Interaction of insulin with SDS/CTAB catanionic Vesicles

Bidisha Tah, Prabir Pal, G.B. Talapatra *

Department of Spectroscopy, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700 032, India

A R T I C L E   I N F O

Article history:
Received 13 February 2013
Received in revised form 28 June 2013
Accepted 10 July 2013
Available online 19 July 2013

Keywords:
Catanionic vesicles
Insulin
Phosphorescence
Surfactant

A B S T R A C T

In the present study, a novel method was used for entrapping the protein, insulin into the catanionic SDS/CTAB vesicle membrane. The anionic SDS and cationic CTAB formed catanionic vesicles at particular concentration (35:65 by volume). In this study, vesicle membrane can be considered as model membrane. The vesicle formation and entrapment efficiency depend on the pH of the aqueous solution. The insulin molecules have attached with the vesicular membrane at pH 7.0. However, at acidic pH, the vesicles were ruptured and the insulin did not entrap into the vesicle membrane, whereas at alkaline pH insulin became fibrillar. The scanning electron microscope (SEM), Dynamic light scattering (DLS), and Zeta potential studies established the self-assembled structure formation of insulin and catanionic vesicles. To know the protein confirmations, Circular dichroism (CD) was also employed. The temperature dependent steady state and time resolved emission spectroscopy show that at room temperature (25 °C), apart from the 305 nm tyrosine fluorescence, a new emission peak at 450 nm was observed only in case of insulin-vesicle system, and was assigned as the tyrosine phosphorescence. This phosphorescence peak is the signature of the entrapment of insulin into the vesicle membrane.

1. Introduction:

Surfactants and protein-surfactant mixtures play important role in many industrial processes such as, stabilization of foam and emulsion, coating process of photographic industry, and in different types of biological applications like drug delivery tool etc [1–3]. However, no prediction can be made concerning the effect of ionic surfactant in the protein solution because in this case inter-ion interaction between surfactant and protein can result in the formation of protein-surfactant complexes [4]. As the aggregation behavior of the surfactant system operating at the molecular level, it can form micelle, vesicle or liposome [5], due to the various non-covalent interactions (such as π–π stacking, H-bonding, Van der Waals interactions etc.). Liposome is intrinsically metastable [6,7].

In our earlier work, we have demonstrated that the stable vesicle can be formed by using anionic sodium dodecyl sulfate (SDS) and cationic cetyltrimethyl ammonium bromide (CTAB) surfactant mixtures in aqueous solution [8]. Strong electrostatic interactions between the oppositely charged head group of the cationic and anionic surfactants are responsible for the formation of vesicle. As a step to understand these interactions, we have used Langmuir monolayer of catanionic surfactant mixtures to mimic the biological membrane and insulin as the protein component [9]. Moreover, the bimolecular interactions in lipid monolayer at the air/water interface has been studied [10,11]. The cationic and anionic surfactants mixture in aqueous media form a variety of microstructures as the solution composition is varied [12–14]. The interaction of protein with surfactant based vesicles can be investigated in equilibrium condition since the surfactant based vesicles are thermodynamically stable [15]. Furthermore, they are charged and they interact with biomolecules in a wide range of pH and ionic strength values. For these reasons, the catanionic vesicles are used as a substitute of costly lipids [14].

We choose insulin as protein in our present work. Insulin, a polypeptide hormone is physiologically important to control glucose level in blood stream, is synthesize and stored in pancreas as a Zn2+ containing hexamer. It contains chain-A (21 residues) and chain-B (30 residues), linked by two disulfide bonds [16,17]. The solution behavior of insulin is complex and dynamic equilibriums of monomers, dimmers, tetramer, hexamers, and oligomers depending on the concentration, pH, ionic strength, temperature, metal ions, and solvent composition [18]. We prefer CTAB and SDS as cationic and anionic surfactant respectively as a model system due to their easy availability. Our main objective is to study the insulin-vesicle complex formation after their interaction, which is very rarely reported. However, the finding of this model system study may also throw some insight about drug binding and delivery system.

