CHAPTER 3

Aggregation studies on an amyloidogenic peptide from human $\beta_2$-microglobulin, $\text{P}\beta_2\text{m}$

$(\text{Ac-DWSFYLLYYTEFT-am})$
3.1 Introduction

Human β2-microglobulin (β2m) is a small (99 residues, 11.8 kDa) protein which constitutes the light chain of major histocompatibility complex antigen class I. Native β2m adopts an immunoglobulin fold with 7-stranded β-sandwich structure both in MHC class I complex (Bjorkman et al., 1987) and as a monomer (Verdone et al., 2002; Trinh et al., 2002; Iwata et al., 2007) (Figure 3.1). Strands A, B, D, and E form one half of the sandwich, while strands C, F, and G form the second half. The two halves are held together by a single disulfide bond between Cys\(^25\) and Cys\(^80\) in strands B and F, respectively (Khan et al., 2000).

In class I MHC complex, β2m interacts non-covalently with the α-chain of the complex, and is essential for expression of the complex on the cell membrane. In adult serum, the concentration of β2m varies between 1-3 mg/liter. During normal catabolism, β2m dissociates from the α-chain of MHC class I complex and is transported to the kidneys, where it gets degraded and excreted (Sundin et al., 1994; Bellotti et al., 1999). In patients with renal dysfunction, β2m removal is not effective resulting in elevated serum concentrations of β2m; up to 60-fold increase in patients with end-stage renal failure (Floegge and Ketteler, 2001; Koch, 1992). As a result of its elevated concentration in serum, β2m forms amyloid aggregates which get deposited largely to osteoarticular sites, particularly synovial membrane (Floegge and Ketteler, 2001; Koch, 1992). In vitro biophysical studies show that at neutral pH, β2m remains monomeric even at concentrations 1000 times higher than those found in the serum of dialysis patients (McParland et al., 2000). However, under acidic conditions (pH < 5.0), the protein partially unfolds populating the structures that assemble to form amyloid fibrils wherein the morphology of the fibrils is highly dependent on pH and ionic strength (McParland et al., 2000; Gosal et al., 2005).

Peptides from β2m have been identified that form amyloid fibrils in isolation (Kozhukh et al., 2002; Jones et al., 2003; Hasegawa et al., 2003). The peptides, β2m\(^{20-41}\) (comprises strand B and C; referred to as peptide K3), β2m\(^{59-71}\) (strand E), and β2m\(^{59-79}\) (comprises strand E, loop between strand E and strand F, and 3 N-terminal residues of strand F) form amyloid fibrils at acidic pH (Kozhukh et al., 2002; Jones et al., 2003). The fibrils formed by β2m\(^{20-41}\) are unstable at neutral pH and depolymerize at pH 8.5 (Kozhukh et al., 2002). However, deletion of 10 C-terminal residues results in fibrils which are stable at neutral pH (Hasegawa et al., 2003). Peptides, β2m\(^{59-71}\) and β2m\(^{59-79}\) form amyloid fibrils both at low (50 mM) and high (400 mM)
Figure 3.1  Crystal structure of human $\beta_2$m at pH 7.0 (Iwata et al, 2007). The $\beta$-strands are labeled from A to G. The two sheets are shown to be connected by disulfide bond between strand B and strand F.
ionic strengths across a wide pH range of 1-7. Unlike the fibrils formed by peptide K3, $\beta_2m^{59-71}$ and $\beta_2m^{59-79}$ form fibrils without any lag phase (Jones et al, 2003). The smaller peptide $\beta_2m^{59-71}$, which exists as $\beta$-strand E in native $\beta_2m$ is more amyloidogenic than the longer peptide $\beta_2m^{59-79}$ (Jones et al, 2003).

Stock solutions of amyloid-forming peptides are often prepared in organic solvents such as DMSO, TFE, and HFIP due to their limited solubility in aqueous buffers (Hirota-Nakaoka et al, 2003; Zhang et al, 1995; Wood et al, 1996). The solvents, TFE and HFIP play an important role in modulating peptide conformation (Rajan and Balaram, 1996; Buck, 1998). They also facilitate dissolution of peptide aggregates (Hirota-Nakaoka et al, 2003; Zhang et al, 1995; Wood et al, 1996). In this chapter, aggregation of the peptide, $\beta_2m^{59-71}$ (denoted as $\beta \beta_2m$ hereafter) was studied. $\beta \beta_2m$ was chosen because it forms fibrils across a pH range of 1 to 7, is more amyloidogenic than the longer peptide, $\beta_2m^{59-79}$, and fibril formation does not have any lag phase. Aggregation of $\beta \beta_2m$ was studied in three alcohols: MeOH, TFE, and HFIP; and in 50 mM aqueous phosphate buffer, pH 7.0. Surface activity of $\beta \beta_2m$ was determined and aggregation at air-water interface was examined by fluorescence microscopy.

3.2 Methods

3.2.1 Peptide synthesis

The peptide, $\beta \beta_2m$ (Ac-DWSFYLLYTEFT-am) was synthesized using standard Fmoc chemistry (Atherton and Sheppard, 1989) as described is Section 2.2. The peptide was synthesized on NovaSyn KR 125 resin (Novabiochem, La Jolla, CA) having a substitution of 0.11 mmole/g. MALDI-MS spectra of the peptide showed m/z value of 1810.94 (calculated mass: 1788.94) indicating sodium adduct of $\beta \beta_2m$.

