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Analytical Methods

Determination of critical aggregation concentration and aggregation number of acid-soluble collagen from walleye pollock (*Theragra chalcogramma*) skin using the fluorescence probe pyrene

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ABSTRACT

The aim of this paper was to investigate the critical aggregation concentration and aggregation number of acid-soluble collagen from walleye pollock (*Theragra chalcogramma*) skin using the fluorescence probe pyrene. Results showed that pyrene was fit for studying the aggregation behaviour of collagen in sodium phosphate buffer at pH 7.2. The plots of the pyrene I_1/I_3 ratio, as a function of the logarithm of total collagen concentration, revealed a typical sigmoidal decrease, the critical aggregation concentration (CAC) from which was determined to be at 0.48 mg/ml. The subsequent transient fluorescence decay study indicated that the aggregation number of collagen was not constant, but varied with different concentrations of collagen. The structure of the aggregates tended to be stable, when the collagen concentration exceeded 1.2 mg/ml.

1. Introduction

Collagen is a fibrous protein composed of three polypeptide chains (α chains) wound together in a right-handed helix. *In vitro*, collagen can be induced to aggregate into fibrils or gels by incubation at 25–37 °C near neutral pH (Huelin, Baker, Poduska, & Merschrod, 2007; Na, Butz, Bailey, & Carroll, 1986). By varying the aggregation conditions, the microfibrillar density and interactions formed within the collagen network can be controlled (Jiang, Hörber, Howard, & Müller, 2004), making it possible to manufacture collagen products for different applications. On the other hand, research has shown that the aggregation behaviour in aqueous solution directly influences the collagen's physicochemical properties (Shi, Ma, Sun, & Wu, 2001). Therefore, the study of the aggregation properties of collagen will provide the theoretical basis for developing collagen products and modifying their functions.

Numerous studies of collagen aggregation *in vitro* have been reported in the past years (Na, Phillips, & Freire, 1989), but the main sources of such collagen are limited to land-based animals, such as bovine or porcine skin and bone. In recent years, marine collagen is being focused on because of its good functionality; additionally, use of marine collagen is appropriate for religious and cultural practices that forbid the consumption of pork products.

Research has revealed that the composition and characteristics of marine collagen are different from those of collagen from land-based animals (Zhong et al., 2006). However, studies on the aggregation behaviour of marine collagens are scarce so far. Sivakumar and Chandrakasan (1998) investigated the turbidity curve for the *in vitro* fibrillogenesis of the cartilage collagens from squid (*Sepia officinalis*) and shark (*Carcharius acutus*); fibril formation and redissolution of collagen from frog (*Rana tigerina*) skin were reported by Sai and Babu (2001). Therefore, the aggregation properties of marine collagens need a more extensive investigation.

Critical aggregation concentration (CAC) and aggregation number (N_{agg}) are the most important parameters in investigations concerning the aggregation behaviour of biological macromolecules. CAC for the *in vitro* aggregation of collagen was observed by Kadler, Hojima, and Prockop (1987). Below the CAC, no aggregation of collagen could be detected by either the turbidity or the centrifugation method. Above the CAC, the collagen aggregated to form large visible aggregates (Na et al., 1986). There are several frequently used methods, like tensiometry, conductometry, fluorimetry and calorimetry, for the evaluation of CAC (Ray, Chakraborty, & Moulik, 2006). At concentrations above the CAC, the aggregation number is an important characteristic for a biopolymer system, revealing the size of the biopolymer aggregates. It is determined by any experimental method as an average value (Yu, Tian, Ho, Ding, & Wohland, 2006). Traditionally, several ap-

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proaches have been used to determine this parameter, including small-angle neutron scattering (SANS), fluorescence, and NMR self-diffusion coefficient measurements (Javadian et al., 2008).

