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Use of Solid-State ^{2}H NMR for Studying Protein–Lipid Interactions at Emulsion Interfaces

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Interactions between myosin and β-casein with lipids at lipid–water interfaces were studied by solid-state ^{2}H NMR using dimyristoylphosphatidylcholine with the four hydrogens at α- and β-positions (DMPC-d_4) and the nine protons at the γ-position substituted by deuterium (DMPC-d_9). Quadrupole splittings and spin–lattice relaxation times were used to describe the amplitude and rate of molecular motion of the choline segment, respectively, in liposomes made of pure labeled dimyristoylphosphatidylcholine or admixed with non-labeled dimyristoylphosphatidylglycerol (DMPG) in a 1:1 mole ratio. No changes were observed in these NMR parameters for the deuterons when increasing amounts of myosin were added to liposomes exclusively made of DMPC-d_9 or DMPC-d_4. However, when DMPG was present, myosin was found to interact electrostatically with the liposomes, and both the quadrupolar splittings and spin–lattice relaxation times of all head-group segments were affected, demonstrating that DMPG was necessary in the liposomes for the interaction to occur. The results suggest that positively charged lysine residues located at the tail domain of myosin provided the necessary sites for the lipid–protein interaction, leaving free the head domain for further structural interaction. On the other hand, β-casein was found to interact both with the charged (with DMPG) and neutral, zwitterionic (DMPC only) liposomes, although this interaction was more pronounced in the charged lipids. In the interaction with charged liposomes, β-casein was able to affect the lineshape of the NMR spectra from DMPC-d_9 deuterons, even at low protein concentration (lipid/protein mole ratio = 30 000:1), indicating its ability to locate at emulsion interfaces. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Most food emulsions are oil-in-water (o/w) or water-in-oil (w/o) discontinuous dispersions where stability is determined by the interfacial tension between the two phases, the characteristics of the adsorbed film, the electrical charge of the globules and the size–surface–volume relationship between the two phases.[1–4] Stabilizing agents for food emulsions are usually proteins, which are amphiphilic molecules and possess unique properties. They adsorb in the emulsion surface, thus providing multiple sites for further binding of important food components. Chemical, microscopic and spectroscopic techniques have been employed to gain structural information about food emulsions, especially about the mechanisms by which they are stabilized by proteins.[5–8] However, detailed information about protein location and orientation at the interface is still needed and would contribute to a better understanding of the process of emulsion formation. Food emulsions are usually made from lipids and proteins that are similar to those of biological membranes. Thus, highly sensitive biophysical methods, such as solid-state NMR, successfully employed to describe protein interactions at membrane interfaces, are likely to be useful to investigate the stability and molecular interactions of such systems.

In high-resolution NMR, where fast molecular reorientation with respect to the external magnetic field occurs, well defined spectra are obtained and NMR properties such as chemical shift, scalar coupling and nuclear Overhauser effect can be used to solve structural problems at a molecular level. In solid-like systems, owing to the restricted slow anisotropic molecular motion, broad signals are observed because of the anisotropy of the dipolar and quadrupolar interactions.

The anisotropy inherent in such broad-line solid-state NMR has been widely used in biological systems in recent years, especially for the description of molecular interactions between lipids and proteins at membrane interfaces.[9] Some specific nuclei can be used as probes, giving detailed information about order, motion, charge and structural rearrangements of the supramolecular assembly of lipids and proteins. The most commonly used nuclei for this type of study are the indigenous ^{31}P present in the phospholipid moiety or chemically

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non-exchangeable ^2H, added to specific positions of the lipid molecule by chemical synthesis. To use ^{31}P to probe a system it must be ascertained that phospholipid is the only source of indigenous phosphorus. The study of lipid–protein interactions of milk caseins that present several phosphoserine residues, for example, should be done with a low concentration of the protein relative to the lipid [lipid-to-protein (L/P) mole ratio > 100] to have practically all the ^{31}P resonance signal coming from the lipid moiety. The use of ^2H as a non-perturbing probe of the lipid moiety presents several advantages as it provides an unequivocal resonance assignment from specific positions of the lipid molecule giving details about the amplitude and rate of segment motion of the lipid and, consequently, lipid–lipid and protein–lipid interactions in biological membranes. Thus, several such interactions have been described employing solid-state ^2H NMR which have helped in an understanding of various interfacial phenomena in biological systems.[9,10–12]

In the present work, we used solid-state ^2H NMR to gain further information at the molecular level about the interactions of myosin and β-casein, which are widely used proteins for the production of food emulsions, with lipids at the emulsion interfaces.