3.2.2 Thioflavin T fluorescence spectroscopy

$\beta \beta_2m$ was dissolved in organic solvents: DMSO, MeOH, TFE, and HFIP and concentration of the peptide was estimated using a molar absorption coefficient of 9550 M$^{-1}$ cm$^{-1}$ at $\lambda = 280$ nm. The concentration obtained was 1.7, 1.7, 1.1, and 1.7 mM in DMSO, MeOH, TFE, and HFIP, respectively. Thioflavin T fluorescence was recorded as described in Section 2.4. 10 $\mu$M ThT in 50 mM phosphate buffer, pH 7.0 was titrated with $\beta \beta_2m$ stock solutions.
3.2.3 CD Spectroscopy

Far-UV CD spectra were recorded on Jasco J-715 spectropolarimeter as described in Section 2.5. 50 µM Pβ2m solutions were prepared in MeOH, TFE, and HFIP. Spectra were recorded immediately after dissolution of the peptides in the solvents and after 24, 48, and 96 hours of incubation at room temperature. CD spectra were also recorded in 50 mM phosphate buffer, pH 7.0 immediately after diluting from the Pβ2m stock solutions in MeOH (1.7 mM), TFE (1.1 mM), and HFIP (1.7 mM). The concentration of organic solvents was less than 5% in aqueous phosphate buffer after dilution. In TFE and HFIP, spectra were recorded from 250 nm – 180 nm. In MeOH and aqueous phosphate buffer, spectra could not be recorded at wavelengths less than 195 nm because of high absorbance of MeOH and buffer at lower wavelengths. Structural components were estimated using CDSSTR program as mentioned in Section 2.5.

3.2.4 Atomic force microscopy

Stock solution of Pβ2m (0.8 mM) was prepared in DMSO and diluted into 50 mM phosphate buffer, pH 7.0 to a concentration of 168 µM. The sample was incubated at 37 °C for 15 hours and AFM imaging was done as described in Section 2.7.

Pβ2m dissolved in organic solvents (1.7 mM in MeOH, 1.1 mM in TFE, and 1.7 mM in HFIP) were deposited onto the freshly peeled mica surfaces and allowed to air dry. Imaging was done as described in Section 2.7. Pβ2m solutions (50 µM) were also prepared in MeOH, TFE, and HFIP and incubated at room temperature. AFM imaging was done immediately after dissolution of the peptides, and after 48 and 96 hours of incubation at room temperature.

3.2.5 Fourier transform infrared spectroscopy

Pβ2m samples at 50 µM and 0.7 mM concentrations were prepared in MeOH, TFE, and HFIP and the solutions were kept at room temperature. ATR-FTIR spectra were recorded after 1 day and 4 days of incubation as described in Section 2.8. The spectrum for 50 µM Pβ2m sample in TFE was also recorded after 5 days of incubation.

3.2.6 Surface activity and aggregation on air-aqueous interface

Aggregation of Pβ2m on air-aqueous interface was studied as described in Section 2.9. Pβ2m stock solutions (0.6 mM) were prepared in MeOH, TFE, and HFIP and kept at room
temperature for 24 hours. Barriers were opened to an area of \( \approx 105 \text{ cm}^2 \) and 70 ml of 50 mM phosphate buffer, pH 7.0 was poured in the trough and left undisturbed. After 10 minutes, a compression-expansion isotherm cycle was recorded as a blank. Pβ2m (6 nmoles) was layered over the subphase (\( \approx 105 \text{ cm}^2 \)) giving molecular area \( \approx 290 \text{ Å}^2 / \text{molecule} \). The set-up was left undisturbed for 30 minutes. Three compression-expansion isotherm cycles were recorded without any delay between two cycles. The set-up was left in expanded form for 2 hours. After 2 hours, 3 more compression-expansion cycles were recorded. One more compression isotherm was recorded and the set-up was left in compressed state (around the collapse pressure) for 2 hours. After 2 hours, expansion isotherm was recorded followed by one compression-expansion cycle.

ThT fluorescence microscopy to study the aggregates formed/modulated/disrupted during compression-expansion of peptide on air-aqueous buffer interface was performed as described in Section 2.9.

3.3 Results

3.3.1 ThT fluorescence spectroscopy

Formation of amyloid fibrils by Pβ2m when transferred to aqueous phosphate buffer from organic solvents DMSO (1.7 mM), MeOH (1.7 mM), TFE (1.1 mM), and HFIP (1.7 mM) at concentrations indicated in parentheses was examined by monitoring ThT fluorescence. Fluorescence emission spectra were recorded from 460–550 nm. Changes in fluorescence intensity at 490 nm as a function of peptide concentration are shown in Figure 3.2. The rise in fluorescence intensity with increasing peptide concentration is suggestive of amyloid fibril formation. However, amyloid fibril formation by Pβ2m strongly depends on the solvent in which peptide was initially dissolved before diluting to aqueous buffer. This suggests that amyloid fibril formation depends on the solvent-history of the peptide. Fluorescence increase is non-linear and all the four curves could be fit to 2nd degree polynomials. Maximum increase was observed when peptide was transferred from TFE to buffer followed by transfer from HFIP, MeOH, and DMSO solutions, respectively. Pβ2m has been shown to form fibrils rapidly (< 2 minutes) across entire acidic pH range when diluted from DMSO solutions (Jones et al., 2003). The results indicate that fibril formation takes place rapidly when diluted from the organic solvents MeOH, TFE, and HFIP.
Figure 3.2  Titration of $10 \, \mu M$ ThT in 50 mM phosphate buffer, pH 7.0 with $\beta_2m$ stock solutions (1.7 mM in DMSO, 1.7 mM in MeOH, 1.1 mM in TFE, and 1.7 mM in HFIP). Fluorescence emission at 490 nm was plotted against peptide concentration and data could be fit to 2nd degree polynomials.
3.3.2 CD spectroscopy

