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AUTHORS: Joseph J. Kehoe and André Brodkorb

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Interactions between sodium oleate and α-lactalbumin: the effect of temperature and concentration on complex formation.

Joseph J. Kehoe and André Brodkorb*

Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

***Corresponding author: andre.brodkorb@teagasc.ie Tel: +353-25-42222**

Abstract

Complexes of α-lactalbumin and oleic acid have previously been shown to be cytotoxic to cancer cells. In this study oleic acid is replaced by the more soluble sodium oleate and complexes of α-lactalbumin and sodium oleate are formed. Dynamic light scattering results showed that there was a small linear increase in the particle size of α-

lactalbumin when it was titrated with sodium oleate. The fluorescence spectra of α-lactalbumin showed a linear increase in the emission maximum when sodium oleate was added up to a molar ratio of 8 to 11 oleate molecules per α-lactalbumin. Differential scanning calorimetry results show that the thermal unfolding of α-lactalbumin is altered by the presence of the sodium oleate. There is a decrease in size of the endothermic peak of apo α-lactalbumin when sodium oleate is added. The temperature at which unfolding occurred decreased for both apo and holo α-lactalbumin. FTIR measurements showed no significant effect of sodium oleate in the amide I region of the α-lactalbumin spectrum indicating the presence of oleate has little or no effect on the secondary structure of α-lactalbumin. The interactions between α-lactalbumin and sodium oleate/ oleic acid are pH dependent, turbidity and dynamic light scattering measurements showed that the association between the two was optimal between pH 6.0 and 8.0.

The results obtained here suggest that α-lactalbumin can bind at least a 20 molar excess of oleate, most likely in a non-specific manner.

Key Words: α-lactalbumin, sodium oleate, oleic acid, BAMLET

Introduction

The formation of a bioactive complex between α-lactalbumin (α-la) and oleic acid known as BAMLET/HAMLET (Bovine/Human α-la made lethal to tumours) has been studied for over a decade now (Svensson, Hakansson, Mossberg, Linse, & Svanborg, 2000). HAMLET/BAMLET has been shown to be cytotoxic to a range of cancer cell

lines at levels where healthy cells are unaffected, the properties and action of the complex have been extensively reviewed (Mok, Pettersson, Orrenius, & Svanborg, 2007; Mossberg, Hun Mok, Morozova-Roche, & Svanborg, 2010).

Several chromatographic methods have been developed to form the complex (Brinkmann, et al., 2011; Svensson, et al., 2000). The chromatographic methods used for the preparation of HAMLET/BAMLET involve preconditioning an ion-exchange column with oleic acid and subsequently passing apo α-la through the column. The active complex can be eluted using a salt gradient. Oleic acid is insoluble in water so it is dissolved in ethanol to facilitate application to the column. It is worth noting however that the buffers used for the chromatography are all above the reported pKa of oleic acid, 7.6 (Hamilton & Cistola, 1986). This means that the majority of the oleic acid may be in the charged oleate form on the column.

The formation of HAMLET/BAMLET has been attributed to a misfolding of α-la and has been purported to be an example the beneficial misfolding of proteins (Svensson, et al., 1999). However, α-la which has been extensively heat denatured still forms tumoricidal complexes (Liskova, Kelly, O'Brien, & Brodkorb, 2010). Furthermore, two other proteins have been used to form cytotoxic complexes; equine lysozyme (Wilhelm, et al., 2009), which is structurally similar to α-la, and bovine β-lactoglobulin (Liskova, et al., 2011), which while being a globular whey protein has very little structural similarity to α-la. Peptides of α-la also form cytotoxic complexes with oleic acid (Tolin, et al., 2010). Recently it has been proposed that oleic acid is the cytotoxic component of these complexes (Permyakov, et al., 2012).

The mechanism of BAMLET action on cancer cells has been widely studied as has the protein component of the complex. In light of the findings outlined above, interest in the oleic acid portion of the complex has increased recently. It had been shown that

simply mixing α-la and oleic acid would not form complexes with cytotoxicity comparable to HAMLET/BAMLET (Svensson, et al., 2002; Svensson, et al., 2000). More recently, several papers have demonstrated that it is possible to form cytotoxic complexes by mixing the components under the correct conditions of pH and temperature (Kamijima, et al., 2008; Knyazeva, et al., 2008). Kamijiama et al. (2008) mixed α-la with a 120 molar excess of oleic acid (14 mM). To form the molten globule of the protein the solution was heated at 50 or 60°C, excess oleic acid was removed by centrifugation. Knyazeva et al. (2008) formed complexes by titrating α-la in solution at pH 8.3 with oleic acid. The concentrations of oleic acid used in the study were below the critical micelle concentration (cmc); the hypothesis behind using the lower concentrations of oleic acid was that structures formed by the oleic acid at higher concentrations would prevent its interaction with the α-la to form a complex. A greater number of oleic acid molecules bound to α-la at higher temperatures, 2.9 at 17°C vs. 9 at 45°C. Agger and Bro (2009) formed cytotoxic complexes by mixing oleic acid and α-la under high shear conditions. Permyakov et al. (2011) titrated α-la at 45°C with oleic acid. The titrations were made in a buffer at pH 12.0 under these conditions the oleic acid is in its deprotonated form, oleate. The study found that up to 34 oleic acid molecules could be bound to the α-la. This is much greater than any values previously reported for BAMLET/HAMLET,

These approaches demonstrate different means to overcome the two central issues in forming complexes between α-la and oleic acid: firstly the α-la protein needs to be partially unfolded and secondly the insoluble oleic acid had to be dispersed and brought into contact with the α-la. The solubility of oleic acid in water is low. In the chromatographic methods it is solubilised in ethanol before being applied to the column where it is dispersed over the resin to make it accessible to α-la. By mixing the oleic

119 acid with the protein at high pHs the oleic acid is deprotonated and more soluble. A
120 method has been developed which uses more soluble sodium oleate (NaOle) to for the
121 formation of complexes (Brodkorb & Liskova, 2010; Liskova, et al., 2011).
122 To unfold the protein two approaches have been used; in the methods using a column,
123 Ca is removed from α-la using a chelating agent, such as EDTA, causing the protein to
124 partially unfold. An alternative method is to unfold the protein by heating; this
125 approach has been used in methods where the two components of the complex are
126 mixed together. The quantity of oleic acid bound to the protein has also been widely
127 debated; initially it was thought that it was a one to one ratio (Svensson, Mossberg,
128 Pettersson, Linse, & Svanborg, 2003). However, more recent studies have shown that
129 the ratio is in fact much higher (Brinkmann, et al., 2011; Pettersson-Kastberg, et al.,
130 2009). Tolin et al. (2010) showed that oleic acid can bind to several different peptides
131 from α-la, meaning that there could be several different binding sites within α-la for
132 oleic acid.
133 To date there is limited information on the interaction between NaOle and α-la and the
134 stability of complexes formed between the two. This paper will investigate the
135 interaction between NaOle and α-la as a function of temperature and NaOle
136 concentration.
137
138 **Material and methods**
139 α-La (95% purity, approximately 3% β-lactoglobulin) was sourced from Davisco Foods
140 International (Eden Prairie, Mn, USA). NaOle (≥95 purity) and phosphate buffered
141 saline (PBS) was from Sigma Aldrich (Arklow, Ireland)
142
143 ***Dynamic light scattering: titration of protein with Na oleate.***