To confirm the self-assembled structure formation of insulin and catanionic vesicles, we used scanning electron microscope (SEM), Dynamic light scattering (DLS), and Zeta potential. Excitation and emission spectroscopy were also used for characterization. To know the protein confirmations, Circular dichroism (CD) was also employed.

* Corresponding author. Tel.: +91 33 2473 4971; fax: +91 33 2473 2805.
E-mail addresses: spgbt@iacs.res.in, gbtalapatra@gmail.com (G.B. Talapatra).

0022-2313/S – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jlumin.2013.07.040

© 2013 Elsevier B.V. All rights reserved.
The results established that the vesicle formation and entrapment efficiency of insulin into the catanionic vesicle depend on the pH of the aqueous solution. At room temperature, apart from the tyrosine fluorescence, a new observed emission peak was assigned as the tyrosine phosphorescence and was identified as the signature of the entrapment of insulin into the vesicle in insulin-vesicle system.

2. Experimental section

2.1. Materials

The anionic surfactant Sodium Dodecyl Sulfate (SDS) and cationic surfactant Cetyl Trimethyl Ammonium Bromide (CTAB) were purchased from Merck and Himedia, (Mumbai, India) respectively. The chloroform (UV Grade) and methanol were purchased from Spectrochem and Sisco Research Laboratory, (Mumbai, India) respectively. The human insulin (Actrapid) was purchased from Abbott India Limited, Mumbai, India.

2.2. Methods

2.2.1. (A) Preparation of stock solution

The stock solution of SDS of concentration \( C_{\text{SDS}} \) of 0.7 mM was prepared in 1:1 chloroform and methanol solvent, whereas the solution of CTAB of concentration \( C_{\text{CTAB}} \) of 0.7 mM was prepared in chloroform solvent. The concentrations of the solution of pure SDS and CTAB were far below than their critical micelle concentration (CMC) [19,20]. In the present study, the specific volume ratio (35:65) of SDS:CTAB was chosen as the catanionic vesicles are formed at the specific volume ratio [8].

2.2.2. (B) Preparation of insulin incubated vesicles

The prepared catanionic solution in organic solvent was heated in water bath at 70 °C to evaporate the solvent. Then the Millipore water was added to form vesicles [8]. The aqueous solution of insulin of concentration 0.4 mg/ml (pH 7.0) was added after the formation of vesicles and sonicated for 10 min.

A schematic diagram of methods used in this work is shown in Fig. 1.

2.2.3. (C) Process of substrate cleaning

All the glass substrates were cleaned in a liquid soap ultrasonic bath followed by repeated rinsing with Millipore water. They were then immersed in acetone in an ultrasonic bath. Finally, they were cleaned, using Millipore water in the ultrasonic bath. A uniform layer of water onto the slide confirmed the hydrophilicity of the slide [21].

2.2.4. (D) Preparations of cast film

The cast films were prepared by drop casting the protein, protein-vesicular aqueous solution onto a hydrophilic substrate [22].

2.2.5. (E) FE-SEM and AFM characterizations

High-resolution field emission scanning electron microscope (FE-SEM, model JEM-6700 F) with a range of 0.5–30 kV and lateral resolution of 1.2–2.2 nm, was employed to extract the surface morphology of all transferred films and on fine hydrophilic glass substrate.

For study the surface morphology of the cast films, AFM (AFM, VECCO diCP-II Model No AP-0100) imaging was used. The tapping mode was used in air to minimize any kind of force exerted on the samples from the scanning tip. Thin phosphorus doped silicon cantilever (with no coating on the front side and 50 ± 10 nm aluminum coating at the backside) of resistivity 1–10 Ω-cm was used for scanning. The thickness of the cantilever ranges form 3.5–4.5 μm with a length of 115–135 μm as well as width of 30–40 μm. The processed images were subsequently analyzed for diameter, height, and surface roughness by Proscan 1.8 and Image analysis 2.1. The line profiles were used to calculate surface roughness. Whereas the height profile were used to calculate the variation between highest peak and lowest valley along the line.

2.2.6. (F) Dynamic light scattering (DLS)

The dynamic light scattering studies were carried out in a Brookhaven BI-200SM Goniometer (Brookhaven Instruments Corporation) with a 35 mW He–Ne laser (633 nm). The mean diameter was determined using the non-negativity constrained least-squares algorithm.