In this paper, we used pyrene as a fluorescence probe to explore the aggregation behaviour of collagen. Pyrene, a typical polycyclic aromatic hydrocarbon, is constituted of four fused benzene rings and contains no functional group otherwise (Siddharth, Redden, Hendricks, Fletcher, & Palmer, 2003). It has earlier been used as a fluorescence marker to study the aggregation behaviour of chitosan and derivatives (Amiji, 1995; Li et al., 2007), and to determine the CAC of some surfactants, such as sodium dodecyl sulphate, dodecyltrimethylammonium bromide, Triton X-100, octaethylene glycol monododecyl ether, etc. (Aguiar, Carpena, Molina-Bolivar, & Ruiz, 2003), and the N_{agg} of binary mixed systems involving a sugar-based surfactant and different *n*-alkyltrimethylammonium bromides (Hierrezuelo, Aguiar, & Ruiz, 2006). Fujimori and Shambaugh (1983) employed pyrene to label collagens from young and old rat tail tendon for studying the cross-linking.

To the best of our knowledge, up to now there have been no reports about studying collagen aggregation behaviour using the fluorescence probe pyrene. The goal of the present study was to elaborate the potential of pyrene fluorescence spectra for studying the critical aggregation concentration and aggregation number of acid-soluble collagen from walleye pollock skin in Na-phosphate buffer at pH 7.2.

2. Materials and methods

2.1. Preparation of collagen

Walleye pollock (*Theragra chalcogramma*) skins were collected from a local fish processing factory in Qingdao. They were transported to the laboratory and stored at −20 °C until used. Collagen was prepared according to the method of Nagai and Suzuki (2000) with a slight modification. The extraction procedure and characterisation of collagen were shown in the paper of Yan et al. (2008).

2.2. Preparation of pyrene solution

Pyrene was purchased from Sigma Chemicals (Sigma–Aldrich, Munich, Germany) and used without further purification. A stock solution (1 mM) of pyrene was prepared in ethanol. The mixture was sonicated to dissolve completely. Pyrene, used as a fluorescence probe, was employed at a final concentration of 2 μM in the aggregate solution of collagen wherein the ethanol concentration was 0.2%. Such a small concentration of ethanol was considered not to affect the spectral and aggregation behaviour of collagen.

2.3. Measurement of fluorescence spectroscopy

Acid-soluble collagen sample was dissolved in 0.1 M acetic acid at 4 °C. Aggregation process was initiated by mixing collagen solution with cooled Na-phosphate/NaCl buffer in an ice bath. After mixing, concentrations were 5 mM Na-phosphate buffer and 60 mM NaCl, collagen covering a range of 0.001–3 mg/ml. The above solutions were homogenised. After checking that the pHs were 7.2, the stock solution of pyrene was introduced at a final concentration of 2 μM and the mixtures were homogenised, and then the samples were immediately placed in a temperature-controlled thermostated water bath at 37 °C in total darkness. Every 12 h, fluorescence measurements were taken using a Hitachi F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), with a 10-mm path length quartz cuvette, according to the method of Li et al. (2007). Pyrene was excited at 343 nm, and the emission spectrum was collected in the range of 360–500 nm at a scanning rate of 300 nm/min. The slit openings for both excitation and emission were fixed at 10 and 2.5 nm, respectively. All measurements were carried out in triplicate.

2.4. Turbidity measurement

The solutions (pH 7.2), containing 5 mM Na-phosphate buffer, 60 mM NaCl, and collagen at concentrations of 0.5, 0.6 and 1.0 mg/ml, respectively, were incubated in a water bath at 37 °C. The aggregation process was examined by recording the increase in absorbance at 313 nm at various time intervals using a Shimadzu spectrophotometer UV-2550 (Shimadzu, Kyoto, Japan). The assays were conducted at least in triplicate on separate occasions.