An α-la solution 424 μM (6 gL^{-1}) was prepared in a 2X concentration of PBS (pH 7.4). A stock solution of 10 mM NaOle was prepared in milliQ® water. Various quantities of the NaOle solution was added to 0.5 mL aliquots of the protein solution and the final volume was adjusted to 1.0 mL, giving solutions containing 212 μM (3 gL^{-1}) α-la containing a molar excess of NaOle ranging from 0 to 20 in a PBS solution. The hydrodynamic diameter of the aggregates was determined using a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). The measurements were carried out at 25ºC measuring the backscatter at 173°. The cumulative method was used to find the mean size of a particle that corresponded to the mean of the volume distribution. The viscosity of the solvent was assumed to be the same as water, given the low concentration of protein. The size of the aggregates present in NaOle solutions in the same concentration range was also measured.

Change in particle size during heating and protein refolding

A solution containing 3 gL^{-1} α-la and 1.06 mM NaOle (5 molar excess) was prepared as outlined above. The particle size of solution was measured at 5°C temperature increments from 20 to 60 °C. The cuvette containing 1 mL of the mixture was equilibrated for 2 minutes at each temperature prior to measuring the particle size as outlined above. A control solution containing 3 gL^{-1} was subjected to the same measurement regime. The solutions were cooled from 60°C back to 20°C and measured again. Solutions that had been heated in a waterbath for 60°C for 1h were also measured at 20°C for comparison.

Samples were also prepared as outlined above containing 0 to 20 molar excess NaOle, the particle size of the solutions was measured and they were heated at 60°C for 1h. After cooling the particle size was measured again at 20°C.

Fluorescence: titration of α-lactalbumin with oleate

Fluorescence measurements were carried out using a Cary eclipse fluorometer (Varian, Inc., USA) with temperature controlled cuvette holder. The measurements were made at an α-la concentration of 25 μM in PBS with various excess of NaOle added. The solutions were equilibrated to the desired temperature while stirring constantly. Emission scans between 300 and 400 nm were taken using an excitation wavelength of 280 nm. The slits were both set to 5 nm. Samples were measured at 10, 25, 35, 45, 55 and 60 °C. The spectra were converted to the wavenumber scale and the curves fitted to a Gaussian distribution to calculate the emission maximum. The emission maximum was plotted against excess NaOle for each temperature.

Fluorescence: the formation of a complex during protein refolding

Solutions containing α-la with various molar excesses of NaOle were prepared as outlined in the dynamic light scattering section. The solutions were heated at 60°C for 1 hour and cooled on ice. For fluorescence measurements an aliquot of the solution taken before and after heating was diluted to 25 μM with PBS and the fluorescence spectrum measured at 20°C as outlined above. The emission maximum was determined as described above.

Differential Scanning Calorimetry (DSC)

The DSC measurements were made using a Steraram DSC III microcalorimeter. PBS was used in the reference cell. A 100 gL^{-1} (7.05 mM) α-la solution in PBS with a 0, 1, 2 or 5 molar excess of NaOle added was placed in the reference cell. The cells contained

~900mg of solution balanced to ±0.5 mg. The solutions were heated from 20 °C to 80°C at 0.5 °C min^{-1}.

FTIR

FTIR measurements were carried out using a Bruker Tensor 27 instrument in ATR mode using a BioATR cell II ™. The spectra used were an average of 300 scans at a resolution of 4 cm^{-1}. A sample was prepared containing 10 gL^{-1} α-la and 3.5 mM NaOle in PBS. A sample containing only 10 gL^{-1} α-la was also prepared. Using a PBS solution as the background a temperature sweep from 20 to 60 °C was made taking readings at 5°C intervals. Using Opus version 5.5 software, the spectra were corrected for the atmospheric conditions (H_2O and CO_2) and solvent conditions (H_2O). The spectra were vector normalized between 1720 and 1480 cm^{-1}. The spectrum of the sample at 20°C were subtracted from those at higher temperatures. The resulting curves highlighted the areas in which changes were occurring during heating.

Effect of pH on α-lactalbumin sodium oleate interactions

Solutions (50 mL) were prepared in distilled water containing 10 gL^{-1} α-la, 10gL^{-1} α-la with 7 mM NaOle (10 molar excess) and 7 mM NaOle. The solutions pH of the solutions was decreased by the addition of 100 mM HCl in 100 μL increments. The pH of the solution was measured as the titration progressed and 1 mL aliquots were removed at intervals. The optical density of the solutions was measured at 600 nm. The particle size of the aliquots which were soluble was measured by dynamic light scattering as outlined above.

Results and Discussion

Dynamic light scattering was used to follow the changes in particle size as α-la was titrated with NaOle. α-La without NaOle had a diameter of 3.6 nm, which compares well with previously published values (Kataoka, Tokunaga, Kuwajima, & Goto, 1997). The size of the particles in the solution increased linearly with the addition of NaOle at room temperature. The increase in the particle diameter was quite small ~0.9 nm per mmol of NaOle added (Figure 1A). No larger particles, which could be attributed to free NaOle forming structures, were detected. The concentrations of NaOle used were all well above the CMC of NaOle which was found to be ~10 μM in PBS solution (results not shown). When NaOle at the concentrations used here is titrated into a PBS solution large aggregates are formed (Figure 1B). PBS was used to maintain a standard pH of 7.4 across all the experiments as oleate will increase the pH of the proteins solutions in a non buffered system. Additionally it standardises the ionic environment that the complex is formed in. The charge of both the protein and fatty acid portions is affected by pH and ionic environment and may contribute to how the complex is formed.

Even though the oleate solutions were turbid no precipitation occurred. The NaOle aggregates distribute themselves over a range of sizes depending on the concentration present. The NaOle aggregates are all much larger than the α-la and the particles present in the α-la NaOle mixtures, making it unlikely that there are two different particles of a similar size present in the α-la NaOle mixtures. The immediate association of the NaOle with the α-la present means that it may not be necessary to unfold the protein to form a complex. Also the size of the aggregates continued to increase up to the 20 fold molar excess that was added.