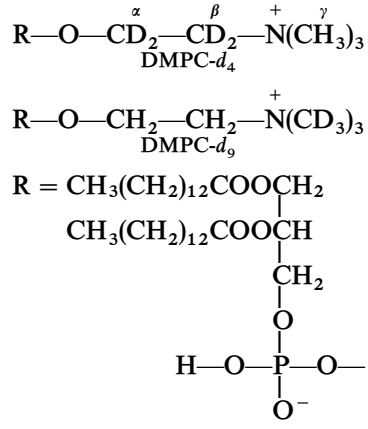
EXPERIMENTAL

Proteins

The β-casein employed was purchased from Sigma, (St Louis, MO, USA) (Lot 25H9550) and purified in a Fast Polymer Liquid Chromatographic (FPLC) system (Pharmacia Fine Chemicals, Uppsala, Sweden) using a Superdex 200 column, resulting in preparations essentially free of other proteins (not detected by gel electrophoresis). Myosin was extracted from bovine lung alveoli and purified by precipitation of an actin–myosin complex, which was dissociated by ATP–Mg^{2+}, and later fractionated with ammonium sulfate to an 11.3- and 2.2-fold enrichment, calculated from Ca^{2+} and K^+, EDTA–ATPase activities, respectively. Lung myosin preparation purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and presented three types of polypeptide chains with molecular weights of 200, 19 and 16 kDa, estimated by SDS-PAGE with addition of β-mercaptoethanol, thus being classified as a Type II myosin.[13–15] Maximum values of enzyme activity were observed at pH 8 and 9 for Ca^{2+} and K^+, EDTA–ATPase activities, respectively.

Deuterated lipids

Deuterated phospholipids were specifically labeled in the head group as indicated below. Dimyristoylphosphatidylcholine with the nine γ-protons substituted by ^2H (DMPC-d_9) was prepared by methylation of 1,2-bis-(dimethylphosphino)ethane (DMPE) with CD_3I[16] and dimyristoylphosphatidylcholine with the two α- and the two β-protons substituted by ^2H (DMPC-d_4) was prepared from the reaction of DMPE-d_4 with CH_3I, the DMPE-d_4 being synthesized from dimyristoylglycerol and perdeuterated ethanolamine,[17] which in turn was produced by catalytic exchange of protonated ethanolamine against D_2O.[18]



Sample preparation

Samples for NMR experiments were prepared from 50 mg ml^{-1} stock standard solutions of each lipid in $CHCl_3$–CH_3OH (2:1, v/v) by mixing suitable amounts of each solution to give the desired composition. Solvent was removed in a stream of nitrogen at ambient temperature and dried under high vacuum overnight ($<10^{-4}$ Torr; 1 Torr = 133.3 Pa). The dried lipid or lipid mixture was then fully rehydrated by vortexing it in an excess of 20 mM Tris–glycine buffer (pH 8.2), 1 mM EDTA and 0.6 M KCl (which was the optimum pH and ionic strength for myosin Ca^{2+} and K^+ EDTA–ATPase activity) for the experiments with myosin, followed by three cycles of freezing under liquid nitrogen and thawing at 37 °C. The water used in this buffer was deuterium depleted. For the β-casein experiments, both the lipid dispersion and protein solution were prepared with pure deuterium-depleted water. This procedure produced liposomes with an average diameter greater than 200 nm (as monitored by electron microscopy), which produced a typical anisotropic NMR signal for each specific nucleus.

