology, amyloidosis, membrane transportation, immunochemistry, and structural genetics.

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Conflict of Interest
The author declares no conflict of interest.

Author Contributions
K. G. reviewed the studies of the VUUVD spectroscopy in structural biology and wrote the manuscript.

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