Initially when BAMLET/HAMLET was made it was reported that there was a 1:1 ratio of oleic acid to protein (Svensson, et al., 2003), this value has been subsequently

discounted and a variety of values have been reported. Typical values for complexes produced using a chromatographic method range from 4 (Brinkmann, et al., 2011; Pettersson-Kastberg, et al., 2009) to 14 (Brinkmann, et al., 2011) oleic acid molecules per α-la. In the present study it can be seen that the size of the particles present continue to increase up to a 20 molar excess of NaOle. The BAMLET samples in previous studies had been extensively dialysed before the oleic acid content was measured; this suggests that while the NaOle initially associates with the α-la it may be loosely bound and disassociate during dialysis. The association of NaOle with α-la prior to heating is significant as it supports the hypothesis that α-la is acting as a solubilisation agent for oleic acid (Permyakov, et al., 2011; Tolin, et al., 2010). Previous authors had assumed that to form complexes the concentration of oleic acid had to be at concentrations below its cmc (Permyakov, et al., 2011). The dynamic light scattering results appear to discount these concerns as the NaOle is associating with the protein when present at concentrations far in excess of its cmc. While the NaOle may associate with α-la at room temperature, unfolding the protein by heating may allow the NaOle to bind to areas that are not exposed at room temperature.

A solution of 3 gL^{-1} (0.21 mM) α-la and 1.06 mM NaOle (five fold excess) was used as an example of a typical mixture used to create protein/NaOle complexes (Brodkorb, et al., 2010; Liskova, et al., 2011). As the solution was heated from 20 to 60 °C there was no measurable change in the diameter of the particles present (Figure 2A.). Subsequent cooling of the mixture to 20°C did not change the particle size either. The same is true of the control sample containing 3 gL^{-1} α-la. These measurements were made while heating the protein solutions *in situ* in the DLS instrument. When samples that had been heated under the conditions previously used for the preparation of complexes (Brodkorb, et al., 2010; Liskova, et al., 2011), 60°C 1h, and cooled were measured the

diameter of the particles were the same as those heated *in situ*. There was a small increase, ~0.3 nm, in the diameter of the control α-la sample when they were heated at 60°C for 1h.

Using the same heating regime, 60°C 1h, the particle size of 3 gL^{-1} α-la with 0 to 20 molar excess (4.2mM) of NaOle added was measured before and after heating. Heating the solutions had no significant effect at any NaOle concentration (Figure 2B.). Statistically the concentration of NaOle had an effect on particle diameter while heating the solution had none. It is possible that the heating and cooling cycle causes some of the NaOle to be trapped within the core of the protein without changing the diameter significantly but leading to some NaOle being bound in the core of the protein molecule.

As discussed above changes in particle size were observed at concentrations of NaOle above the quantities previously found bound to α-la in BAMLET. Intrinsic fluorescence was used to probe whether the tertiary structure of α-la was affected by the binding of NaOle and the temperature dependence of the binding. Due to differences in the sensitivity of the techniques much lower concentrations were used for the fluorescence measurements, however in all cases the NaOle concentration was above the cmc of NaOle in PBS (pH 7.4). At 10°C the addition of a 20 molar excess of NaOle brought about a 6.9 ± 1.9 nm red shift in the emission wavelength (Figure 3). At 25 and 35 °C the addition of NaOle had a larger shift in the emission wavelength, 10.8 ± 0.9 and 9.9 ± 0.9 nm respectively. At higher temperatures where the protein is more unfolded, the addition had a smaller effect on the emission wavelength, only a 3.1 ± 1.4 nm shift at 45°C. At 55 and 60 °C the addition of NaOle did not significantly affect the emission max (data not shown). The increase in the emission was not linear over the range studied. The maximum fluorescence wavelength as a function of NaOle molar excess

produced curves with two linear regions. Initially there was a rapid increase in the emission maximum as NaOle was added, after which further addition of NaOle did not significantly affect the emission maximum. Straight lines were fitted to these regions and the intercept calculated. The intercept of the two lines was found to be at a molar excess of 11.2, 8.3, 8.9 and 8.1 at 10, 25, 35 and 45 °C respectively. These values are significant as they are close to the binding stoichiometries previously reported for the production of HAMLET/BAMLET using similar approaches to those employed here (Knyazeva, et al., 2008; Permyakov, et al., 2011). However, the dynamic light scattering data in Figure 1 suggests that NaOle continues to bind or at least be associated with α-la above these quantities. It is possible that the NaOle is binding in at least two different sites or by two different means. Initially the NaOle is binding in a location close to the tryptophan residues, as the quantity of NaOle increases the NaOle is binding in locations that no longer affect the tryptophan fluorescence possibly loosely associated at the surface of α-la. In the preparation of HAMLET/BAMLET a dialysis step is applied, in the chromatographic methods this is to remove the salts present; however, loosely bound NaOle may also be removed during this step leaving only the more tightly bound NaOle. This could explain the discrepancies between the dynamic light scattering and fluorescence results reported here and the quantity of oleic acid found in the preparation of HAMLET/BAMLET in other studies.

At 60°C α-la partially unfolds; therefore it is surprising that a larger change in the fluorescence spectrum is not observed during the titration of α-la with NaOle at 60°C. During the preparation of BAMLET using NaOle solutions were heated to 60°C for 1 hour and rapidly cooled upon ice to refold the protein (Brodkorb, et al., 2010). The re-folding may trap the NaOle within the core of the α-la forming the complex, making the cooling step the most important in the formation of BAMLET. This means that even if

318 there are no changes observed in the fluorescence spectrum at 60°C some may still
319 occur during cooling. To see if this was the case mixtures of α-la and NaOle were
320 heated at 60°C for 1 hour and then cooled on ice. The fluorescence spectrum before and
321 after heating was measured at room temperature. The addition of NaOle prior to heating
322 brought about a red shift in the emission spectrum. As was observed with the
323 fluorescence spectra at other temperatures, λ_{max} did not change significantly after the
324 addition of 10 or more molar excess of NaOle (Figure 4A). The same can be said for
325 the intensity at λ_{max} (Figure 4B). Heating the solutions and cooling them only brought
326 about a very small increase in the λ_{max}, <3 nm. The increases in intensity are also very
327 small, < 15%. In fact the largest change in intensity occurs in solution which contained
328 no NaOle. Overall the quantity of NaOle added had a more significant effect on the
329 fluorescence than heating the samples. There are four tryptophan residues in α-la
330 located at positions 26, 60, 104 and 118. In the native conformation these are buried in
331 the core of the protein. Quenching studies have shown that two of the residues are more
332 readily accessible (Lala and Kaul, 1992), it is likely that it is the fluorescence of these
333 residues that is most affected by the presence of oleate. At temperatures above 35°C
334 apo a-la is partially unfolded which may allow oleate access to the remaining
335 tryptophan residues. However, the intercept of the two linear regions of the max
336 wavelength plot (Figure 3) does not increase significantly at 35 and 45°C. Thus it
337 seems unlikely that additional oleate molecules bind in the region close to these
338 residues. Furthermore Figure 4. shows that unfolding the protein in the presence of
339 oleate by heating it to 60°C and cooling does not have a large affect on the
340 fluorescence, suggesting that any oleate trapped in the core of α-la by this process does
341 not bind in locations close to the tryptophan residues (if at all).