Measurements

NMR spectra were obtained on either a Bruker MSL 400, a Nicolet 360 or a Bruker DPX300 spectrometer. Depending on the spectrometer and set-up conditions used, either short single $\pi/2$ pulses of 4–6 μs or the quadrupole echo sequence $[(\pi/2)_x$–τ–$(\pi/2)_y$–2τ–t_2(acquire)], where τ = delay in NMR pulse sequences, were used with static samples, resulting in typical broad-line solid-state spectra. The quadrupole echo sequence, as expected,[19] gave the best results for further phasing the transformed spectra. Spin–lattice relaxation times (T_1) were obtained by the inversion–recovery technique with the pulse sequence π–τ_{vd} [variable delays (a series of distinct delays) used in an NMR pulse sequence for T_1 determination followed by $\pi/2$ or quadrupole echo pulse sequence for acquisition. Values of T_1 were obtained from the dependence of the relative intensities against the τ_{vd} delays, which fitted an exponential function, $M_z = M_0[1 - A \exp(-\tau/T_1)]$,

where M_z is the observed magnetization, A is a fitting parameter ≤ 2 and τ is the variable delay used.[20] Spectra were either processed in the spectrometer where they were obtained or transformed and processed off-line with the programs FELIX (Biosym Technologies, USA) or nmrPipe (NIH, Bethesda, MD, USA).[21] All NMR experiments were performed at 303 K.

RESULTS AND DISCUSSION

The observed size (300–350 nm) of the liposomes, measured by electron transmission microscopy (not shown), revealed that they are in the size range of droplet particles usually observed in food emulsions (300–1500 nm).[2] The liposomes provide a charged or zwitterionic interface, depending on their composition, where the polar head group of the lipid moiety is located and can be used as a model of an interface similar to that observed in food emulsions.

Figures 1–5 present the broad and spherically averaged ^{2}H spectra of phosphatidylcholine deuterated in the α- and β-(DMPC-d_4) and γ-positions (DMPC-d_9) in pure and mixed liposomes, with and without the presence of myosin and β-casein. Each chemically distinct deuteron presents a double resonance separated by the quadrupole splitting ($\Delta\nu_Q$), which is the result of the strong quadrupolar interaction of deuterons with the applied field that dominate the solid-state spectra.

The broad ^{2}H NMR spectrum is obtained when liposomes are prepared with sizes greater than 200 nm, where slow macromolecular tumbling in solution does not average the anisotropy of the quadrupolar magnetic interaction that would be averaged out for small vesicles (<200 nm) when rapid tumbling occurs. These typical ^{2}H NMR powder patterns arise from the partial restriction of the phospholipid head groups, which are able to rotate only at the axis perpendicular to the liposome interface, giving rise to partially averaged anisotropy reflected by the amplitude of the deuterium motion relative to the liposome interface. The assignments of deuterons at each specific position was based on their motional behaviour.[12] The γ-deuterons of the methyl groups of DMPC-d_9, which possess a greater amplitude of head-group motion, result in an averaged quadrupolar splitting, $\Delta\nu_Q$, of *ca.* 1 kHz, whereas the more constrained α- and β-deuterons of DMPC-d_4 result in a $\Delta\nu_Q$ of *ca.* 4 and *ca.* 10 kHz, respectively.[10,11] These assignments are shown in Figs 1–5. The choline head group of DMPC behaves as a 'molecular voltmeter'[21,22] reporting any change in electrical charge at the liposome surface, which in turn causes a reorientation of the phospholipid head group. This reorientation is reflected in the molecular order parameter

