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Physicochemical properties of microemulsion analogues of low density lipoprotein containing amphiphatic apoprotein B receptor sequences

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Abstract

Low density lipoprotein (LDL) has been proposed as a drug targeting vector in cancer chemotherapy, however, research has been limited due to the necessity to isolate material from plasma. In this study, the physicochemical properties of synthetic lipid microemulsions containing an amphiphatic version of the apoprotein B receptor binding sequence have been examined. The effect of peptide sequence length, lipid anchor type and location along with microemulsion lipid composition were investigated via changes in particle size and zeta potential. Size increases were related to the amphiphatic peptides lipophilic portion and too a lesser extent by amino acid sequence length. Two lipophilic anchors, retinoic acid and cholesterol, produced large size increases whilst a single anchor (retinoic acid) did not affect size. The amphiphatic peptide reversed measured zeta potential from negative to positive values in a concentration dependent manner. This was related to peptide structure and could be effected by changes in pH, indicating that the peptide was surface located and responsive to the external environment. Alteration of microemulsion lipid composition also affected physicochemical properties but to a lesser degree than changes in the amphiphatic peptide. These novel systems may represent a useful synthetic alternative to native LDL for a variety of applications.

Keywords: LDL; Apoprotein B; Zeta potential; Size; Amphiphatic peptides; Drug targeting

1. Introduction

Low density lipoprotein (LDL) is a normal constituent of plasma whose function is the in vivo transport of cholesterol. It is a spherical particle (diameter 20–24 nm) consisting of an internal core of cholesterol esters solubilised by a monolayer of phospholipid which contains the receptor protein, Apoprotein B (Apo B) (Spady,

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1991). The function and interaction of LDL with mammalian cells was elucidated by Goldstein and Brown in a series of classical experiments (Brown and Goldstein, 1986). This research demonstrated that LDL was assimilated by cells via a receptor dependent pathway involving Apo B and a cell surface receptor located in coated pits, and illustrated the importance of the LDL pathway in familial hypercholesterolaemia and related diseases (Myant, 1993).

Further studies have elucidated a role for LDL in certain types of cancer where tumour cells assimilate the material at elevated rates when compared with similar normal cells (Hynds et al., 1984). This has led to the suggestion that LDL may be a useful drug targeting vector for cancer chemotherapy (Gal et al., 1981). However, LDL must be isolated from fresh plasma using a relatively prolonged technique (Eley et al., 1990), which only yields small quantities of the material. Once isolated LDL is both chemically unstable and has a tendency to aggregate in solution a property related to Apo B. These restrictions have limited research into LDL to in vitro experiments (Eley et al., 1990) or small scale studies in vivo (Filipowska et al., 1992).

In order to circumvent LDL supply problems various apoprotein free microemulsions employing lipid constituents similar to those of native LDL have been produced (Ginsburg et al., 1982; Halbert et al., 1984; Owens and Halbert, 1995). These systems have proved useful for studying lipid interactions but have a limited biological utility since they do not posses the receptor apoprotein. This may be grafted on to for example liposomes after production (Lundberg et al., 1993) but this technique still requires a source of native apoprotein, usually from plasma. In the case of Apo B, its size and physical properties make grafting a difficult proposition.

The primary structure of Apo B, which was resistant to classical protein analysis techniques, was determined from cDNA analysis (Knott et al., 1986; Yang et al., 1986) and a putative receptor site identified. This consisted of a nine amino acid sequence (Table 1), which is located near the molecule's C terminal end. Previous studies have demonstrated that amphiphatic molecules will spontaneously interact with LDL in solution (Tucker and Florence, 1983) and this can be detected through changes in particle diameter. In addition suitable amphiphatic molecules can impart different receptor binding status on LDL after insertion into the particle's surface layer (Vanberkel et al., 1985). It is proposed that by employing suitably modified amphiphatic versions of the Apo B receptor sequence in combination with the lipid microemulsions described above it may be possible to prepare synthetic LDL (sLDL) particles, without the requirement to isolate from natural sources.