2.2.7. (G) Spectroscopic characterizations

The fluorescence emission and excitation spectra of pure insulin and insulin-vesicle system were recorded using a cuvette having a path length of 1 cm by a fluorescence spectrophotometer (Hitachi F-4500). The temperature dependent emission spectra were recorded by controlling the temperature below room temperature using liquid nitrogen as coolant. Phosphorescence decay profiles were observed by this spectrometer also.

The fluorescence lifetime measurements were done in a time correlated single photon counting (TCSPC) system. The samples were excited at 280 nm using a picoseconds diode laser (IBH Nanoled-07) in an IBH Fluorcube apparatus. The repetition rate was 1 MHz. The fluorescence decays were collected on a Hamamatsu MCP photomultiplier (C487802). The fluorescence decays were analyzed using IBH DAS6 software.

The CD spectra of pure insulin and insulin-vesicle bioconjugate in aqueous medium were recorded at room temperature on a JASCO J-815 CD spectrometer (Model no. J-815-150S) with 1 mm path length. In all the solutions, concentration of insulin was 0.4 mg/ml. All spectra were collected from 190 to 250 nm and average of three scans was adopted to increase the signal-to-noise ratio of the CD spectra.

2.2.8. (H) Zeta potential measurements

Zeta potential measurements were performed using a Malvern Zetasizer Nano ZS (Part no.: ZEN3600) instrument at 25 °C at scattering angle (175°) and detection angle (12.8°) with a He–Ne laser (power 4 mW, wavelength 632.8 nm and beam diameter 0.63 nm (1/e²) respectively). The machine has size measurement range 3.8–100 nm and having no practical range of zeta potential measurement. The Vesicular solutions and insulin-Vesicle system were placed in standard Malvern zeta potential disposable capillary cells for zeta potential measurements. All measurements were repeated three times.
3. Results and discussion

3.1. Surface morphology

We have done FESEM imaging to know the surface morphology of the drop casted film of simple vesicular solution and insulin mixed vesicular solution on hydrophilic glass substrate. Fig. 2 shows such results. Panel A and B represent the surface morphology of the vesicles’ membrane before and after adding of insulin respectively. Inset shows magnified single vesicle structure. The sizes of the vesicles are in the range of 200–600 nm. Surface of the vesicle without insulin is found smooth (Panel A), whereas, insulin makes the surface of the vesicles become rough (Panel B). The globular insulin is appeared to bind with vesicle membrane. Panel-C of Fig. 2 shows the AFM image recorded for the drop-casted film of insulin-vesicle system at pH 7.0 on a hydrophilic glass substrate and Panel-D represents the high magnified image at same condition. Panel-E shows the height profile of the indicated line. The side bar is a color representation of height.

The FESEM images of insulin-vesicle system at pH 12.0 (Panel-A) at pH 3.0 (Panel-B) are shown in Fig. 3. It is interesting to note that at acidic (pH 3.0) and alkaline (pH 12.0) aqueous media the insulin entrapment is not successful. At pH 3.0, the vesicles have been broken and the numbers of vesicles become reduced. On the other hand, at pH 12.0, the insulin is being fibrillated and no vesicles are formed. From the previous literature we know that the tyrosine have three pKa values i.e. 2.2, 9.11, 10.07 [23]. At higher alkaline pH (above 10), hydrogen bond of tyrosine are broken and tyrosinate ions are formed [24]. This tyrosinate formation may facilitate the formation of fiber. The FESEM image suggests that the sizes of the protein granules are larger than the crystal structure of insulin monomer mentioned in PDB file. These observations suggest that the insulin molecule make smaller globular aggregates at pH 3 and pH 7.0. Insulin binds to vesicles as oligomeric form.

To confirm the incorporation of insulin and its status (whether it is denatured or not) we have done the followings.

3.2. DLS and zeta potential study

Panel-A of Fig. 4 shows particle size distribution curves resulted from DLS measurements of catanionic vesicle (SDS:CTAB=35:65)
without and with insulin at pH 7.0. The result shows that the particle size of the insulin-vesicle system increases compared to pure vesicular system. This size increment may be due to the attachment of insulin. This result also matches with the dimension of FESEM images.