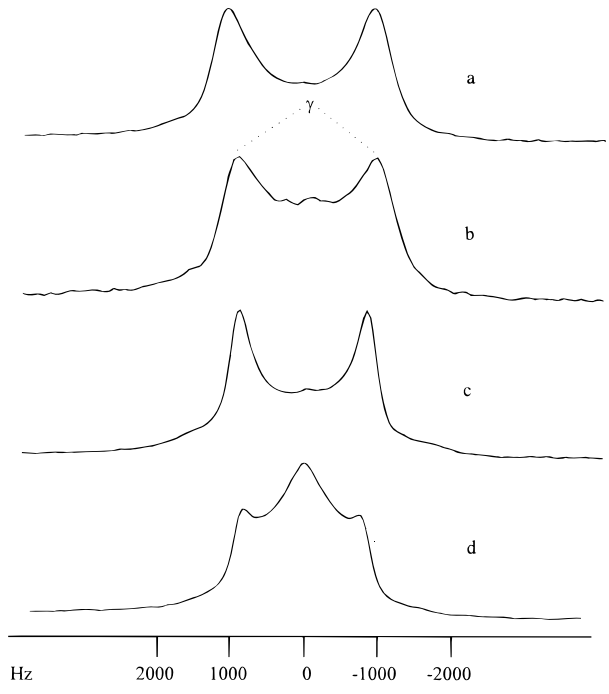


Figure 1. ^{2}H NMR spectra of liposomes made from DMPC-d_9 (a) without and (b) with added myosin to an L/P ratio of 8000:1, and liposomes made from an equimolar mixture of DMPC-d_9 and DMPG (c) without and (d) with addition of myosin to an L/P ratio of 8000:1. The assignments of γ-deuterons are indicated. Static spectra obtained at 55.28 MHz with a Nicolet 360 wide-bore spectrometer, using single pulse sequence.

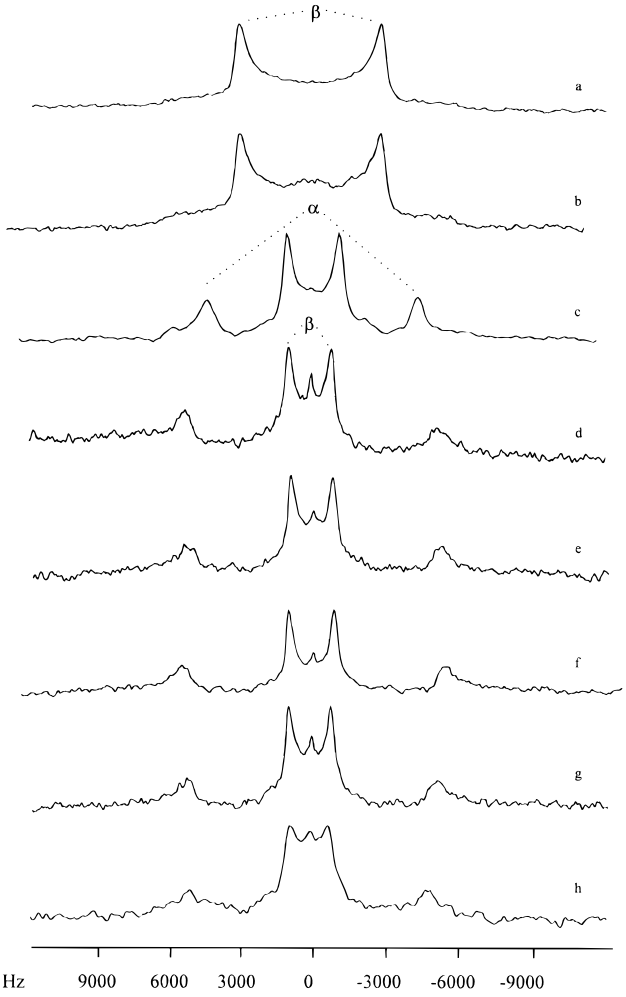


Figure 2. ^{2}H NMR spectra of liposomes made from pure DMPC-d_4 (a) without and (b) with added myosin to an L/P ratio of 8000:1, and liposomes made from an equimolar mixture of DMPC-d_4 and DMPG (c) without and with addition of myosin to L/P ratios of (d) 30000:1, (e) 20000:1, (f) 15000:1, (g) 10000:1 and (h) 8000:1. The assignments of α- and β-deuterons are indicated. Static spectra obtained at 61.42 MHz with a Bruker MSL 400 wide-bore spectrometer, using quadrupole echo sequence.