In this paper, various amino acid sequences and two different hydrophobic lipid end groups have been employed to create amphiphatic Apo B receptor sequences, see Table 1. The effect of these amphiphatic sequences on the particle size and zeta potential of LDL like lipid microemulsions has been measured and related to changes in peptide concentration, external medium pH, lipid composition and the lipophilic anchors employed. Previously similar particles have been shown to be capable of supporting the growth of U937 cells in vitro (Baillie et al., 1994) indicating that this system does posses a degree of receptor competency.

Table 1
Peptide structures used in the preparation of sLDL

Peptide	N terminal	Peptide sequence[a]	C terminal
PEP1	Retinoic acid	Leu-Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu	–Cholesterol
PEP2	Retinoic acid	Gly-Thr-Thr-Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu	–OH
PEP3	Retinoic acid	Tyr-Lys-Leu-Glu-Gly-Thr-Thr-Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu-Ala-Thr-Ala-Leu-Ser	–Cholesterol

[a] Underlined, proposed apoprotein B receptor sequence.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (>99% pure, PC) was purchased from Lipid Products, Surrey, UK and used without further purification. Tris(hydroxymethyl)-methylamine (Tris), hydrochloric acid (HCl), dimethylsulfoxide (DMSO) and *t*-butanol (Analar grade) were obtained from Merck Ltd, Glasgow, UK. Triolein (99% pure, TO), cholesteryl oleate (98% pure, CO), unesterified cholesterol (UC), sphingomyelin (SM), phosphate buffered saline tablets and the reagents for cholesterol testing were purchased from the Sigma Chemical Company, Dorset, UK. Polycarbonate extrusion filters were obtained from Costar Corporation, Buckinghamshire, UK. Sterile 0.2 μm polysulfone filters were purchased from Whatman Ltd, Maidstone, UK. Sterile distilled water complying with the European Pharmacopoeia's water for injection monograph for was purchased from Steripak Ltd, Runcorn, UK. All polypeptides were synthesised by Thistle Peptide Services at the Department of Biochemistry, University of Glasgow. Peptide structures are listed in Table 1, material was supplied at greater than 90% purity and used as received.

2.2. Preparation of synthetic low density liporpotein particles

Appropriate mixtures of lipids were dissolved in chloroform/methanol 2:1 (v/v) and the solvent removed under a nitrogen stream. The lipid film was re-dissolved in *t*-butanol and lyophilised for 24 h (EF4 Modulyo Freeze Dryer, Edwards High Vacuum, Crawley, UK), then re-suspended in 0.01 M Tris–HCl buffer (pH 8.0) to give a final concentration of 7–8% w/v. The lipid dispersion was sonicated under a stream of N_2 for 2 h using a 250 W sonicator, centrifuged at 10 000 rpm for 60 min (MSE Superspeed 75 Ultracentrifuge, MSE Ltd, London, UK) and then transferred to the Extruder vessel (Lipex Biomembranes Inc., Vancouver, Canada). The lipid mixture was successively extruded (at 50–55 °C) through polycarbonate filters of pore size 0.1 and 0.05 μm using two stacked filters and at least four extrusions under 60 psig nitrogen pressure (Owens and Halbert, 1995).

The resultant lipid microemulsion was diluted with Tris–HCl buffer to give a CO concentration of approximately 1 mmol/l and heated to 55 °C in a water bath. Aliquots of peptide dissolved in DMSO were added under the surface of the stirring microemulsion, control experiments were performed with DMSO alone. The volume of DMSO added in each case was kept below 20 μl/ml of microemulsion. The peptide–microemulsion complex was incubated at 55 °C for 15 min then dialysed overnight against 0.01 M Tris–HCl buffer. The resulting sLDL preparation was filter sterilised (0.2 μm) and stored at 4 °C under N_2 before use.

2.3. Analysis for cholesterol content

Microemulsions were assayed for cholesteryl ester content using the method of Allain (Allain, 1974). Cholesterol reagent (cholesterol oxidase, cholesterol esterase, horseradish peroxidase (HRP), 4-aminoantipyrene, *p*-hydroxybenzenesulfonate and buffer) was warmed to 30 °C in a water bath. To 1.0 ml of reagent was added 0.01 ml of sample, blank (distilled water) or standard (Cholesterol 200 mg/100 ml). Reagent-sample mixtures were allowed to incubate at 30 °C for 10 min. The absorbance of each sample was measured spectrophotometrically at 500 nm. All readings were completed within 30 min after the end of the incubation period. Cholesteryl oleate content was calculated by reference to the cholesterol standard.