The heat stability of α-la was studied using DSC. The 100 gL^{-1} α-la solution showed two endotherms centred at 38°C and 66°C (Figure 5A). These correspond to published values for apo and holo α-la unfolding (Boye, Alli, & Ismail, 1997). The addition of NaOle brought about a decrease in the size of the endothermic peak; initially the less thermally stable, more unfolded, apo α-la is affected (Figure 5B). There was a linear decrease in the enthalpy of apo α-la, albeit over a relatively low concentration range. Extrapolation of the graph would suggest that 2.8 NaOle molecules would be required to stabilise the apo α-la fully. There was also a decrease in the peak top temperature for both apo and holo α-la as the concentration of NaOle increased, suggesting that NaOle decreases the thermal stability of both apo and holo α-la (Figure 5C). A previous study had found one binding site for oleic acid on apo α-la but no effect in holo α-la (Barbana, et al., 2006); the results for DSC support this to an extent with a decrease in the size of the apo α-la endotherm. The decrease in the denaturation temperature of holo α-la shows that there may be an interaction occurring between it and NaOle also. Both the particle size data and the fluorescence show that unfolding is not necessary for an interaction to occur between the protein and oleate. However, the decrease in T_m suggests that oleate is binding to α-la in its unfolded state, again raising the possibility of oleate molecules binding in the core of a-lactalbumin without significantly affecting tryptophan fluorescence or particle size.

The DSC results show that NaOle has an effect on the unfolding of α-la; FTIR spectroscopy was used to see if this effect could be observed in the secondary structure of the protein. As the protein solution was heated changes in the in the amide I region, similar to those observed by other studies during the heat denaturation of α-la, occurred (Figure 6A). The presence of a five molar excess of NaOle did not significantly effect this unfolding as a function of temperature (Figure 6B). While some structural changes

could be observed while the protein NaOle mixtures were being heated, all the changes were reversible upon cooling. Mixtures of α-la and NaOle which had been heated to 60°C for 1 hour and cooled on ice showed no structural differences between the samples pre-heating and post heating and cooling (Results not shown).

The nature of the interactions between NaOle/oleic acid with α-la are unclear. Given the possible negative charge on the NaOle it is possible that the interactions could arise from electrostatic attraction. Any charge related interactions would be highly influenced by pH. The results in Figure 7 show that as a mixture of α-la and NaOle is titrated with HCl, changes are observed in the turbidity (O.D. 600 nm). At high pHs the NaOle solution is clear; reducing the pH below 8.5 causes an increase in turbidity, the NaOle solution remains turbid as the pH is further decreased. The α-la solution is clear at pHs above 5.5. At values close to the pI of α-la the solution becomes turbid but once the pH is decreased below 4.5 the solution becomes clear again. This turbidity is due to the reversible aggregation of the apo α-la present. Mixtures of α-la and NaOle are clear above pH 5.9 but become turbid below this pH and remain turbid as the pH further decrease to pH 2.0. There is a small increase in turbidity between pH 7.0 and 6.0, which may be due a rearrangement of the complex as the negatively charged oleate becomes protonated to form oleic acid. An increase in the particle size can also be observed in this pH range (Figure 7C) The particle size (dynamic light scattering) of the α-la and the mixture of α-la and NaOle were measured at intervals during the titration. The regions where the turbidity was highest were not soluble so no particle size measurements were made at these pHs. The α-la solution shows an increase in particle diameter at the pHs closest to the pI but the particle reduces back to the original size as the pH is further decreases. In the case of the α-la NaOle mixture a similar increase in

particle size is observed but as the pH is decreased below the pI there is only a small decrease in particle size.

These results indicate that there is an interaction between α-la and NaOle at between pH 5.8 and 8.5. It is worth noting that even though the net charge of α-la is negative in this pH range, there are many amino acids with positively charged residues (Arg, Lys and His) located on the protein surface available to participate in electrostatic interactions with negatively charged NaOle. Furthermore these charged amino acid residues may not be located near the tryptophan or tyrosine residues. Therefore, binding of oleate would have little or no effect on intrinsic fluorescence, which could go some way towards explaining the differences observed between the fluorescence study and the dynamic light scattering study. The pI of α-lactalbumin is approximately 4.5 (Zittle, 1956). In this pH region the solution is turbid and it is not possible to determine if any interaction is occurring between the protein and NaOle/oleic acid. Below the pI, the α-la/NaOle mixture remains turbid and the particle size is large. Below ~pH 3.5 the turbidity and particle size of the mixture is less than that of α-la alone, indicating that there may still be some interactions occurring between the protein and oleic acid. In this region the protein would have an overall positive charge but the NaOle is fully converted to the non charged, poorly soluble oleic acid form. If these solutions are let to stand the oleic acid phase separates out of the solution.

Conclusions

The results outlined in this study show how α-la and oleic acid or its salt oleate can interact under various conditions. The results provide further evidence α-la/oleate complexes can have various structures and stoichiometries, thereby changing the possible bio-function of the complex, such as those demonstrated for BAMLET/HAMLET. α-La also seemed to facilitate the solubilisation or dissociation of

large aggregates of fatty acid salts, which are present at concentrations above the critical micelle concentration. The interaction between the protein and oleate appears to be highly pH dependent, pointing towards the importance of electrostatic interactions, most likely combined with hydrophobic interactions. As fatty acids are known cytotoxic compounds, globular proteins such as β-lactoglobulin (Liskova, et al., 2011) and α-la could be seen as a means of dissolving higher amounts of fatty acid and thereby acting as delivery vehicles to the cells, a hypothesis that needs to be substantiated by more details cytotoxicity studies using complexes such as those presented in the current study.

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Acknowledgements

This work was supported by the Food Institutional Research Measure (FIRM, project no. 08RDTMFRC650) of the Department of Agriculture, Fisheries, and Food, Ireland. DR. Seamus O'Mahony and Avril McCord, School of Food and Nutritional Sciences, University College Cork, Ireland are gratefully acknowledged for their assistance with the differential scanning calorimetry experiments.

Figure legends

Figure 1. (A) Hydrodynamic diameter of the particles present in a 3 gL^{-1} (0.2 mM) α-la solution titrated with NaOle. Insert shows the volume distribution of particle diameters within the samples. **(B)** The volume distribution of particle diameters within NaOle at the concentrations used in figure 1A in the absence of α-la. All measurements were made in PBS.