Far-UV CD spectra of 50 µM Pβ2m were recorded in MeOH, TFE, and HFIP. Spectra were also recorded in 50 mM phosphate buffer, pH 7.0 when diluted from stock solutions prepared in MeOH (1.7 mM), TFE (1.1 mM), and HFIP (1.7 mM). The CD spectra are shown in Figure 3.3. Left panels represent spectra recorded in organic solvents while right panels represent those recorded in buffer. Panels A, B, and C represent the CD spectra of Pβ2m in MeOH, TFE, and HFIP, respectively, after 96h incubation at room temperature. CD spectra were also recorded immediately, 24, and 48 hours after dissolution of the peptides in the three organic solvents. The peptide adopts helical conformation in all the three alcohols and spectra of the peptide did not exhibit time dependent changes in any of the solvents. Deconvolution of the spectra shown in Figure 3.3A-C using CDSSTR program indicated that the helix content was 73.7%, 78.2%, and 74.8% while beta sheet content was 11.1%, 9.6%, and 8.4% in MeOH, TFE, and HFIP, respectively. The analysis indicates that Pβ2m adopts predominantly helical conformation in all the three alcohols. Panels D, E, and F show the CD spectra of Pβ2m in 50 mM phosphate buffer, pH 7.0 diluted from MeOH, TFE, and HFIP solutions, respectively. When diluted into phosphate buffer, the spectra show minimum ≈ 216 nm, characteristic of β-structure. Although the concentrations of the peptide are same, greater negative ellipticity is observed when diluted from TFE and HFIP solutions as compared to MeOH.

3.3.3 Atomic force microscopy

AFM images were recorded for 168 µM Pβ2m solution prepared in 50 mM phosphate buffer, pH 7.0 from 0.8 mM stock solution in DMSO. The sample was kept at 37 °C for 15 hours and AFM imaging was done as mentioned in Section 2.7. Figure 3.4 shows that Pβ2m forms extensive fibrils under these conditions.

AFM images of Pβ2m, after deposition and drying on mica, from stock solutions in MeOH (1.7 mM), TFE (1.1 mM), and HFIP (1.7 mM) are shown in Figure 3.5. Panel A indicates amorphous-like aggregates for the MeOH sample. Similar amorphous-like aggregates were also observed from HFIP (panel B). When Pβ2m was deposited on mica from TFE, distinctive ring like structures are clearly seen (panels C-F). Panel G shows height measurement of the ring-like structures shown in panel D. Using “Section” tool in the Nanoscope software, a line was drawn passing through the two rings. Heights of individual
Figure 3.3  Far-UV circular dichroism spectra of 50 μM Pb2m. Left panels show spectra in organic solvents after 96 hours of incubation at room temperature. (A), MeOH; (B), TFE; (C), HFIP. Right panels show spectra in 50 mM phosphate buffer, pH 7.0 immediately after diluting from MeOH (D), TFE (E), and HFIP (F) stock solutions.
Figure 3.4  AFM imaging of 168 μM Pβ2m in 50 mM phosphate buffer, pH 7.0 diluted from 0.8 mM stock solution in DMSO and incubated at 37 °C for 15 hours. Scale bar represents 1 μm.
Figure 3.5 AFM imaging of Pβ2m from stock solutions made in organic solvents. (A), MeOH, 1.7 mM; (B), HFIP, 1.7 mM; and (C-F), TFE, 1.1 mM. Panel E represents the 3-dimensional view of panel D. Panels A-E represent the imaging of peptides on mica from undiluted Pβ2m stock solutions while panel F represents the imaging of peptide 20 fold diluted in TFE from stock solution. Panel G represents the measurements of tube lengths using Nanoscope software. Scale bars represent 1 μm.
rings were measured by placing cursors on the rings and their hollow centers. The heights indicate that the structures are in fact short tubes with a hollow interior and length to diameter aspect ratios less than 1. Panels C-E are images of Pβ2m in TFE that was deposited on mica without any dilution. Tubes of lengths ranging from 10 nm to more than 50 nm are observed. Although, there is significant variation in length, the longest tubes are ≈ 50-60 nm in length. AFM imaging does not give as good resolution in x-y direction as in z-direction and lateral resolution depends on tip morphology (Kowalski et al, 2003). Therefore, it is not possible to accurately measure the diameters of these tubes but their outer diameters are ≈ 200-400 nm. Panel F shows imaging of Pβ2m aggregates when stock solution was diluted 20-fold in TFE prior to deposition on mica. The tube morphology is severely affected and tube length in general is ≤ 30 nm, suggesting that the tubular structures formed by Pβ2m are not very rigid and stable. However, these tubes give an insight into the mechanism of tube formation by Pβ2m in TFE. The rings appear to be made up of various modules of small rod shape aggregates self-assembling laterally to give tubular structures.

Self-association of Pβ2m at a much lower concentration of 50 μM in all the three alcohols was monitored after different incubation periods by AFM. In MeOH (Figure 3.6), the major population is that of very small globular aggregates with very few fibrillar aggregates. But with increasing time of incubation, a population of elongated aggregates is observed (Figure 3.6B and C). Images recorded after 96 hours incubation show protofibril-like structures that are < 200 nm in length (panel C). In TFE (Figure 3.7), aggregates at early time points have a fibrillar morphology wherein the fibrils have tapering ends (panels A and B). With time, these fibrillar species appear to circularize to give the tubular aggregates (panels C-E), thus suggesting an alternative mechanism of tube formation apart from the lateral association of rod like aggregates as observed at higher Pβ2m concentrations (Figure 3.5F). Pβ2m shows small globular aggregates in HFIP even at early time points (Figure 3.8A-C) but with increasing time, forms fibrillar aggregates with tapering ends which circularize to give rings/tubes which are quite different from those observed in TFE (panels D and E). These tubes are ≈ 15-20 nm in length, and unlike the tubes obtained in TFE, these tubes have thinner walls and larger (= 2 fold) inner diameter. Apart from the tubes, a large population of globular aggregates (≈ 10-40 nm) is also present (panels D and E). It is observed that even at a low concentration of 50 μM, Pβ2m has the ability to form aggregates when dried on mica from all the 3 alcohols even after short incubation periods. In order to examine the conformation of
Figure 3.6  Time-dependent AFM imaging of 50 μM Pβ2m in MeOH incubated at room temperature for 4 days. Aliquots were removed at different time points and samples prepared for AFM imaging. Panels A, B, and C represent the images recorded immediately after dissolution, 48, and 96 hours of incubation. Scale bars represent 1 μm.
Figure 3.7 Time-depandant AFM imaging of 50 μM Pβ2m in TFE incubated at room temperature for 4 days. Aliquots were removed at different time points and samples prepared for AFM imaging. Panels A-B, C, and D-E represent the images recorded immediately after dissolution, 48, and 96 hours of incubation. Scale bars represent 1 μm.
Figure 3.8  Time-dependant AFM imaging of 50 µM Pβ2m in HFIP incubated at room temperature for 4 days. Aliquots were removed at different time points and samples prepared for AFM imaging. Panels A, B-C, and D-E represent the images recorded immediately after dissolution, 48, and 96 hours of incubation. Scale bars represent 1 µm.
Pβ₂m aggregates in the solid-state, FTIR spectra of Pβ₂m were recorded after drying from MeOH, TFE, and HFIP at 50 μM and 0.7 mM concentrations.