2.4. Photon correlation spectroscopy

Particle size analysis was carried out using photon correlation spectroscopy (Zetasizer® 4, Malvern Instruments, Malvern, UK). Before analysis samples were diluted with Tris–HCl buffer (0.01 M) and filtered (0.2 μm). Sizing measurements were carried out at a fixed angle of 90°. The correlator was operated in parallel mode and the cumulants method of analysis was used to calculate the mean sample size weighted according to

the intensity of scattered light (z-average diameter). Since this diameter is weighted strongly in favour of large particles, Rayleigh theory was used to convert intensity distributions into number distributions (Washington, 1992). All results are the mean and standard deviation (S.D.) of at least ten measurements on the sample.

2.5. Zeta potential measurement

Samples were diluted 1 in 5 with 0.01 M Tris buffer (pH 8.0) and Zeta potential measured at 25 °C using a Zetasizer® 4 (Malvern Instruments). The applied voltage was 150 V in each case and duty cycling was used to limit the cell current to 20 mA. All results are the mean and S.D. of at least ten measurements on the sample.

3. Results and discussion

It is known that the lipid microemulsions utilised in this study are not homogeneous and may contain up to 50% of vesicular structures (Owens and Halbert, 1995). Peptide incorporation during preparation would, therefore, lead to 'entrapped' material on the inner vesicular surfaces. In addition, the amphiphatic peptide sequences employed are not soluble in water and, therefore, cannot simply be mixed with the pre-formed microemulsion. To circumvent these problems, the amphiphatic peptides were dissolved in a water miscible solvent (DMSO) before addition to the preformed aqueous microemulsion. To facilitate peptide–microemulsion interaction, a temperature above the highest lipid transition temperature was also utilised.

In Fig. 1, the effect on microemulsion particle diameter and zeta potential of incubation time and concentration of amphiphatic peptide added is shown. Both diameter and zeta potential increase immediately on amphipahtic peptide addition in a concentration dependent manner. Diameter further increases with time to a maximal plateau value at around 2 h whilst control preparations do not change during the observation period. Zeta potential decreases during incubation and the control containing DSMO behaves in a similar fashion to peptide containing microemulsions. Significantly, the control microemulsion without DSMO has a stable zeta potential during the observation period. It is known that solvents have a destabilising effect on the diameter of lipid emulsions (Owens et al., 1991) but no information is available on zeta potential. It is possible that the effects noted in Fig. 1b are due to chemical degradation of microemulsion constituents, which is exacerbated by the presence of DMSO. Since the initial effects occur rapidly, evident immediately after peptide addition, and size changes stabilise with time incubation at 55 °C was restricted to 15 min to limit the effect on zeta potential.

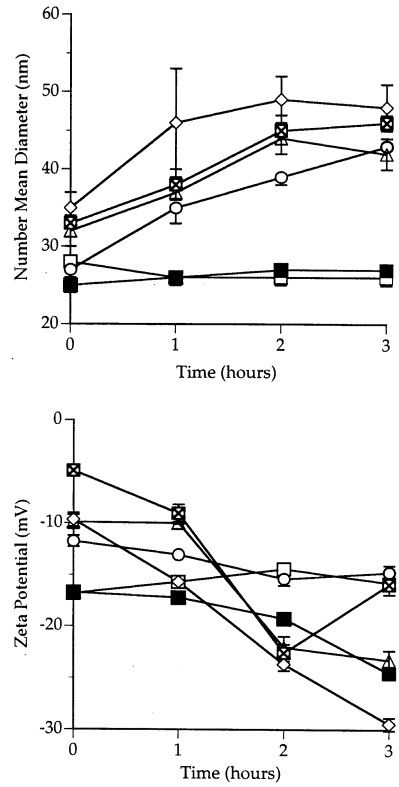

Fig. 1. (a) The effect of PEP1 on microemulsion diameter during incubation at 55 °C. (b) The effect of PEP1 on microemulsion zeta potential during incubation at 55 °C. All preparations PC:TO:CO, 3:2:1 (molar ratio). The amount of DMSO added was maintained at 4.4 μl/ml for each sample. Controls subjected to same incubation conditions. (□) Untreated control; (■) DMSO control; (○) 0.01 mole PEP1/mole CO; (△) 0.02 mole PEP1/mole CO; (⊠) 0.03 mole PEP1/mole CO; (◇) 0.04 mole PEP1/mole CO. Mean ± S.D., $n = 10$.