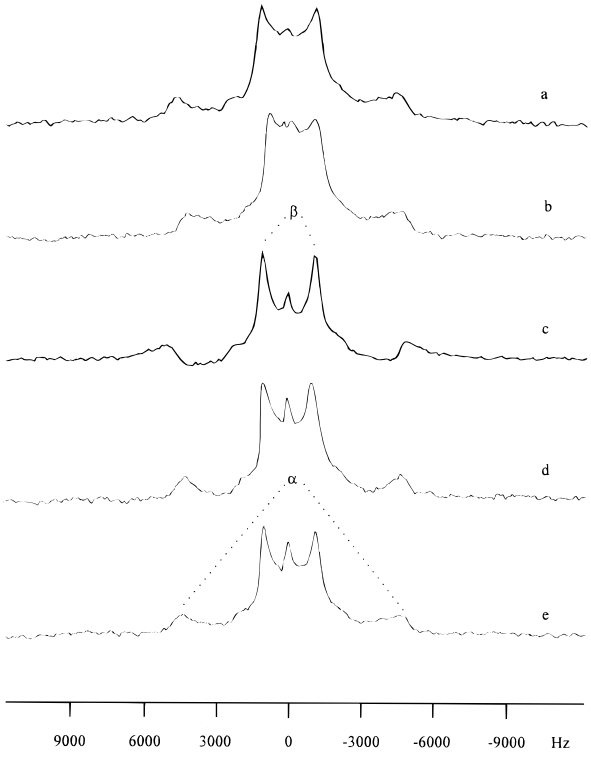


Figure 3. ^{2}H NMR spectra of liposomes made from an equimolar mixture of DMPC-d_4 and DMPG (a) without and with added β-casein to L/P ratios of (b) 109 000 : 1, (c) 25 000 : 1, (d) 12 000 : 1 and (e) 5000 : 1. The assignments of α- and β-deuterons are indicated. Static spectra obtained at 46.07 MHz with a Bruker DPX300 narrow-bore spectrometer fitted with a CP/MAS probe, using quadrupole echo sequence.

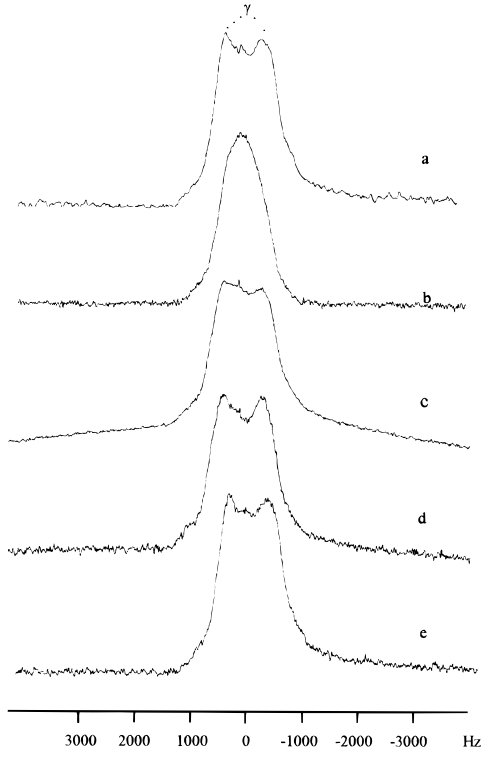


Figure 4. ^{2}H NMR spectra of liposomes made from an equimolar mixture of DMPC-d_9 and DMPG (a) without and with added β-casein to L/P ratios of (b) 30 000 : 1, (c) 20 000 : 1, (d) 10 000 : 1 and (e) 5000 : 1. The assignments of γ-deuterons are indicated. Static spectra obtained at 46.07 MHz with a Bruker DPX300 narrow-bore spectrometer fitted with a CP/MAS probe, using quadrupole echo sequence.