### 3.3.4 Fourier transform infrared spectroscopy

FTIR spectra of Pβ₂m in the amide I region, which is sensitive to secondary structure (Surewicz et al., 1993; Haris and Chapman 1995; Pelton and McLean 2000), were examined. FTIR spectra of Pβ₂m, dried from MeOH, TFE, and HFIP are shown in Figure 3.9. Samples were prepared from stock solutions in which peptide concentrations were 50 μM and 0.7 mM. Spectra were recorded 4 days after dissolution of the peptide. The spectrum for the TFE sample at 50 μM was also recorded after 5 days. Samples prepared from MeOH solutions showed peaks at 1627 cm⁻¹ (Figure 3.9A and B), which is characteristic of amide I band of peptides adopting β-structure. The peak positions in the spectra from TFE solutions at 1628 cm⁻¹ and 1653 cm⁻¹ (corresponding to amide I band of peptides in helical conformation) indicate that β-structure is formed when dried from dilute solutions whereas α-helical conformation predominates when dried form 0.7 mM solution (Figure 3.9C and D). When samples were prepared from HFIP, peaks at 1653 cm⁻¹ (Figure 3.9E and F) indicate helical conformation irrespective of the concentration of the stock solution. Trifluoroacetate counterion was not exchanged for any of the samples prepared for FTIR spectra. Trifluoroacetate gives an absorption band around 1673 cm⁻¹ (Surewicz et al., 1993). A low intensity shoulder at ≈ 1673 is present in the spectra recorded on films dried from 50 μM Pβ₂m solutions which can be attributed to TFA. The intensity of TFA band is further diminished in the spectra recorded on films obtained from 0.7 mM Pβ₂m samples (Figure 3.9B, D, and F). The intensity of TFA band was too less to interfere in the assignment of the amide I band.

The FTIR spectra recorded after 4 days of incubation in MeOH and HFIP were identical to 1 day old samples. However, time dependent changes in spectra were observed when the peptide was dried from 50 μM TFE. Peaks for the sample from TFE were observed at 1646 cm⁻¹, 1635 cm⁻¹, and 1628 cm⁻¹ for 1, 4, and 5 day old samples. The peak at 1646 cm⁻¹ indicates unordered structure (Pelton and McLean, 2000) at early time points and β-structure after 5 days incubation.
Figure 3.9 FTIR spectra in the amide I region for Pβm dried from 50 μM (left panels) and 0.7 mM (right panels) stock solutions. The peptide was dried on ZnSe crystal from MeOH (A, B); TFE (C, D); and HFIP (E, F). Panel C represents the spectrum recorded after 5 days of incubation at room temperature. All other panels are for samples incubated for 4 days.
3.3.5 Surface activity and aggregation on air-aqueous interface

Aggregation of Pβ2m on air-aqueous interface was studied using Wilhelmy film balance and ThT fluorescence microscopy. Surface pressure-Area (π-A) isotherms are routinely recorded using Wilhelmy film balance (Maget-Dana, 1999). The spread monolayer can be compressed or expanded at desired rate using movable barriers, and surface pressure and surface area are continuously recorded. The changes in surface pressure with area imply elasticity in the monolayer films. The equilibrium elasticity is related to compressibility coefficient. By analogy with the compressibility for a bulk material, monolayer compressibility is defined as:

\[ C_s = -\frac{1}{A} \left( \frac{\partial A}{\partial \pi} \right)_{P,T,n} \]

where \( A \) = area/molecule, \( \pi \) = surface pressure, \( n \) = number of moles of the material.

Limiting molecular area \( (A_0) \) is the minimum area occupied by each molecule in the monolayer at zero surface pressure. Limiting area can be derived by extrapolating the steep portion of the isotherm to the area \( (\text{Area/molecule}) \) axis. The compressibility, at limiting molecular area is given by

\[ C_{s0} = -\frac{1}{A_0} \left( \frac{\partial A}{\partial \pi} \right)_{P,T,n} \]

It is more convenient to use the compressibility modulus, \( C_s^{-1} \) (elastic modulus of area compressibility), which is reciprocal of the compressibility, to analyze the physical states of the monolayers. The compressibility modulus provides a measure of the compressional elasticity of a monolayer and can be used to characterize the phases of monolayers (Davies and Rideal, 1963). The compressibility modulus of a clean surface is zero, increasing with the amount of surfactant. In general, \( C_s^{-1} \) depends on the state of the film, being greater for the more condensed films. According to Davies and Rideal, compressibility modulus of peptide monolayers ranges from 12.5-50 mN/m for liquid expanded phase, while for liquid condensed phase, it lies between 100 and 250 mN/m (Davies and Rideal, 1963).