After this period, the systems were dialysed to remove DMSO and then sterilised by filtration. Previous studies have demonstrated native LDL surfactant interactions are instantaneous and stable at ambient temperatures (Tucker and Florence, 1983). The results indicate that the amphiphatic peptide sequence employed is interacting with the lipid microemulsion but that the size changes are not instantaneous and require time to reach equilibrium. However, it is not possible from the results to determine the proportion of the amphiphatic peptide, which interacts with the microemulsion particle.

The effects of increasing amphiphatic peptide concentration on diameter and zeta potential are presented in Fig. 2. All the peptides act to change measured zeta potential from a negative to a positive value but produce markedly different effects on particle diameter. Below 0.6 mole/mole CO PEP1 has no effect on size but produces an almost linear increase in zeta potential with concentration. Higher concentrations finally produce size increases but zeta potential plateaus at around +17 mV. PEP2 at low concentrations provides a dramatic reversal in zeta potential from around −15 to +15 mV without any change in size. Further concentration increases above 0.3 mole/mole CO do not increase zeta potential, which remains constant at +15 mV but size also remains constant. Around 0.1 mole/mole CO of PEP3 also produces a reversal of zeta potential without affecting measured diameter, whilst at higher concentrations, diameter almost doubles and zeta potential decreases but remains positive. Concentrations of PEP3 above 0.4 mole/mole CO were not measured since the measured diameter and zeta potential was markedly different from the starting values and the effects were equal to those of higher PEP1 or PEP2 concentrations.

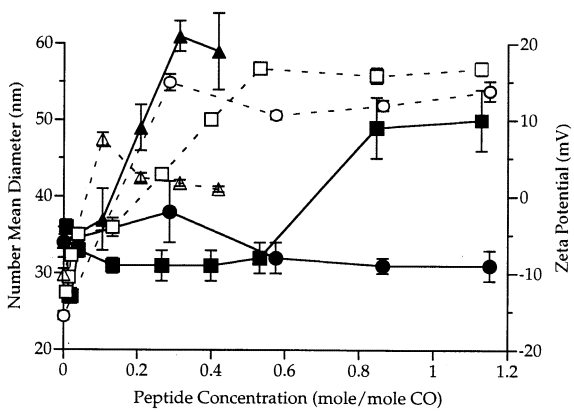

Fig. 2. The effect of increasing amphiphatic peptide concentrations on microemulsion diameter and zeta potential. Closed symbols and solid line diameter, open symbols and dashed line zeta potential. (□) PEP1 (PC:TO:CO, 3:2:1); (○) PEP2 (PC:UC:TO:CO, 6.2:1:4.2:2.1); (△) PEP3 (PC:UC:TO:CO, 6.2:1:4.2:2.1). Mean ± S.D., $n = 10$.

Zeta potential is a measure of surface charge and the observed results indicate that the amphiphatic peptides are spontaneously locating at the microemulsion particle surface. Probably because of electrostatic attraction, since the microemulsion particle is initially negatively charged, and also due to the peptide's amphiphatic nature. Since the lipid anchors are electrically neutral, they should not contribute to the observed changes in zeta potential, which can only be due to the associated peptide. At low peptide concentrations (below 0.2 mole/mole CO), where diameter remains relatively unchanged, the zeta potential increases produced by PEP1 and PEP2 are roughly equal whilst PEP3 has a considerably greater effect. This could be related to the greater size of the peptide fragment in PEP3 (22 amino acids vs. 13). The amino acid charge in PEP3 is not greater than PEP1 or PEP2 so this effect may be linked to the increased chain length permitting an easier interaction of the charged amino acids with the external environment.