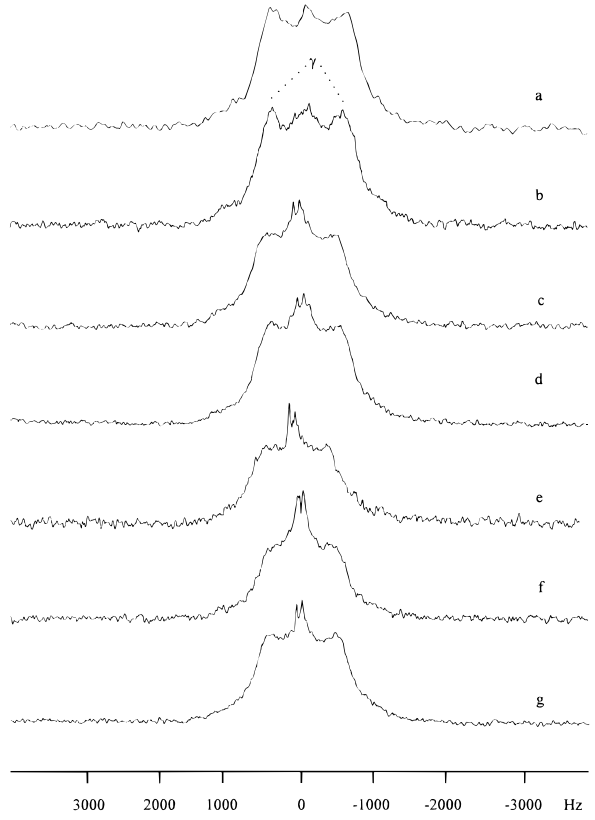


Figure 5. ^{2}H NMR spectra of liposomes made from pure DMPC-d_9 (a) without and with added β-casein to L/P ratios of (b) 57 000 : 1, (c) 19 000 : 1, (d) 14 000 : 1, (e) 11 500 : 1, (f) 8000 : 1 and (g) 6300 : 1. The assignments of α- and β-deuterons are indicated. Static spectra obtained at 46.07 MHz with a Bruker DPX300 narrow-bore spectrometer fitted with a CP/MAS probe, using quadrupole echo sequence.

(S_{zz}), that is a measure of the degree of wobbling of the choline segment. The relationship between the observed quadrupole splitting and this order parameter is

$$\langle \Delta\nu_Q \rangle = \frac{3}{2}\frac{e^2qQ}{h} S_{zz}$$

where $\Delta\nu_Q$ = observed quadrupole splitting (Hz), e^2qQ/h = quadrupole coupling constant for a C—D bond in the solid state ($\approx$167 kHz) and $S_{zz} = \langle 3\cos^2\Theta - 1\rangle$ = molecular order parameter about the z axis, where Θ is the angle between C—D bonds and the magnetic field.[11,23,24]

Thus, observation of changes in the lineshape of broad-line solid-state spectra reflects changes in the molecular order parameter of the lipid moiety and allows semi-quantitation of the quadrupolar splitting that describe molecular motion on the head group on interactions with proteins in mixed phospholipid bilayers.[9]

The ^{2}H spectra shown in Figs 1–4 are from the non-exchangeable deuterons of the head group lipid moiety that were chemically added by synthesis. The results showed a significant effect of dimyristoylphosphatidylglycerol (DMPG) addition to the liposomes compared with those of pure DMPC, that can be seen in the spectra of pure and mixed liposomes (Figs 1 and 2) where the order of the lipid head group is dramatically decreased, as shown previously.[12] This effect is more pronounced for the α- and β-deuterons in DMPC-d_4,

and has been reported in literature for describing lipid–lipid interactions at liposome surfaces and reflects changes in the orientation of the choline segment due to changes in either the electrical charge at the liposome surface or structural perturbations, or both.[12,22,23–28]

Figures 1 and 2 show the effect of addition of myosin to charged liposomes prepared from DMPC-d_9 or DMPC-d_4 and DMPG in a 1:1 mole ratio. Increasing the concentration of added myosin produced a decrease in the lipid order of β- and γ-deuterons due to myosin-selective interaction with DMPG. On the other hand, the order of α-deuterons increased or remained unchanged with added protein. These results are characteristic of electrostatic interaction similar to protein–lipid interactions already described for biological systems.[29–31]