Figure 3.10 shows compression-expansion cycles of 6 nmoles of Pβ2m layered on subphase from 0.6 mM Pβ2m solution prepared in MeOH, TFE, and HFIP. Figure 3.11 represent the compression-expansion cycles of Pβ2m (6 nmoles) layered on subphase from 50 μM Pβ2m prepared in 50 mM phosphate buffer, pH 7.0 from 0.6 mM Pβ2m solution prepared
Figure 3.10 Compression-expansion isothermal cycles of 6 nmoles Pβ2m on aqueous subphase (50 mM phosphate buffer, pH 7.0) at room temperature. The peptide was layered on the surface from 24 hours old 0.6 mM Pβ2m in MeOH, TFE, and HFIP as mentioned in the figure. Repeated compression-expansion cycles were recorded between zero surface pressure and collapse pressure.
Figure 3.11 Compression-expansion isothermal cycles of 6 nmoles Pβ2m on aqueous subphase (50 mM phosphate buffer, pH 7.0) at room temperature. The peptide was diluted into 50 mM phosphate buffer, pH 7.0 from 24 hours old 0.6 mM Pβ2m in MeOH, TFE, and HFIP to a final concentration of 50 μM and kept at room temperature for 24 hours. The peptide was layered on the air-buffer interface and compression-expansion cycles were recorded between zero surface pressure and collapse pressure.
in MeOH, TFE, and HFIP. Limiting molecular areas (Ao), collapse pressures (πc), and compressibility moduli at limiting molecular areas (Cs0⁻¹) for first compression are shown in Table 3.1.

When Pb2m was layered from MeOH, the limiting molecular area calculated from first compression isotherm was 286 Å². When peptide was layered from buffer into which peptide was diluted from MeOH, a significant decrease in limiting molecular area is observed. This suggests a rearrangement within the molecule and/or intermolecular interactions causing reduction in average area of the peptide or peptide aggregates. However compressibility and collapse pressure of the peptide are not significantly affected.

Limiting molecular areas (Ao) and compressibility moduli at limiting molecular area (Cs0⁻¹) of Pb2m depend on the solvents used for dissolution of peptide. Compressibility moduli of the peptide layered from TFE and HFIP are significantly lower than that layered from MeOH. This shows that the peptide layered from fluorinated alcohols TFE and HFIP is more compressible as compared to that layered from MeOH. When the peptide was diluted in phosphate buffer from TFE and HFIP, and incubated for 24 hours prior to layering on subphase, the isotherms show an increase in limiting molecular areas. The peptide layered from buffer, into which it was diluted from TFE, becomes significantly less compressible as compared to that layered from TFE.

Figure 3.12 shows plots of compressibility modulus against molecular area for all the six samples. The compressibility moduli were calculated from the first compression isotherms. Briefly, the isotherms were plotted in Origin® software and surface pressure was differentiated with respect to molecular area, i.e. dπ/dA was calculated at all the molecular areas. The Cs⁻¹ values were obtained by multiplying dπ/dA values with negative of corresponding molecular areas. The Cs⁻¹ was plotted against the molecular area. Panels on left side show Cs⁻¹ against molecular area plots of Pb2m layered from organic solvents: MeOH (panel A), TFE (panel B), and HFIP (panel C). Above 200 Å²/molecule, Pb2m layered from MeOH shows large compressibility moduli as compared to that layered from TFE and HFIP, where compressibility moduli are < 30 mN/m. For peptides layered from TFE and HFIP, the maximum compressibility moduli (≈ 120 mN/m) are at ≈ 158 Å²/molecule (panels B and C). The data clearly show that the peptide layered from TFE and HFIP is in liquid expanded state (Cs⁻¹ ≤ 50 mN/m) up to molecular area of 180 Å². Further compression is accompanied by
Table 3.1  Limiting molecular areas ($A_0$), collapse pressures ($\pi_c$), and compressibility moduli at limiting molecular areas ($C_{a0}^{-1}$) of the peptide films, calculated from first compression isotherm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$A_0$ (Å$^2$/molecule)</th>
<th>$\pi_c$ (mN/m)</th>
<th>$C_{a0}^{-1}$ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_2m$ in MeOH</td>
<td>286.0</td>
<td>34</td>
<td>75.6</td>
</tr>
<tr>
<td>$\beta_2m$ in Buffer from MeOH</td>
<td>242.1</td>
<td>34</td>
<td>69.8</td>
</tr>
<tr>
<td>$\beta_2m$ in TFE</td>
<td>191.5</td>
<td>37</td>
<td>43.6</td>
</tr>
<tr>
<td>$\beta_2m$ in Buffer from TFE</td>
<td>203.8</td>
<td>34</td>
<td>58.3</td>
</tr>
<tr>
<td>$\beta_2m$ in HFIP</td>
<td>186.8</td>
<td>35</td>
<td>49.9</td>
</tr>
<tr>
<td>$\beta_2m$ in Buffer from HFIP</td>
<td>227.4</td>
<td>33</td>
<td>50.3</td>
</tr>
</tbody>
</table>
Figure 3.12  Plots of Pβ₂m compressibility modulus against molecular area, calculated from first compression isotherms. Left panels show plots when peptide was layered at air-aqueous interface from organic solvents. (A), MeOH; (B), TFE; and (C), HFIP. Right panels show plots when peptide was layered from 50 mM phosphate buffer, pH 7.0 into which it was diluted from MeOH (D), TFE (E), and HFIP (F) stock solutions.
increase in compressibility moduli, and the films become more condensed (Davies and Rideal, 1963). Decrease in $C_s^{-1}$ at molecular areas less than 150 Å² suggest inter/intra-molecular rearrangements at higher surface pressures. Panels D-F show $C_s^{-1}$ against molecular area plots for peptide layered from phosphate buffer, pH 7.0, into which peptide was diluted from MeOH (panel D), TFE (panel E), and HFIP (panel F). When layered from buffer into which it was diluted from TFE and HFIP, $P_2m$ films are more compressible than that was diluted into buffer from MeOH. For $P_β_2m$, that was diluted into buffer from MeOH and TFE prior to layering at air-water interface, transient decrease in $C_s^{-1}$ is observed at molecular areas ≈ 220 Å² and ≈ 185 Å², respectively, suggesting structural reorganizations around these molecular areas. This suggests some inter/intra-molecular rearrangements going on at high molecular areas too.