The measured Zeta potentials of insulin-surfactant system and simple catanionic surfactant system at pH 7.0 are 27.6 mV and 32 mV respectively. Since CTAB has the volume percent of 65, the vesicular solution is CTAB rich. Due to excess cationic surfactant (CTAB), the zeta potential of vesicular solution is found positive. According to the previous literature the isoelectric point (pI) of insulin is nearly 5.3 [25] and normally at pH 7.0, zeta potential of insulin become negative. In our experiment, we also observed that the zeta potential of insulin solution at pH 7.0 is −11.8 mV. However, in case of insulin-surfactant system at pH 7.0, negatively charged insulin may try to neutralize the system and decrease the value of zeta potential from 32 mV to 28 mV, which also depicts the fact that, insulin particles are incorporated into the vesicle membrane.

3.3. CD spectroscopy study:

CD spectroscopy is a very efficient tool to know about the structural change in protein due to unfolding and folding. It is also very informative about any structural change in secondary structure of protein. In this regards our motivation is to find out whether insulin undergo any structural changes or denaturation in the presence of catanionic vesicular solution. Fig. 4 (Panel-B) shows the minima at 224 nm and 208 nm, and a maxima peak at ~195 nm, which are typically for α-helix configuration [26–28]. It means the protein do not denature in presence of catanionic vesicle. Moreover, to get the detail information about the secondary structure of the system we have fitted the CD data by CDpro (SELCON 3) [28] program and the amount of different conformational element are listed in Table 1.

It is found that the amount of α-helix increase in case of insulin-vesicle system than pure insulin. This result once again depict the fact that insulin molecule may be entrapped into the catanionic vesicle membrane and being compact or folded.

3.4. Steady state and time resolved emission spectroscopy

Intrinsic fluorescence of protein is a sensitive technique that has been exploited in studying the structural, physicochemical, and functional properties of proteins [29]. The emission of proteins and polypeptides is the aggregated contributions from the three aromatic amino acids: tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). Each amino acid has two major absorption bands between 200 and 300 nm. The lower energy bands for Trp, Tyr, and Phe occur near 280, 277 and 258 nm respectively, and their extinction coefficients at these wavelengths are in the ratio 27:7:1. As a result, Trp generally dominates the absorption and emission spectra of proteins.
vesicle, intensity of the long wavelength band increases and studies reveal (not shown in Fig. 4) that with increasing amount of emission peak at 305 nm arises strongly. Concentration dependent the freeze-thaw process, some vesicles may break also. Hence, the may frees some attached insulin into the vesicle. Moreover, due to thaw process, there may be some changes in vesicle structure that comparable to the pure insulin. The reason of this observation is emission spectra show enhanced intensity of 305 nm peak (curve-e)pared with free tyrosine[24].

characteristic of tyrosine is observed but at reduced yields com-

insulin, which contain tyrosine but no tryptophan, the emission fluorescence of tyrosine. It should be noted that in proteins like uorescence arises due to the rigid geometry of the tyrosine insu,

phorescence could happened when the tyrosine side chain inclusion may be due to restriction of1Lb vibration. This observa-

Thus, the fluorescence quantum yield of tryptophan is much greater than that of tyrosine and phenylal alanine.

Insulin contains four tyrosine located at A-14, A-19, B-16, and B-26 on two chains connected by disulfide bridges and three phenylalanine residues [30]. However, it does not have any tryptophan residue. Thus, for insulin, tyrosine dominates in the absorption/emission process in absence of tryptophan [23,31].

Fig. 5 represents the peak normalized emission and excitation spectra of pure insulin (at pH 7.0) and insulin in the presence of catanionic vesicle at 25 ºC. For the case of pure insulin (curve-b), a single 305 nm peak in emission spectra is observed. This emission is fluorescence of tyrosine. It should be noted that in proteins like insulin, which contain tyrosine but no tryptophan, the emission characteristic of tyrosine is observed but at reduced yields compared with free tyrosine [24].