3. Results and discussion

3.1. Theory of determining the aggregation number

The values of aggregation number can be obtained by a transient fluorescence decay method, using pyrene as the fluorescence probe (Jiang, Ye, & Wu, 1992). Our approach was based on the assumption that pyrene molecules randomly distribute amongst the aggregates, which is accurately described by the Poisson distribution. N_{agg} can be determined by measuring the total fluorescence intensity of pyrene monomer at the decay time, I_M^t. The fluorescence decay is described by a double exponential function:

$$I_M^t = I_M^0 e^{\left[\langle S\rangle\left(e^{-k_E t}-1\right)-k_1 t\right]}, \tag{1}$$

where I_M^0 corresponds to the fluorescence intensity of pyrene monomer at decay time zero, k_1 and k_E are the fluorescence decay rate constants of pyrene monomer and excimer, respectively, and $\langle S\rangle$ is defined as the average occupied degree of pyrene in the aggregates, a parameter related to the distribution of pyrene in the aggregates, which is given by

$$\langle S\rangle = \frac{c_{Pyrene}}{c_{Aggregate}}, \tag{2}$$

where c_{Pyrene} refers to the concentration of pyrene in the whole system, and $c_{Aggregate}$ can be written as

$$c_{Aggregate} = \frac{c_{Collagen} - CAC}{N_{agg}}. \tag{3}$$

It follows from Eqs. (2) and (3) that:

$$N_{agg} = \frac{\langle S\rangle\left(c_{Collagen} - CAC\right)}{c_{Pyrene}}. \tag{4}$$

In the case of $e^{-k_E t} \to 0$, or using the linear part in the double exponential decay curve, Eq. (1) can be written as

$$I_M^t = I_M^0 e^{-(\langle S\rangle + k_1 t)}, \tag{5}$$

or

$$\ln\frac{I_M^t}{I_M^0} = -\langle S\rangle - k_1 t, \tag{6}$$

$\langle S\rangle$ can be obtained by extending the linear part to the *y*-axis in the plots of $\ln(I_M^t/I_M^0)$ *versus* time, and then N_{agg} is calculated by fitting Eq. (4) to the data.

3.2. Selection of the probe

Pyrene was used as a fluorescence probe to detect the aggregation behaviour of collagen in Na-phosphate buffer at pH 7.2. It has the following feature: the ratio of intensities of the first and third

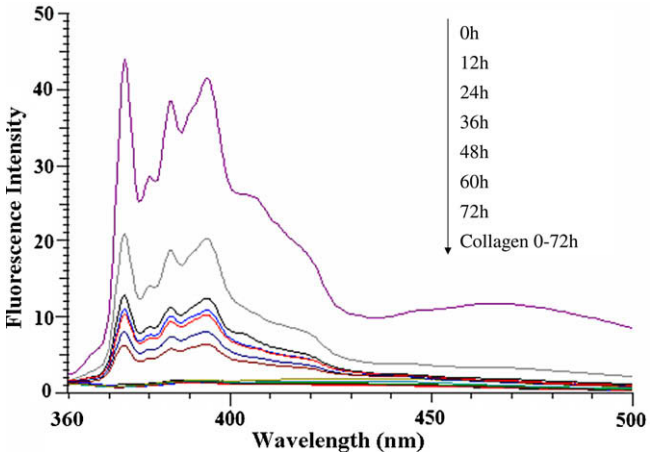

Fig. 1. Fluorescence spectra of pyrene (2 μM) and acid-soluble collagen from walleye pollock skin (0.6 mg/ml) in the collagen/Na-phosphate buffer system at 37 °C.