Compression isotherms are sufficient to obtain limiting molecular area, collapse pressure, and compressibility of the monolayer, but recording complete compression-expansion cycle provides information about irreversible processes going on while compression or expansion (Lepere et al, 2007). $P_β_2m$ clearly shows hysteresis in the isotherms for all the 6 samples, suggesting either rearrangements of molecules or intermolecular interactions in the peptide films. The compression isotherms show significant shift towards lower molecular areas after successive compressions while no significant shift was observed in expansion isotherms. This suggests the presence of irreversible processes while compression.

Aggregation status of $P_β_2m$ at different surface pressures was studied using ThT fluorescence microscopy as mentioned in Section 2.9. The peptide was layered on the aqueous subphase and compression-expansion cycles were recorded as discussed for Figure 3.11. After these cycles, one more compression-expansion cycle was recorded and images were recorded from $P_β_2m$ compressed to different surface pressures. Figure 3.13 is a representative compression-expansion isotherm showing how samples were prepared for fluorescence imaging studies. Briefly, the monolayers were subjected to compression-expansion cycle. A circular glass cover-slip, held using tips of the forceps, was gently touched to the monolayer as soon as desired surface pressure was reached. These coverslips were then mounted on glass slides having 20 µl of 20 µM ThT in water. The labels A, B, C, and D indicate the points at which the glass coverslips were touched to the monolayers. Microscopic slides prepared at these points were called slides A, B, C, and D, respectively.
Figure 3.13  A representative compression-expansion cycle for preparing microscopic slides of Pβ₂m compressed to different surface pressures. The labels A, B, C, and D represent the points at which slides were prepared by touching microscopic cover glass to the peptide film.
When imaging was done for the peptide layered on subphase directly from organic solvents, images obtained using ThT showed very little fluorescence. When Pβ2m was layered from phosphate buffer, ThT-positive structures were observed. Although Pβ2m compression-expansion isotherms, when layered from buffer, are similar to those when peptide is layered from organic solvents, the latter give weakly ThT-positive aggregates. It is shown that Pβ2m forms ThT-positive structures immediately after diluting into the aqueous solution (Figure 3.2). These ThT-positive aggregates are modulated on layering at air-water interface and through compression-expansion cycles. However, as CD spectra clearly show, Pβ2m adopts α-helical structure in organic solvents (Figure 3.3). Although, diluting the peptides into aqueous buffer from organic solvents forms β-structured fibrils, layering at air-aqueous interface as well as compression-expansion cycles fail to modulate the peptide into ThT-binding cross-β structures. Figure 3.14 shows the aggregation of peptide which was diluted into buffer from MeOH stock solution and kept for 24 hours. ThT fluorescence images at different regions of the slide recorded from this sample are shown in panels A-F. ThT positive fibrillar aggregates are clearly observed. The fibrils are straight, rod-like and most of them are 2-8 μm long (as indicated by arrows), but smaller fibrils (<1 μm in length) are also present to small extent. Panels G-L in Figure 3.14 represent the images recorded from slide A i.e. at zero surface pressure (C_s⁻¹ = 0 mN/m) (Figure 3.13). Needle-like structures that cause large increase in ThT fluorescence are clearly observed. Careful analysis reveals that these needle-like structures are formed by small, ThT-positive modules that are aligned to give needle-like structures, up to 15 μm long (indicated by arrows). As compared to the fibrils formed in solution (panels A-F), these structures cause significantly large increase in ThT fluorescence. The peptide film was compressed and slide B was prepared at ≈ 25 mN/m (C_s⁻¹ ≈ 120 mN/m). Needle-like structures are present but ThT fluorescence is slightly less intense (panels M-R), as compared to that obtained from samples prepared at zero surface pressure i.e. at maximum compressibility (panels G-L). At further higher surface pressure (slide C, surface pressure ≈ 42 mN/m, C_s⁻¹ ≈ 10 mN/m), the fibrillar morphology is severely compromised and very few ThT-positive aggregates could be observed, and fluorescence was very weak (panels S-X). The compressibility modulus at 42 mN/m surface pressure is very low (high compressibility). This suggests that the peptide film is severely affected by the surface pressure, making it further compressible. When peptide layer was expanded to zero surface pressure (C_s⁻¹ = 0 mN/m), fibrillar morphology of the aggregates is restored (panels Y-ZD). Rod-like
Figure 3.14 ThT fluorescence imaging of Pβ2m when layered on air-aqueous interface from buffer into which it was diluted from MeOH. Prior to layering on subphase (panels A-F); after repeated compression-expansion cycles and recorded at zero surface pressure ($C_s^{-1} = 0$ mN/m, panels G-L), $\approx 25$ mN/m ($C_s^{-1} = 120$ mN/m, panels M-R), $\approx 42$ mN/m ($C_s^{-1} = 10$ mN/m, panels S-X), and again at zero pressure ($C_s^{-1} = 0$ mN/m, Y-ZD). Scale bars represent 10 μm. The arrows are described in the text.
Figure 3.14 continued... ThT fluorescence imaging of Pβ2m when layered on air-aqueous interface from buffer into which it was diluted from MeOH. Prior to layering on subphase (panels A-F); after repeated compression-expansion cycles and recorded at zero surface pressure ($C_s^{-1} = 0$ mN/m, panels G-L), $\approx 25$ mN/m ($C_s^{-1} = 120$ mN/m, panels M-R), $\approx 42$ mN/m ($C_s^{-1} = 10$ mN/m, panels S-X), and again at zero pressure ($C_s^{-1} = 0$ mN/m, panels Y-ZD). Scale bars represent 10 μm. The arrows are described in the text.
aggregates, up to 8 μm long (panel Y, as indicated by arrows) and needle like structures, up to 15 μm long (panels Z-ZD, as indicated by arrows) could be observed.