Myosin is a characteristic protein with several remarkable structural and functional properties. Muscle myosins have a similar structure, being a dimeric molecule of high molecular weight (*ca.* 200 kDa for each monomer unit), and with each unit forming a long rod that makes a super coiled-coil with the unit rod from the other molecule. At the end of the dimer rod is a globular head, which is free for interactions and responds for the enzymatic ATPase activity and interaction with actin.[13] It is an important biological motor responsible for motion in several systems, especially muscle contraction and relaxation. More recently, a whole family of unconventional non-muscle myosins have been described, which are able to interact directly with biological membranes moving organelles within the cells.[15] Myosin is also important for the stability of meat emulsions produced in the preparation of comminuted meat products. The results shown in Fig. 1 for myosin liposome interaction are similar to others already described for peripheral membrane proteins,[32–34] and account for an electrostatic type of interaction where positively charged residues from the protein molecule interact with the anionic phospholipid (DMPG). This type of interaction would occur preferably at the rod portion of the molecule where positively charged lysine residues are located, leaving free the head domain. This molecular assembly can explain the remarkable ability of meat emulsions in comminuted meat products to form stable gels on cooking, as the head domain of myosin contains cysteine residues that can participate in disulfide bridges, rendering a flexible and resistant gel network, as observed in pure myosin.[7]

Addition of both myosin and β-casein to liposomes that contain DMPG in a 1:1 mole ratio affects both lineshape (spin–spin relaxation time, T_2) and T_1 values for the choline segment. The determined spin–lattice relaxation time constants (T_1) are presented in Table 1. The interaction of protein with the liposomes altered the re-orientational correlation time of the lipid segment and hence T_1. For myosin addition, T_1 values increased up to a maximum of 51 ms, decreasing at higher concentrations. For β-casein, a steady increase was observed when this protein interacted with charged liposomes. These changes in motional rate for the lipid head groups are most probably due to conformational changes of head groups, which result in altered re-orientational correlation times and, probably, changed amplitudes caused by the binding of protein.

Table 1. Spin–lattice relaxation times (T_1) for β-casein and myosin interaction with charged liposomes (made from an equimolar mixture of DMPC-d_9 and DMPG)

	T_1 (ms)	
L/P ratio	β-Casein	Myosin
∞ (pure liposome)	49 ± 2.3[a]	47 ± 1.24[a]
30 000:1	54 ± 1.03[b]	51 ± 0.54[c]
8000:1	61 ± 0.15[d]	43 ± 0.28[e]

[a–e] Different superscript letters indicate significant differences ($p < 0.05$) through analysis of variance.

The changes in spectral lineshape on addition of β-casein to pure and mixed liposomes (Figs 3 and 4) showed that interaction may occur in both bilayers, being more pronounced in the liposomes that contain DMPG than in DMPG-free liposomes. Reduction in the lipid order occurred in both cases, although this was more effective in charged bilayers. At low protein concentrations (L/P mole ratio = 30 000:1), using DMPC-d_9 as a probe of the liposome surface, the quadrupole splitting is almost lost and lateral phase separation might be occurring. This type of interaction has already been described for mellitin and myelin basic protein[29] and resulted in a complete lateral phase separation of DMPG induced by the strong electrostatic interaction between this lipid and the protein. β-Casein is a protein for which structural information is still controversial.[35] It has 209 amino acid residues with a large number of Pro, spaced regularly at the molecule after His-50, and for this reason it is thought to have a completely disordered structure from this amino acid onwards. However, several workers have reported the presence of significant amounts of β-sheet and α-helices by Raman, IR and CD spectroscopy,[36,37] that supports some predictions obtained from molecular modeling resulting in a tentative three-dimensional structure that would account for these ordered structures.[35] In this predicted structure there are several charged amino acid residues available in distinct domains of the protein (mainly Lys, Ser, Arg and Ser-phosphate) to participate in a strong electrostatic interaction with charged liposomes, as indicated by the ^{2}H NMR results in Fig. 4. Besides this ability for electrostatic interaction, β-casein also possesses hydrophobic domains that could account for the observed neutral interaction with uncharged liposomes, although less intensely than in the former case, as shown in Figs 3 and 4. This dual characteristic of this protein makes it highly suitable for emulsion stabilization since it can locate at emulsion interfaces of a wide range of electric surface charge produced by emulsifiers other than proteins.

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