Figure 2. Effect of temperature and NaOle concentration on particle size. **(A)** Diameter during heating *in situ* 3 gL^{-1} α-la (squares), 3 gL^{-1} (0.212 mM) α-la 1.06mM NaOle (5 molar excess) (circles). Shapes not filled represent samples which had been heated to 60°C *in situ* and cooled to 20°C (for representation purposes, these two data points were off-set by 2 degrees). Gray shapes represent samples heated at 60°C for 1h in a waterbath. **(B)** Diameter at 20°C of 3 gL^{-1} α-la with various excesses of NaOle added, before (dotted bars) and after (stripped bars) heating at 60°C for 1h and cooling on ice. All experiments carried out in PBS solution and the error bars represent the standard deviation of three replicates.

Figure 3. Fluorescence of 25 μM α-la titrated with NaOle at a range of temperatures, excitation wavelength 280nm. The graph shows the wavelength of the point of maximum intensity when the fluorescence spectrum is fitted to a Gaussian curve.10°C (diamonds), 25°C (circles), 35°C (squares) and 45°C (triangles). The lines represent possible linear fits to the two regions of the curve.

Figure 4. The maximum wavelength **(A)** and intensity **(B)** from a Gaussian fit of the fluorescence spectrum (20°C, excitation wavelength 280 nm) for α-la with various

546 excesses of NaOle added. Samples were measured prior to heating (dotted bars) and
547 after heating at 60°C for 1 hour and subsequent cooling (striped bars).
548
549 **Figure 5.** DSC profiles of 100gL^{-1} α-la in PBS with various quantities of NaOle added.
550 **(A)** Heat flow profiles, the curves have been off-set on the vertical axis to aid in the
551 visualisation of the data. **(B and C)** Change in enthalpy and peak top temperature as a
552 function of NaOle concentration apo α-la (squares) and holo α-la (circles).
553
554 **Figure 6.** Changes in the amide I region of the FTIR spectra of **(A)** 10 gL^{-1} α-la and **(B)**
555 10 gL^{-1} α-la with 3.5 mM NaOle (5 fold molar excess) as a function of temperature.
556 The spectra shown have the spectrum of the sample at 20°C subtracted, to highlight the
557 regions where the changes are occurring.
558
559 **Figure7.** The titration of solutions of α-la, NaOle and mixtures of the two with HCl.
560 **(A)** The turbidity (O.D. 600 nm) of the solutions as a function of pH. 10 gL^{-1} α-la
561 (triangles), 10 gL^{-1} a-la 7mM NaOle (circles), 7mM NaOle (squares). Particle size
562 (dynamic light scattering) of 10 gL^{-1} α-la **(B)** and 10gL^{-1} α-la 7mM NaOle **(C)** at a
563 range of pHs.
564
565
566
567
568

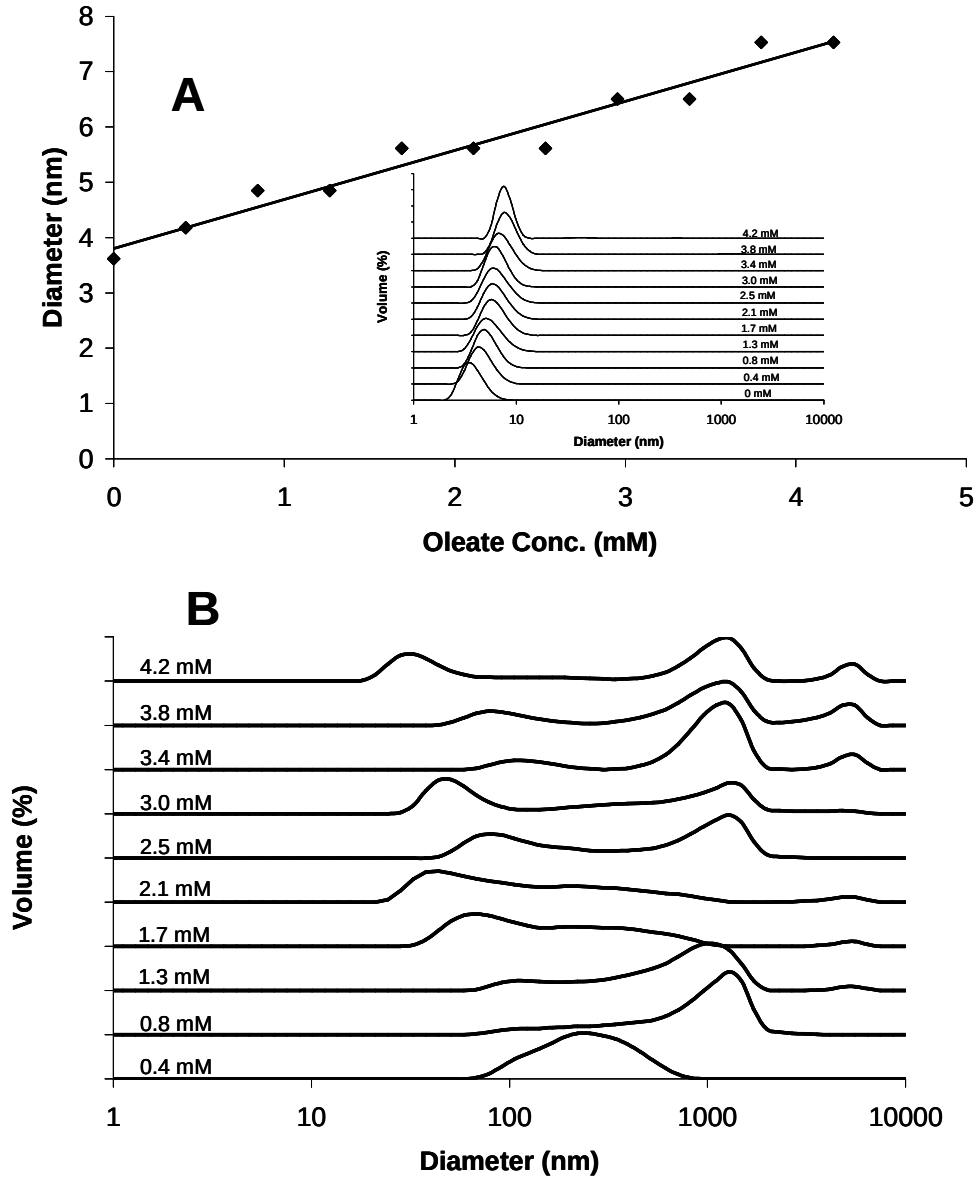
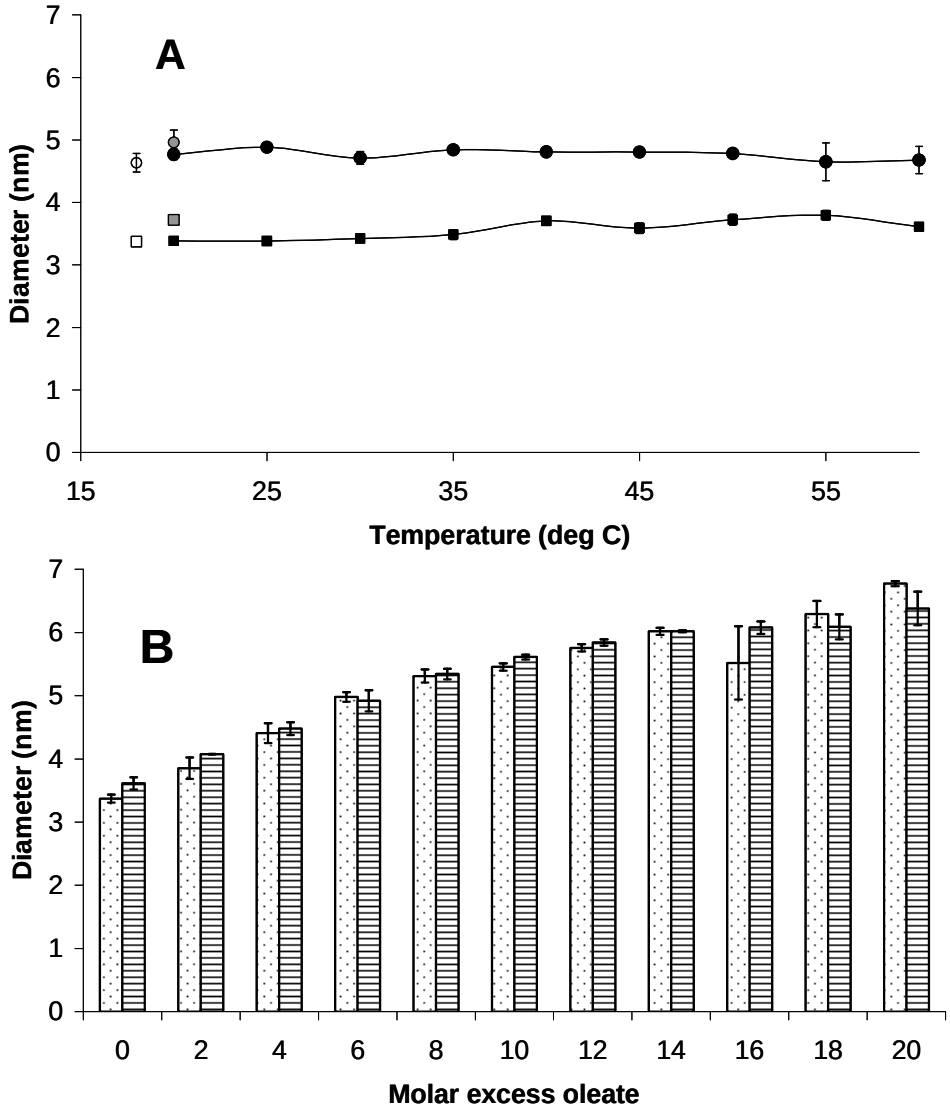