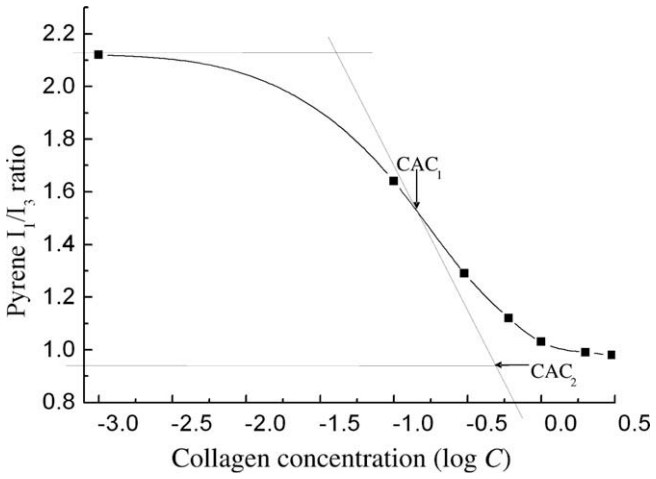

Fig. 2. Plots of pyrene I_1/I_3 ratio *versus* the logarithm of different concentrations of acid-soluble collagen from walleye pollock skin in Na-phosphate buffer at pH 7.2 after incubation for 72 h at 37 °C.

vibronic peaks, I_1/I_3, is often considered to estimate the polarity level of the microenvironment where pyrene is located (Ray et al., 2006), and, as a result, the conformation change of collagen was deduced from the changes of polarity in the solution. The emission spectra of pyrene in the collagen/Na-phosphate buffer system at 37 °C are shown in Fig. 1. It could be found that in the period of collagen aggregation, the emission spectra of pyrene all showed four peaks at 374, 380, 385 and 394.5 nm, similar to those in methanol and water in Ray's report (Ray et al., 2006), suggesting that pyrene exhibited stable fluorescent properties in the collagen/Na-phosphate buffer system at 37 °C. However, a continuous decrease in the fluorescence intensity was observed. The reason might be that with collagen self-aggregating, pyrene molecules moved into the hydrophobic microdomains of the aggregates so that the collisions of pyrene and collagen were increased, resulting in the decline of pyrene fluorescence intensity (Qiao & Jin, 2000). From Fig. 1, we could also find that pyrene I_1/I_3 ratio decreased with collagen aggregation, revealing a gradual reduction in the polarity of the solution.

Collagen has autofluorescence, due to the presence of some phenylalanine and tyrosine residues. To minimise the disturbance due to autofluorescence of collagen, an appropriate excitation wavelength should be selected at the site where the chromophore of collagen has very little absorbance. Additionally, an appropriate emission wavelength should also be selected at the site where the chromophore of collagen has little emission (Shi et al., 2001). In our experiment, pyrene was excited at 343 nm, and the emission spectrum was collected over the range of 360–500 nm. Under the studied conditions, pyrene was well excited, while the chromophore of collagen showed little emission, as shown in Fig. 1. Besides, the emission spectra of collagen did not change significantly in the period of aggregation, and the fluorescence intensity was near to zero. Therefore, the disturbance of collagen autofluorescence could be avoided when the excitation wavelength of pyrene was selected at 343 nm. From the abovementioned results, it could be concluded that the fluorescence probe pyrene is suitable for studying the aggregation behaviour of acid-soluble collagen from walleye pollock skin in Na-phosphate buffer at pH 7.2.

3.3. Determination of the critical aggregation concentration of collagen

Plots of pyrene I_1/I_3 ratio *versus* logarithm of collagen concentration are shown in Fig. 2. Analysing by curve fitting, the pyrene I_1/I_3 ratio data for the studied collagen are well fitted by a Boltzmann function:

$$y = 0.94 + \frac{1.18}{1 + e^{(x+0.86)/\Delta x}} \left(R^2 = 0.99956\right),$$

where the variable y is the pyrene I_1/I_3 ratio, and the independent variable x is the logarithm of collagen concentration. It was shown that at lower collagen concentrations, pyrene I_1/I_3 ratio value corresponded to a polar environment; as the collagen concentration increased, the pyrene I_1/I_3 ratio decreased rapidly, indicating that pyrene was sensing a more hydrophobic environment, and then levelled off with further increase in collagen concentration, due to the incorporation of the probe into the hydrophobic region of the aggregation (Aguiar et al., 2003).