Figure 3.15 shows the aggregation of Pb2m which was diluted in buffer from TFE stock solution and kept for 24 hours. Panels A-F show the images recorded from this sample. Very faint fluorescence is observed. After repeated compression-expansion cycles, at zero surface pressure (slide A, C5-1 = 0), the peptide forms fibrillar aggregates, which cause large increase in ThT fluorescence (panels G-L). These fibrils range between 2-6 μm in length, but lateral assembly of short ThT-positive modules appear to form flat sheet-like structures (panels J-L). When compressed to higher surface pressure (surface pressure ≈ 18 mN/m, C5-1 ≈ 120 mN, panels M-R), the fibrillar morphology is compromised and few weakly ThT-positive needle-like structures, up to 8 μm in length, were observed (shown by arrows). At further higher surface pressure, few ThT-positive aggregates were observed but ordered structures are completely lost (≈ 35 mN/m, C5-1 ≈ 75 mN/m, panels S-X).

Figure 3.16 shows the aggregation of Pb2m which was diluted in buffer from HFIP stock solution and kept for 24 hours. Panels A-F represent the images recorded from this sample. Short rod-like structures (< 3 μm in length, indicated by arrows) are observed that cause appreciable increase in ThT fluorescence. After repeated compression-expansion cycles, at zero surface pressure (slide A, C5-1 = 0 mN/m), the peptide forms sheet like structure which appears to be composed of fibrillar aggregates (panels G-I). In panel H, fibrils (up to 8 μm long) were also observed over ThT-positive peptide film (indicated by arrows). The peptide was compressed on air-buffer interface and slides were prepared at ≈ 8 mN/m (slide B, C5-1 ≈ 80 mN/m, panel J) and ≈ 18 mN/m (slide C, C5-1 ≈ 95 mN/m). Panel J shows very few, amorphous-like aggregates while nothing was observed on slide C (no image shown). Panels K and L show imaging when peptide is expanded back to zero surface pressure (slide D, C5-1 = 0). The fibrillar morphology, along with ThT binding property is restored.

The ThT imaging shows that at low molecular areas, the peptide monolayers are highly compressible, and form ThT-positive fibrillar/needle-like structures. As the films are compressed, their compressibility decreases with increasing surface pressure leading to disruption of fibrillar morphology. At pressures, where C5-1 ≥ 100 mN, suggested as liquid-condensed state (Davies and Rideal, 1963), fibrillar morphology is severely compromised and further compression causes reduction in compressibility modulus suggesting structural
Figure 3.15  ThT fluorescence imaging of Pβ2m when layered on air-aqueous interface from buffer into which it was diluted from TFE. Prior to layering on subphase (panels A-F); after repeated compression-expansion cycles and recorded at zero surface pressure ($C_s^{-1} = 0 \text{ mN/m}$, panels G-L), $\approx 18 \text{ mN/m}$ ($C_s^{-1} = 120 \text{ mN/m}$, panels M-R), and $\approx 35 \text{ mN/m}$ ($C_s^{-1} = 75 \text{ mN/m}$, panels S-X). Scale bars represent 10 μm. The arrows are described in the text.
Figure 3.15 continued... ThT fluorescence imaging of Pβ2m when layered on air-aqueous interface from buffer into which it was diluted from TFE. Prior to layering on subphase (panels A-F); after repeated compression-expansion cycles and recorded at zero surface pressure ($C_s^{-1} = 0$ mN/m, panels G-L), $\approx 18$ mN/m ($C_s^{-1} = 120$ mN/m, panels M-R), and $\approx 35$ mN/m ($C_s^{-1} = 75$ mN/m, panels S-X). Scale bars represent 10 μm. The arrows are described in the text.
Figure 3.16  ThT fluorescence imaging of Pβ2m when layered on air-aqueous interface from buffer into which it was diluted from HFIP. Prior to layering on subphase (panels A-F); after repeated compression-expansion cycles and recorded at zero surface pressure ($C_{s^{-1}} = 0 \text{ mN/m}$, panels G-I), ≈8 mN/m ($C_{s^{-1}} = 80 \text{ mN/m}$, panel J), and again at zero pressure ($C_{s^{-1}} = 0 \text{ mN/m}$, panels K-L). Scale bars represent 10 μm. The arrows are described in the text.
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reorganization. Interestingly, expansion of the peptide films restores the fibrillar/needle-like morphology.