The observed increases in particle diameter are probably attributable to the amphiphatic peptide's lipid anchor portion. Peptides containing both anchors (PEP1 and PEP3) produce increases in diameter at low concentrations (0.5 and 0.2 mole/mole CO, respectively) whilst PEP2 with only one anchor has no effect at any concentration. The bulky nature of the extra steroidal cholesterol anchor in PEP1 and PEP3 or the combination of two anchors, therefore, appears to be producing significant particle disruption. The two anchors may also be capable of cross-linking particles to produce size increases. Similar effects were noted in native LDL where surfactant in-

duced size increases could be related to the molecular size of the lipophilic portion (Tucker and Florence, 1983). However, absence of the cholesterol anchor does not appear to hinder peptide insertion into the particle since the zeta potentials obtained for PEP1 and 2 are similar. The increased peptide chain length in PEP3 also appears to have an effect on diameter, which increases at lower concentrations than PEP1. Since both peptides have the same anchor groups this must be related to an effective increase in hydrodynamic diameter produced by the larger peptide 'halo'. This effect may be similar to that seen after the absorption of block co-polymer surfactants onto nanoparticles (Illum et al., 1982). It is not clear why zeta potential eventually reaches a plateau or even falls at higher peptide incorporation values. For PEP1 and PEP3, this event corresponds to an increase in diameter with concomitant increasing surface area and possibly a reduction in effective surface concentration. It is also conceivable that surface saturation occurs and excess peptide forms micelles in solution although no direct evidence for this has been observed. This latter explanation is unlikely since micellar concentrations would potentially solubilise the microemulsion and reduce diameter (Tucker and Florence, 1983). The results suggest that the hydrophobic lipid anchors become buried in the microemulsion particle's outer phospholipid layer whilst the relatively hydrophilic peptide remains on the outer surface to alter zeta potential.

If amphiphatic peptide after interaction is exposed to the microemulsion's aqueous phase then it should respond to changes in this environment. In Fig. 3a, the effect of pH alterations on the measured zeta potential of various PEP1 systems is presented. All exhibited a shift to negative values with increasing pH with a marked change from positive to negative values for the highest peptide concentration tested. Control microemulsion particles without peptide also exhibits this trend -10 mV over 3 pH units, which can be attributed to changes in buffer ionic strength due to pH adjustment with sodium hydroxide. At 0.532 mole PEP1/mole CO, the zeta potential change is -20 mV and at 0.106 moles PEP1/mole CO -16 mV, both greater than the control. This

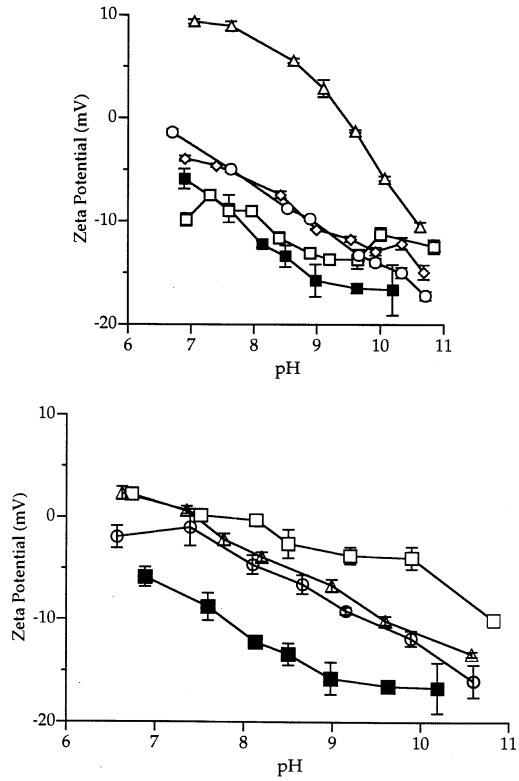

Fig. 3. (a) The effect of pH on the zeta potential of various PEP1 microemulsion preparations. All preparations PC:TO:CO, 3:2:1. (■) DMSO treated control; (□) 0.02 mole PEP1/mole CO; (◇) 0.039 mole PEP1/mole CO; (○) 0.106 mole PEP1/mole CO; (△) 0.532 mole PEP1/mole CO. Mean ± S.D., $n = 10$. (b) The effect of pH on the zeta potential of various microemulsion amphiphatic peptide preparations. All preparations PC:TO:CO, 3:2:1. (■) DMSO treated control; (□) PEP1 0.119 mole/mole CO; (○) PEP2 0.112 mole/mole CO; (△) PEP3 0.111 mole/mole CO. Mean ± S.D., $n = 10$.