A
Diameter (nm)
Oleate Conc. (mM)
Volume (%)
Diameter (nm)
4.2 mM
3.8 mM
3.4 mM
3.0 mM
2.5 mM
2.1 mM
1.7 mM
1.3 mM
0.8 mM
0.4 mM
0 mM
B
Volume (%)
Diameter (nm)
4.2 mM
3.8 mM
3.4 mM
3.0 mM
2.5 mM
2.1 mM
1.7 mM
1.3 mM
0.8 mM
0.4 mM

Figure 1.



573

574 **Figure 2.**

575

576

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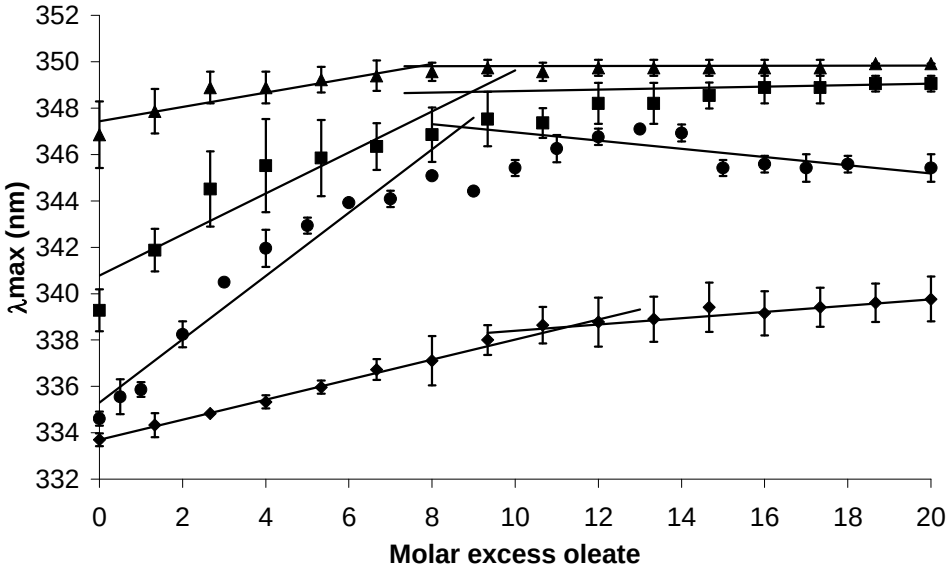