We adopted the term "critical aggregation concentration (CAC)" to describe the effect of collagen concentration on the aggregation behaviour. By using this term, no assumption about the nature, the size, or the shape of the aggregates are made: the aggregates can be of finite size (micellar system) or infinite (phase separation) (Garofalakis, Murray, & Sarney, 2000). The CAC for acid-soluble collagen aggregates was graphically determined from the plots of pyrene I_1/I_3 ratio *versus* the logarithm of collagen concentration in this study, as shown in Fig. 2 (Aguiar et al., 2003). It was found that the CAC value of acid-soluble collagen from walleye pollock skin in Na-phosphate buffer of pH 7.2 at 37 °C might be 0.138 mg/ml (CAC_1) or 0.48 mg/ml (CAC_2). On the other hand, the aggregation kinetics of collagen could be detected by absorbance at 313 nm (Sivakumar & Chandrakasan, 1998). The higher absorbance indicated more or larger collagen aggregates. As shown in Fig. 3, absorbance at 313 nm showed a marked increase in the aggregation kinetics of the collagen solutions at 0.6 and 1.0 mg/ml, while a rise in the aggregation kinetics of collagen solution with a concentration of 0.5 mg/ml was not found. Thus it could be concluded that the CAC of collagen was in the range of 0.5–0.6 mg/ml. The CAC determined by the plot of pyrene I_1/I_3 ratio *versus* the logarithm of different collagen concentrations was 0.48 mg/ml, a slight deviation from the range of CAC obtained by the aggregation kinetics perhaps showing the difference between theoretical and experimental value. The CAC of collagen from pollock skin was close to the threshold concentration of the collagen aggregation from porcine skin in 0.1 M acetic acid reported by Shi et al. (2001), but much higher than that of pC-collagen from the medium of cultured fibroblasts from normal human skin found by Kadler et al. (1987). The reasons were as follows. Firstly, the aggregation behaviour of col-

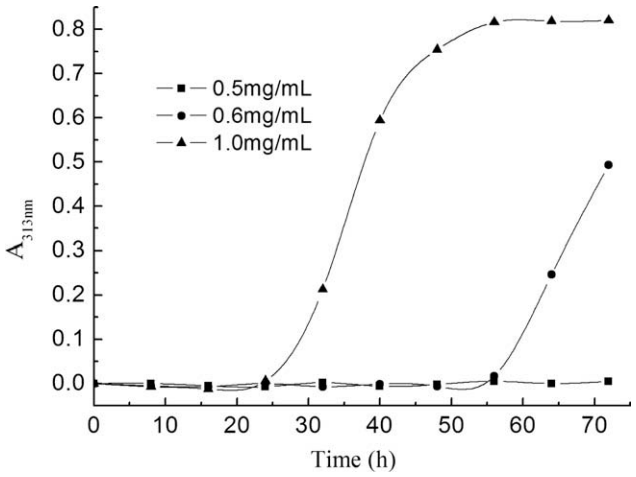

Fig. 3. Aggregation kinetics of collagen from walleye pollock skin at different concentrations in Na-phosphate buffer at pH 7.2 and 37 °C (closed square, 0.5 mg/ml; closed circle, 0.6 mg/ml; closed triangle, 1.0 mg/ml).

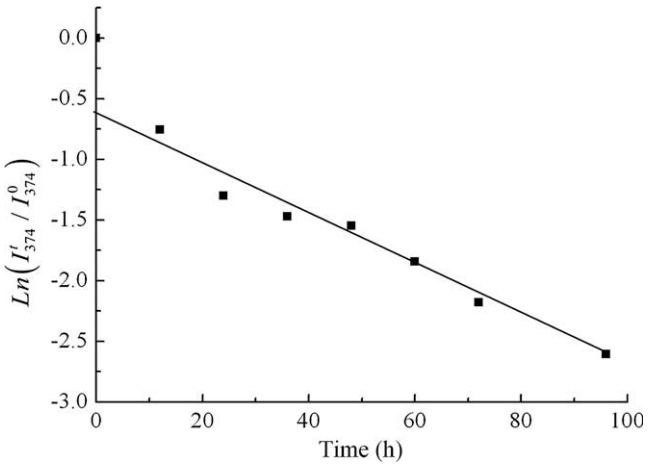

Fig. 4. Plots of the natural logarithm of pyrene I^t_{374}/I^0_{374} *versus* time in the acid-soluble collagen (0.6 mg/ml)/Na-phosphate buffer system at 37 °C.