result can be explained in terms of decreasing amino acid side chain group ionisation resulting in loss of positive charge and subsequent shift in zeta potential. This would be expected from the amino acid sequence since the major ionic charge is provided by positively charged amino side chain groups. At the extreme alkaline pH tested, all systems have similar zeta potential values indicating that peptide ionisation has been suppressed and the measured value is probably close to that of the microemulsion. This pH–zeta potential effect is reversible, if the system is returned to acidic pH values (data not shown) and overall the

results support the hypothesis that the amphiphatic peptide is exposed on the microemulsion particle's surface.

Results for three different sLDL preparations at similar peptide concentrations are presented in Fig. 3b, all display the trend presented in Fig. 3a with some subtle differences. At the majority of pH's, the most positive zeta potential is provided by PEP1 followed by PEP3 and then PEP2. Based on the results and discussion of Fig. 2, this rank order of effect in Fig. 3b is interesting. If pH induced alterations are related to ease of amino acid group interaction with the external environment then the results indicate that a small amphiphatic peptide with two anchors (PEP1) is restrained more (i.e. less able to interact with the external environment) than a large peptide with two anchors (PEP3) and only one anchor (PEP2) allows the amphiphatic peptide greatest freedom. Support for this hypothesis would require experiments at higher peptide concentrations where pH effects on zeta potential are more easily discerned (Fig. 3a) and may be enzymatic degradation studies. Overall the results indicate that the amphiphatic peptide is attached to the particle's surface and responsive to changes in the external environment.

In Fig. 4, the effect of alterations in the microemulsion's lipid components are presented with the lipid mixtures employed at proportions relative to