583

584 **Figure 3.**

585

586

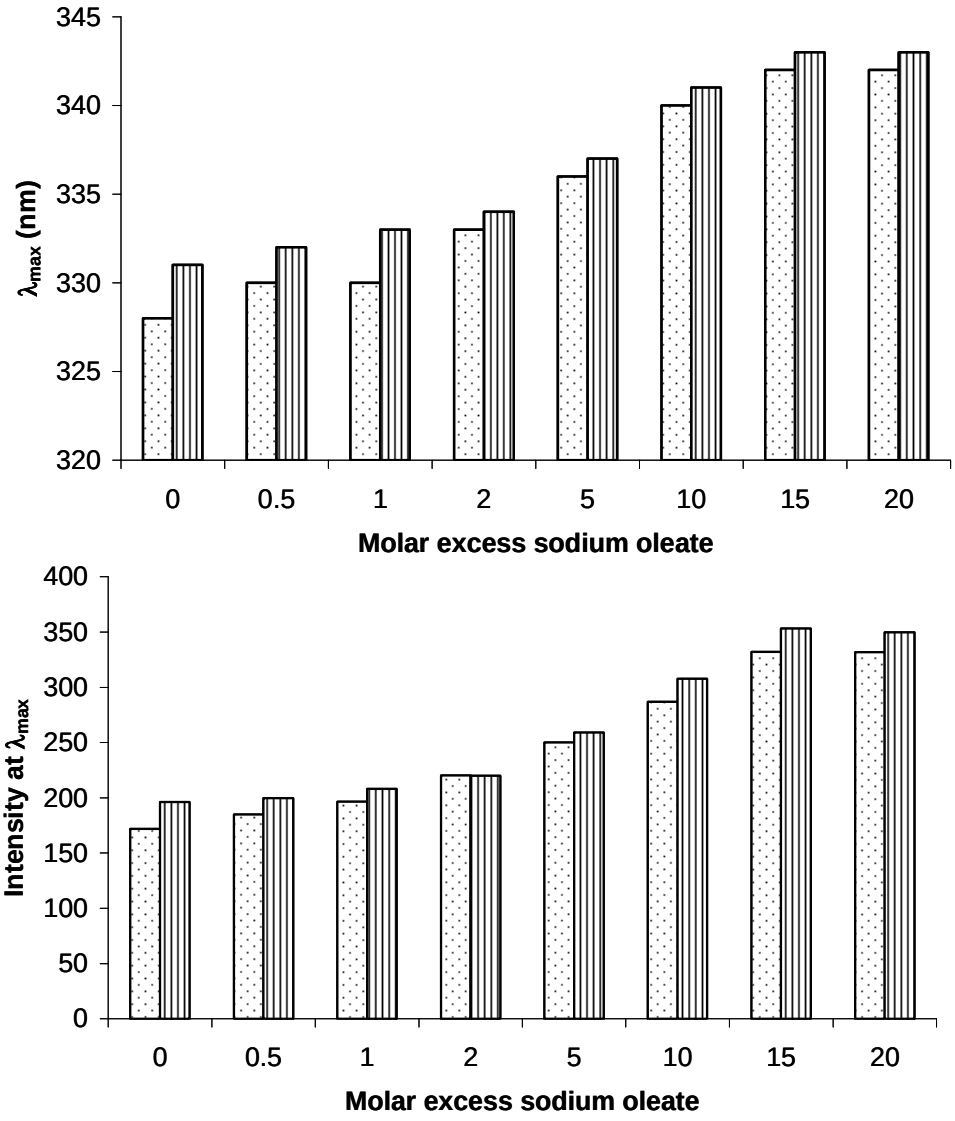
345
340
335
330
325
320
λmax (nm)
0
0.5
1
2
5
10
15
20
Molar excess sodium oleate
400
350
300
250
200
150
100
50
0
Intensity at λmax
0
0.5
1
2
5
10
15
20
Molar excess sodium oleate

Figure 4.

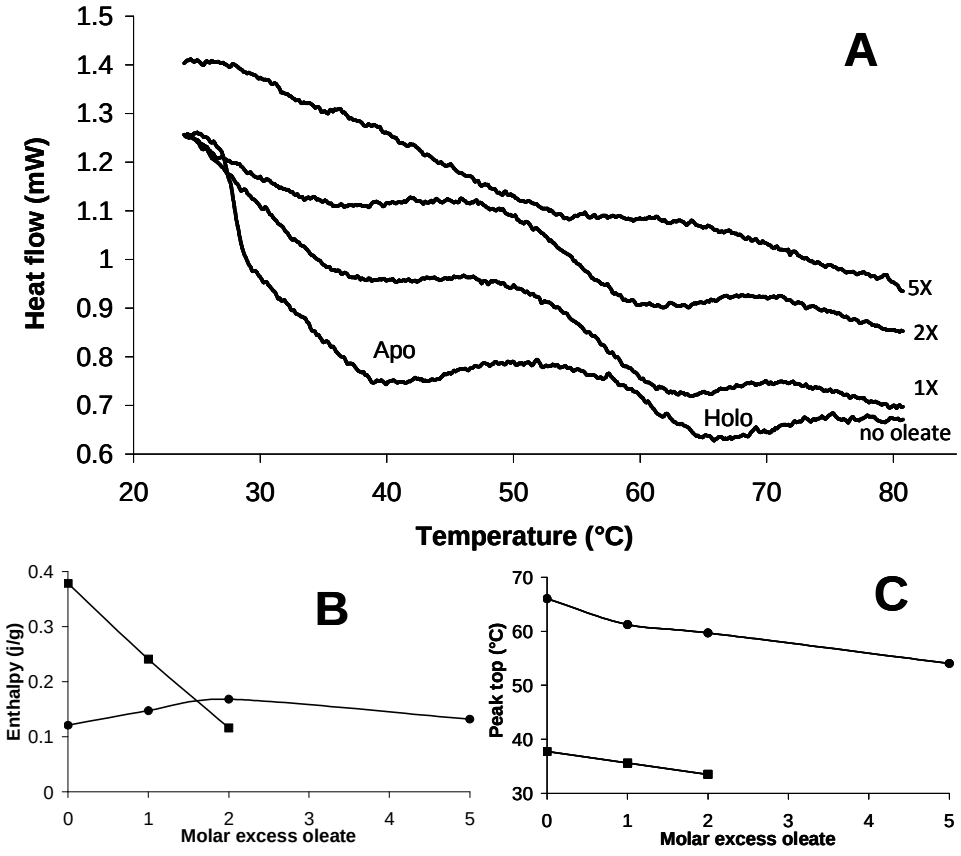
A
Heat flow (mW)
Temperature (°C)
5X
2X
1X
Apo
Holo
no oleate
B
Enthalpy (j/g)
Molar excess oleate
C
Peak top (°C)
Molar excess oleate

Figure 5.