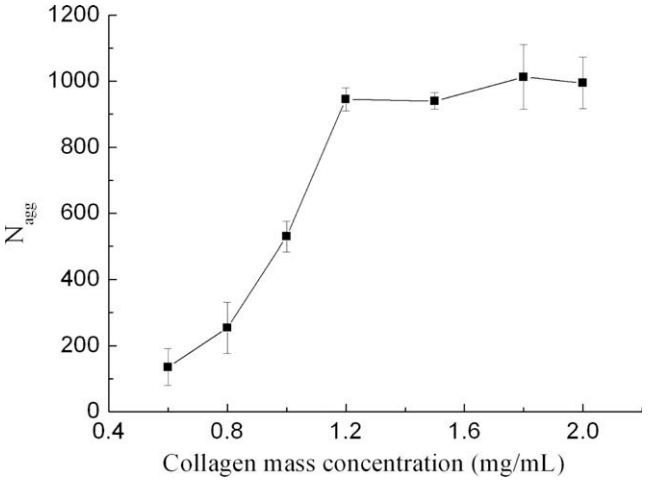

Fig. 5. Aggregation numbers of acid-soluble collagen from walleye pollock skin at different concentrations in Na-phosphate buffer at pH 7.2 and 37 °C.

lagen was closely related to its cross-linking. Collagen from walleye pollock skin shows rather less cross-linking than those from mammals (Liu, Li, & Guo, 2007; Yan et al., 2008), resulting in a much higher CAC. Secondly, the long lag time in the aggregation kinetics of collagen (Fig. 3) revealed that the collagen was mainly in the monomeric form, which also led to a much higher CAC. Thirdly, ionic strength and temperature could affect the aggregation of collagen, too. Generally, increasing the ionic strength in collagen solution resulted in a higher CAC for collagen, while increasing the temperature induced a lower CAC for collagen (Na et al., 1989). However, we believe that the higher CAC of collagen from walleye pollock skin was mainly ascribed to the lower cross-linking and the higher monomer content of collagen in the study.

Collagen self-aggregation in aqueous solution can be explained by its molecular structure. The regular three-strand helical structure of collagen can be destroyed after extraction. In neutral solution, there was no repelling action in collagen molecular chains for the net charge near to zero. At lower collagen concentrations, few aggregates appeared in the solution, due to less combination between the carbonyl and amide groups. When collagen concentration was increased to the CAC, the carbonyl groups in collagen chains, which could combine with the amide groups in neutral solution, increased. Thus the action of the intra- and intermolecular hydrogen bonds increased, which resulted in the formation of irregular coil structures and hydrophobic microdomains in the solution (Shi et al., 2001). As collagen concentration was further increased, the majority of carbonyl and amide groups participated in the formation of hydrogen bonds. Simultaneously, the hydrophobic interactions of the hydrophobic amino acid residues in the polypeptide chains became stronger, and the actions of the intermolecular hydrogen bonds posed by the hydroxyl groups of hydroxyproline in the polypeptide chains enhanced. Besides, the hydroxyl groups of hydroxyproline also interacted with water, to produce the hydrogen bonds. So the aggregates were formed, which induced a roughly constant value of pyrene I_1/I_3 ratio.