3.4 Discussion

The results described in this chapter indicate that the amyloidogenic peptide spanning the β-strand E of wild-type human β2m can form amyloid fibrils when diluted from MeOH and helical-structure promoting solvents such as TFE and HFIP into aqueous buffer. In aqueous buffer, the peptide adopts β-conformation, although in organic solvents, helical conformation predominates. The fibril formation in buffer is rapid and is similar to the observation when the peptide was diluted from DMSO in which it would be unstructured. Irrespective of the structure or lack of it in organic solvents, the peptide Pf3 2 m adopts β-structure in aqueous medium and forms fibrils. Effects of increasing concentrations of HFIP and TFE on a monomeric peptide spanning residues 20-41 of β2m indicated the ability of the solvents to promote fibril formation (Yamaguchi et al, 2006). While helical conformation was observed initially in the organic solvent-aqueous mixtures, β-structure was observed on prolonged incubation in the solvent mixtures. Maximum fibril formation was observed at 20% TFE and 10% HFIP (Yamaguchi et al, 2006). The presence of organic solvent in water would result in decreased hydrophobic interactions between peptide molecules. In neat fluorinated organic solvents such as TFE and HFIP, the hydration shell would be replaced by alcohol molecules followed by hydrogen bond and secondary structure formation such as helix or β-hairpin (Rajan and Balaram, 1996; Buck, 1998; Blanco et al, 1994). Pf3 2 m structures with varying morphologies were observed when peptide was dried on mica from neat MeOH, TFE, and HFIP. In these solvents, the peptide adopts predominantly helical conformation. The rapid evaporation of alcohols on the mica surface would result in favorable hydrophobic interactions and interactions between the adjacent aromatic residues resulting in the formation of self-assembled structures other than fibrils. Although, no time-dependent changes in secondary structure of Pf3 2 m were observed, the time dependent changes in morphology observed from TFE and HFIP solutions suggest a degree of self-association even in these solvents. Although Pf3 2 m adopts predominantly helical structure in the organic solvents, FTIR spectra indicate that samples dried from MeOH adopt β-structure in the solid-state. Also, samples from TFE show time-dependent changes in conformation when prepared from solution at low peptide concentration. Initially, random conformation is observed. After 5 days, the peak position at 1628 cm⁻¹ is characteristic of amide I band of peptides adopting β-
structure. When the sample was prepared from a concentrated stock, β-structure is not observed. Hence, the ring-like structures shown in Figures 3.5C-E arise from self-association of helical structures rather than β-structures. In fact, when β-structures are formed as in the sample from 50 μM TFE, the ring-like structures are less prominent as shown in Figure 3.7D and E. Unordered structure in the solid-state gives rise to elongated structures shown in Figure 3.7A and B.

The surface-activity of peptides and proteins that form amyloid-fibrils have been reported (Soreghan et al, 1994; Schladitz et al, 1999; Maltseva et al, 2005; Lepere et al, 2007). These studies indicate that the peptides are surface-active and insert into lipid monolayers. In fact, there is a report that the fibrillar form of hIAPP was not surface active as compared to the monomer (Engel et al, 2006). Modulation of self-association on air-water interface by multiple compression-expansion cycles have not been investigated extensively. Three-dimensional structures and nanodomains have been observed for a 12-residue peptide that forms amyloid fibrils in aqueous solution when the air-water interface was examined during compression-expansion cycles by Brewster angle microscopy and AFM (Papanikolopoulou et al, 2005; Lepere et al, 2007). The studies on Pβ2m indicate that fibrillar structures are formed on air-water interface whose dimensions and morphologies are dependent on the extent of compression and expansion. The morphologies also appear to be influenced by “solvent-history” i.e. the organic solvents the peptide was dissolved in before the samples were prepared for monolayer experiments. Surface activity experiments show that Pβ2m is highly surface active as evident from large limiting molecular areas. Collapse pressure lies within 34-37 mN/m but compression around this pressure does not result in monolayer collapse suggesting inter/intra-molecular rearrangements within the monolayer at high surface pressures. Repeated compression-expansion cycles shift the compression isotherm to lower molecular areas but expansion isotherms show same profiles. ThT fluorescence imaging of the peptide taken from monolayers at different surface pressures shows that repeated compression-expansion cycles modulate the aggregates. At zero surface pressure i.e. at liquid expanded state (Cs-1 ≈ 0 mN/m), fibrillar aggregates are present; at surface pressures that correspond to liquid condensed state (Cs-1 ≈ 100 mN/m), the fibrillar morphology is severely compromised along with decrease in ThT fluorescence caused by the aggregates. An interesting observation is the reversibility of fibril formation, wherein compression causes distortion of the fibrils while expansion restores the fibrillar morphology.
β2-microglobulin forms amyloid fibrils at acidic pH, and morphology of the fibrils is highly dependent on pH and ionic strength (McParland et al., 2000; Gosal et al., 2005). The protein is composed of seven β-strands arranged in two β-sheets. At pH 3.6, one of the amyloidogenic conditions for β2m, the N and C terminal strands of amyloid precursor monomer are substantially destabilized while other five strands are stable (McParland et al., 2002). H/D exchange studies with β2m fibrils formed at pH 5 show that strands A and G are weakly protected from exchange (Hoshino et al., 2002). Jones et al. studied the fibril forming ability of individual β-strands and showed that strand E (Pβ2m) forms stable amyloid fibrils whereas all other strands were non-amyloidogenic under the conditions tried (Jones et al., 2003). Pβ2m (strand E) is unusually rich in aromatic residues and forms fibrils over a wide pH range of 1-7 suggesting π-π stacking as the possible interactions in the self assembly of Pβ2m. The fibrils formed are long and straight in morphology. Although highly amyloidogenic (forms fibrils immediately in aqueous solutions), the aggregation behavior of Pβ2m has not been extensively studied. Amyloidogenic peptides can be modulated to obtain non-amyloid nanostructures using solid surfaces, organic solvents, and organic solvent-water co-mixtures (Li et al., 2007; Krysmann et al., 2008b). Organic solvents modulate the structure and self-assembly of peptides (Shen and Murphy, 1995; Li et al., 2007) and amyloid forming peptides can be modulated to self-assemble into non-amyloid nanostructures.

Different morphologies were observed when Pβ2m was incubated for varying time periods in organic solvents. The aggregates obtained from organic solvents do not show amyloid fibrillar morphology. Ring-like structures are prominent when Pβ2m was dissolved in helix-promoting solvents TFE and HFIP. Analysis of the CD spectra of Pβ2m indicated the presence of both helical and β-conformations. It is likely that this feature favors self-assembly with varying morphologies on the mica surface. Since short peptides often exist in an ensemble of conformations, it should be possible to generate self-assembled structures with varying morphologies by appropriate choice of surfaces and solvents for dissolution of peptides, particularly those having the property to self-associate such as amyloid-forming peptides.