In presence of vesicle at 25 ºC (curve-d), the emission spectra drastically differ from that of pure case (curve-b). Apart from the relatively reduced intensity of 305 nm peak, a new broad structured band centering at 450 nm appears. It is interesting to note that when the insulin-vesicle complex system was dipped into liquid nitrogen temperature and then thawed at 25 ºC, the emission spectra show enhanced intensity of 305 nm peak (curve-e) comparable to the pure insulin. The reason of this observation is not very clear to us. However, in the second case due to the freeze-thaw process, there may be some changes in vesicle structure that may frees some attached insulin into the vesicle. Moreover, due to the freeze-thaw process, some vesicles may break also. Hence, the emission peak at 305 nm arises strongly. Concentration dependent studies reveal (not shown in Fig. 4) that with increasing amount of vesicle, intensity of the long wavelength band increases and 305 nm tyrosine fluorescence band decreases.

We measured the average decay lifetime of tyrosine fluorescence peak (305 nm) and other parameters. Fig. S1 in SI shows the tyrosine fluorescence decay of insulin with lamp profile. The fluorescence decay shows that it is multi exponential in nature. We have tried to fit the decay profiles using the double exponential function using Eq. 1 [23].

\[
\begin{align*}
\frac{f(t)}{\text{exp}(-t/t_i)} = \sum_{i=1}^{2} B_i \exp(-t/t_i)
\end{align*}
\]

where, \(B_i\)'s and \(t_i\)'s are the relative contributions and the lifetimes of the different components to the total decay. The average lifetime of fluorescence decay is calculated using Eq. 2. All the values are reported in Table S1 in SI.

\[
\langle \tau \rangle = \sum_{i=1}^{2} \frac{B_i t_i^2}{\sum_{i=1}^{2} B_i t_i}
\]

Mean lifetime of tyrosine fluorescence for pure insulin is about 1 ns. Moreover, the lifetime value decreases with increasing amount of catanionic vesicle (shown in the inset of Fig. S1 in SI). This decrement may be due to quenching or intersystem crossing.

Two excitation peaks (at 224 nm and 277 nm) are found for pure insulin (curve-a) and insulin in presence of catanionic vesicle (curve-c), represent the absorption transitions of the tyrosine phenol ring. It is to be noted that in presence of vesicle, two excitation peak positions remain almost same, but the relative intensity changes appreciably. Similarly observation is also found for the excitation spectrum (Fig. S2 in SI) of insulin-SDS system. According to Hooker and Schellman, [32] the lowest energy peak (277 nm) is due to the \(1L_a\) transition, whereas the highest energy peak (224 nm) is due to the \(1L_b\) of tyrosine phenol ring. The \(1L_a\) and \(1L_b\) transition vectors are almost perpendicular to each other. Between these two bands, the higher energy band (224 nm) is stronger for pure insulin. Relative intensity change due to vesicle inclusion may be due to restriction of \(1L_b\) vibration. This observation supports the information in previous literature [23].

The origin of 450 nm centered broad band of insulin in presence of vesicle is not yet clear to us. However, this peak of insulin could be due to (1) the formation of tyrosinate, (2) phos-
phorescence arises due to the rigid geometry of the tyrosine molecule entrapped into the vesicle membrane, and (3) tyrosine excimer formation. The absence of an excitation peak at 294 nm (Fig. 5) depicts the nonexistence of tyrosinate molecule [33]. Therefore, the first possibility does not stand for this phenomenon.

As we know from the previous literatures that the phosphorescence of the tyrosine could happened when the tyrosine side chain is in the rigid, protective environment [34].

Truong et al. [24] observed a broad weakly resolved tyrosine phosphorescence peak approximately from 350 to 500 nm of insulin (at pH 7.0) at 77 K. But our experiment at 25 ºC shows similar observation of broad band centered at 450 nm as observed by Truong et al. [24] at 77 K. To confirm whether it is phosphorescence or ground/excited state complex, we measure the emission of insulin in SDS, in CTAB and in SDS-CTAB catanionic vesicle system. Results are shown in Panel A of Fig. 6. Panel B of Fig. 6 displays the temperature dependent emission spectra around 450 nm and its lifetime. For insulin-SDS system, emission peak is blue shifted and broadened compared to pure insulin. This blue shift may be due to the decrement of effective polarity of the media. Alignment of hydrophobic tail of SDS with the insulin hydrophobic part may be responsible for that.