3.4. Determination of the aggregation number of collagen

We have used the well-established transient fluorescence decay method to determine the mean aggregation numbers of acid-soluble collagen from walleye pollock skin. In this study, pyrene was used as a fluorescence probe, and the decay experiments were analysed using Eqs. (4) and (6). We had carried out the experiments in aggregate solutions with different collagen concentrations. Similar results were found in all cases that the natural logarithm of pyrene I^t_{374}/I^0_{374} decreased drastically as the aggregation went on, and then decreased linearly (Fig. 4). At the same time, good linearity ($r^2 > 0.9$) was achieved in all cases after some aggregation time. From the interception obtained by extending the linear part of the pyrene fluorescence decay curve to the *y*-axis, as shown in Fig. 4, and using the CAC value above, we had determined the N_{agg} of collagen aggregates according to Eq. (4). Fig. 5 shows that at lower collagen concentrations, N_{agg} rose sharply, due to increases in concentration, and when collagen concentration reached 1.2 mg/ml and above, it rose slightly. The result was similar to the changes of aggregation numbers of nonaethylene glycol monododecyl ether (C12E9) with concentration increase (Yu et al., 2006), but different from those of sodium dodecyl sulphate, sodium dodecyl sulphonate (Fang et al., 2001) and quaternary trimeric surfactant (Li et al., 2005). From the results, we could conclude that N_{agg} of acid-soluble collagen from walleye pollock skin was not constant, but varied with different concentrations of collagen, whereas the aggregation numbers obtained for *N*-dodecyllactobionamide show no clear dependence on surfactant concentration (Kjellin, Reimer, & Hansson, 2003). N_{agg} increasing with increases in the collagen concentration showed that the aggregates might be incomplete in structure, but when the concen-

tration reached 1.2 mg/ml and above, N_{agg} rose slightly, indicating that the structure of aggregates tended to be stable. We presume that N_{agg} shows no significant dependence on collagen concentration above 1.2 mg/ml, in good agreement with Kjellin's conclusion (Kjellin et al., 2003). N_{agg} at the CAC could not be determined using transient pyrene fluorescence decay. Usually, it is obtained by extrapolating the N_{agg} *versus* collagen concentration experimental curve (Fang et al., 2001). In the experiment, N_{agg} at the CAC of collagen from pollock skin was found to be about 68.

The results above verified that the fluorescence probe pyrene could be applied to the study of the aggregation behaviour of acid-soluble collagen from walleye pollock skin. The advantage of using the fluorescence probe was that the concentration of the probe used could be very small, which minimised the possibility of any probe–probe interaction that might occur. The method was fast, simple and accurate, and also showed high sensitivity and good selectivity. Also, both the critical aggregation concentration and aggregation number of collagen could be acquired by this method; tensiometry, conductometry and calorimetry can only evaluate the critical aggregation concentration (Ray et al., 2006), while small-angle neutron scattering (SANS) and NMR self-diffusion coefficient measurements only determine the aggregation number (Javadian et al., 2008). Thirdly, the fluorescence probe pyrene was more convenient in the study on the collagen aggregation behaviour, because pyrene could be used directly in the experiment, whereas the fluorescence probe 3-methoxy-4′-*N*,*N*-dimethylamino flavone derivative employed by Shi et al. (2001), in the study on the aggregation behaviour of collagen from pig skin must be synthesised.

4. Conclusion

The results presented here clearly suggested that acid-soluble collagen from walleye pollock skin could self-aggregate in Na-phosphate buffer at pH 7.2 above a certain concentration. We have introduced an alternative method to determine the critical aggregation concentration and aggregation number of collagen by fluorescence spectroscopy of pyrene. The CAC of collagen was found to be at 0.48 mg/ml graphically from the plots of pyrene I_1/I_3 ratio *versus* the logarithm of collagen concentration. Transient fluorescence decay study revealed that N_{agg} was not constant, but varied with different concentrations of collagen. The structure of aggregates tended to be stable, when the collagen concentration exceeded 1.2 mg/ml.

In summary, the fluorescence probe pyrene was suitable to describe the aggregation behaviour of acid-soluble collagen from walleye pollock skin. We believe that these results will aid further study on collagen aggregation behaviour.

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