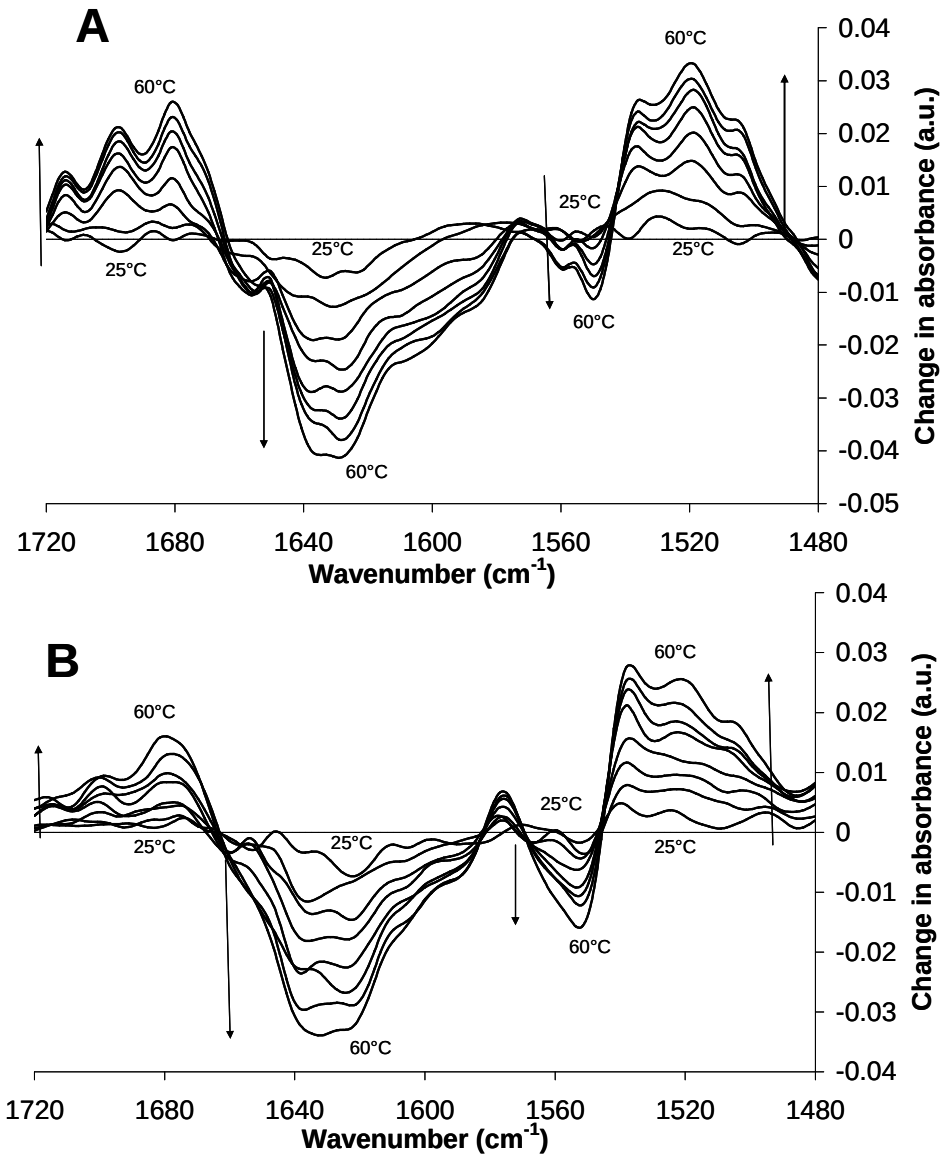
A
60°C
25°C
25°C
60°C
25°C
60°C
25°C
60°C
0.04
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
-0.04
-0.05
Change in absorbance (a.u.)
1720
1680
1640
1600
1560
1520
1480
Wavenumber (cm-1)
B
60°C
25°C
25°C
60°C
25°C
60°C
25°C
60°C
0.04
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
-0.04
Change in absorbance (a.u.)
1720
1680
1640
1600
1560
1520
1480
Wavenumber (cm-1)

Figure 6.

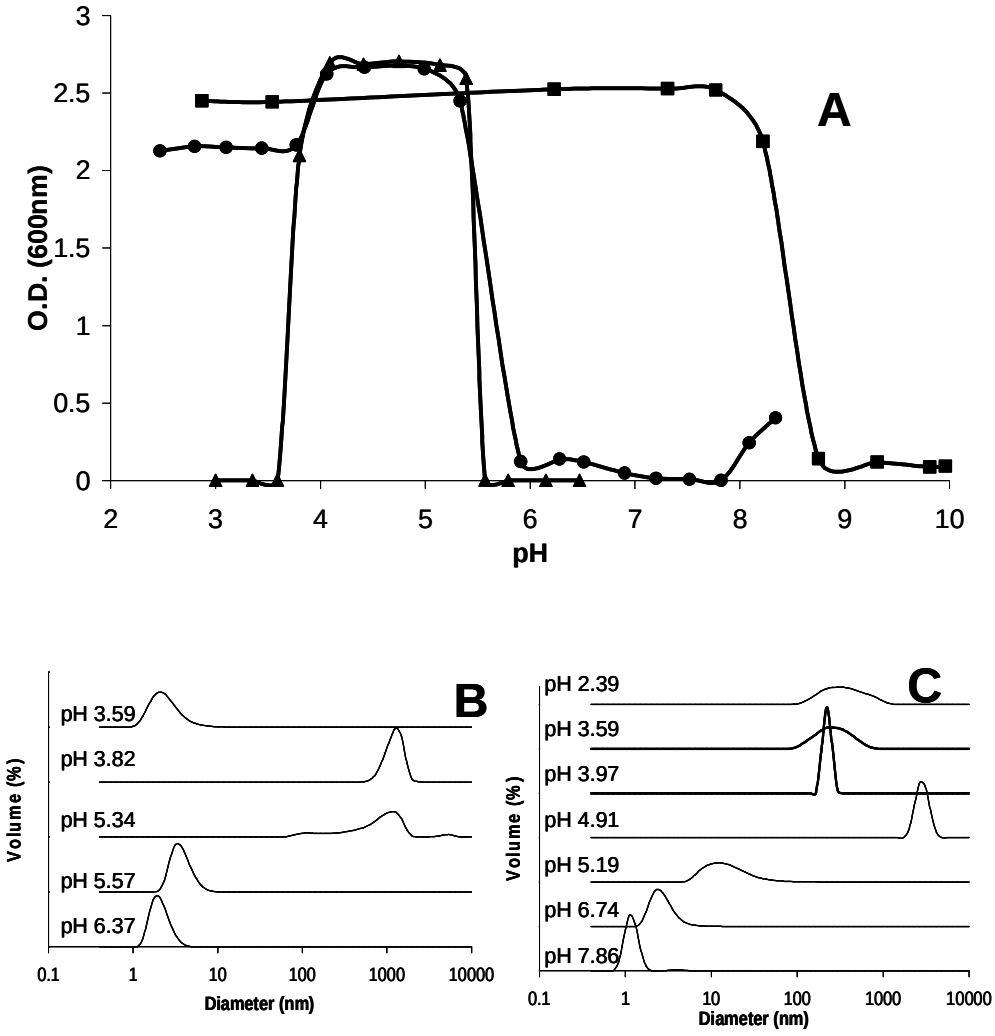
A
O.D. (600nm)
pH
B
pH 3.59
pH 3.82
pH 5.34
pH 5.57
pH 6.37
Volume (%)
Diameter (nm)
C
pH 2.39
pH 3.59
pH 3.97
pH 4.91
pH 5.19
pH 6.74
pH 7.86
Volume (%)
Diameter (nm)

Figure 7.