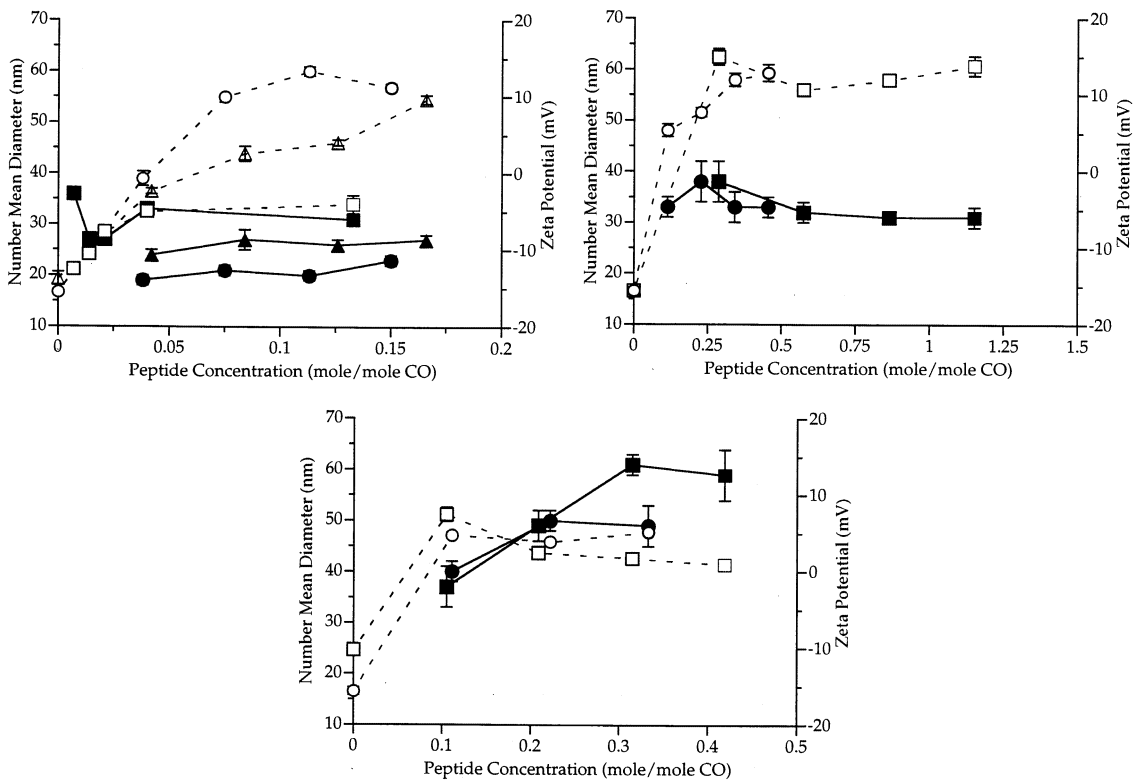

Fig. 4. (a) The effect of microemulsion composition on the diameter and zeta potential of PEP1 microemulsion preparations. Closed symbols and solid line number mean diameter, open symbols and dashed line zeta potential. (□) PC:TO:CO, 3:2:1; (○) PC:SM:TO:CO, 2.5:1:2.4:1.2; (△) PC:SM:UC:TO:CO, 4.4:1.8:1:4.2:2.1. Mean ± S.D., $n = 10$. (b) The effect of microemulsion composition on the diameter and zeta potential of PEP2 microemulsion preparations. Closed symbols and solid line number mean diameter, open symbols and dashed line zeta potential. (□) PC:UC:TO:CO, 6.2:1:4.2:2.1; (○) PC:SM:UC:TO:CO, 4.4:1.8:1:4.2:2.1. Mean ± S.D., $n = 10$. (c) The effect of microemulsion composition on the diameter and zeta potential of PEP3 microemulsion preparations. Closed symbols and solid line number mean diameter, open symbols and dashed line zeta potential. (□) PC:UC:TO:CO, 6.2:1:4.2:2.1; (○) PC:SM:UC:TO:CO, 4.4:1.8:1:4.2:2.1. Mean ± S.D., $n = 10$.

those found in native LDL (Skipski et al., 1967). The results generally follow the same trends reported in Fig. 2 with some interesting deviations. Both PEP1 and PEP2 show a reversal of zeta potential with little change in diameter whilst PEP3 induces large alterations in both parameters. Within different lipid mixtures only PEP1 exhibited a significant difference in zeta potential between the lipid formulations even although all have similar values before incorporation. This effect may be related to the cholesterol anchor since in liposomal systems it is known that different interactions occur between either PC or PC/SM mixtures and cholesterol. Solubilisation of cholesterol by phospholipids is critically dependent on van der Waals interactions between the steroid ring and the acyl chains (Darke et al., 1972) and is favoured by saturated hydrocarbons. The interaction between cholesterol and SM is, therefore, greater than cholesterol and PC, as the only unsaturated acid in SM is nervonic acid compared with oleic, palmitoleic or linoleic in PC. SM formulations may, therefore, promote interaction resulting at any given concentration in a greater magnitude of change to zeta potential. The incorporation of UC into the formulation would mitigate this effect and the results in Fig. 4a seem to support this hypothesis. When retinoic acid only is employed as the lipid anchor, PEP2 Fig. 4b, this effect is not evident and no difference in interaction is seen. The results of PEP3 do not follow this trend probably because the measured diameter also changes dramatically on peptide addition. The results suggest a similar behaviour of the amphiphatic peptides to apoproteins where interaction can vary depending upon phospholipid composition. However, this will require further investigation to confirm the magnitude of the interaction.

4. Conclusions

The synthetic amphiphatic peptides employed in this study incorporate into the surface of lipid microemulsions in a concentration dependent manner. The interaction results in changes in particle size and zeta potential, which can be related to the amphiphatic peptide's chemical structure notably the number and type of lipid anchors and length of peptide sequence employed. Once attached to the microemulsion particle's surface, the amphiphatic peptide is responsive to changes in the external environment and zeta potential is pH dependent. If the amphiphatic peptide contains cholesterol as a lipid anchor the interaction is also influenced by the microemulsion particle's lipid composition. The resultant particles have physicochemical properties, which are comparable to native LDL, with slightly larger diameter but a controllable zeta potential. The results presented illustrate that a synthetic analogue of LDL containing a receptor binding sequence from Apo B can be prepared using this simple technique. Further studies will now be conducted to determine the biological competency of these systems for the LDL receptor.

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