In insulin-CTAB system, there is a drastic change in emission spectrum compared to pure insulin. Apart from 305 nm tyrosine fluorescence peak, a new monotonous broadband appears in the long wavelength region (Panel A in Fig. 6). CTAB is positively charged. Since the isoelectric point of insulin is 5.3, [35] insulin is negatively charged at pH 7.0. Strong electrostatic interaction may lead to a ground state charge transfer complex showing broad long wavelength band. Time resolved fluorescence study of this long

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Unordered</th>
<th>Turn</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS/CTAB-INS</td>
<td>0.261</td>
<td>0.158</td>
<td>0.405</td>
<td>0.171</td>
</tr>
<tr>
<td>INS</td>
<td>0.188</td>
<td>0.352</td>
<td>0.258</td>
<td>0.220</td>
</tr>
</tbody>
</table>

Table 1 Secondary structure confirmation from fitted the CD data by CDpro (SELCON 3) program.
When insulin bound to the membrane of the catanionic vesicle, the frozen state insulin molecule is different from each other. Due to this reason at complex become glassy structure, and the orientation of every not show the structural broad peak. At the frozen condition the phosphorescence. The emission spectrum at low temperature does 1.4 ms. This long lifetime actually con 0.14 ms whereas at liquid nitrogen temperature, it increases to band increases drastically. At room temperature lifetime is about in Fig. 6 (panel B). With decreasing temperature, intensity of the long wavelength band of insulin-CTAB reveals average lifetime is few ns. Drastic change in excitation spectra (Fig. S2 in SI) also supports the idea of ground state CT complex formation.

In insulin-vesicle system, not only the intensity of tyrosine fluorescence decreases a lot, but also a new broad structured long wavelength band centered at 450 nm is appeared. This structured band is quite different from that obtained for insulin-CTAB system. This band looks like phosphorescence. To confirm the fact, we conducted the temperature dependent steady state and time resolved emission of this long wavelength band. Results are shown in Fig. 6 (panel B). With decreasing temperature, intensity of the band increases drastically. At room temperature lifetime is about 0.14 ms whereas at liquid nitrogen temperature, it increases to 1.4 ms. This long life time actually confirms that this emission is phosphorescence. The emission spectrum at low temperature does not show the structural broad peak. At the frozen condition the complex become glassy structure, and the orientation of every insulin molecule is different from each other. Due to this reason at the frozen state fine structures of broad peak are not resolved well. When insulin bound to the membrane of the catanionic vesicle, movement of tyrosine in side chain is restricted. In this restricted environment, intersystem crossing become more efficient and we observe phosphorescence at room temperature.

4. Conclusion

In conclusion, we have successfully developed a novel method for entrapping the essential protein, insulin, into the catanionic vesicle membrane. The vesicle formation and entrapment efficiency depend on the pH of the aqueous solution. The insulin molecules have attached with the vesicular membrane at pH 7.0. However, at acidic pH, the vesicles were ruptured and the insulin did not entrap into the vesicle membrane, whereas at alkaline pH insulin became fibrillar. We have also studied the steady state and time resolved emission spectroscopy. Due to entrapment of insulin in vesicle membrane, a new emission peak at 450 nm, assigned as phosphorescence of tyrosine has appeared. The experiment revealed that with the increment of vesicle concentration, the fluorescence emission of tyrosine at 305 nm was decreased. We have also immobilized the insulin molecule in its native state by drop-cast method. This insulin-vesicle system may be useful and effective in the field of drug delivery, biosensor, and bioengineering.

Acknowledgment

We thank DST, Government of India (Project No.—SR/S2/CMP-0079/2010 (G)) for partial financial support. Thanks also go to the authority of the IACS for providing central instrumental facilities of FE-SEM, AFM, DLS, and CD systems.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jlumin.2013.07.040.

References