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Development of lipid microbeads for delivery of lipid and water-soluble materials to *Artemia*

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Abstract

Lipid spray beads (LSB) containing high concentrations of phospholipids were produced in order to improve their dispersion in both fresh and saltwater. The beads were developed to deliver both fat-soluble and water-soluble micronutrients to *Artemia* and other suspension feeders. LSB were prepared by spraying molted lipid into a chamber that was cooled with liquid nitrogen in order to solidify the lipid beads. Addition of soy lecithin to LSB did not affect retention of glycine when the beads were suspended in distilled water. There was an initial loss of 80% incorporated glycine after LSB were suspended in water for 20min. *Artemia* readily ingested riboflavin-containing LSB and their full guts were evident within 30min of feeding. The riboflavin content of *Artemia* could be increased from 55± 0.6 mg kg^{-1} (dw) to 329±62 mg kg^{-1} (dw) after 1h enrichment. LSB prepared with phospholipids are promising vehicles for enrichment of suspension-feeding organisms used as feed for larval marine fish and crustaceans as well as other suspension feeders.

Keywords: Lipid beads; Phospholipids; Amino acid; Nutrient; Glycine; Riboflavin; Oxytetracycline; *Artemia*

1. Introduction

Compared with natural zooplankton prey, such as copepods, *Artemia* are deficient in several water-soluble micronutrients that may be necessary for high growth and survival of marine fish larvae (Van der Meeren, 2003; Moren et al., 2006; Hamre et al., 2007). Enrichment of *Artemia* with water-soluble micronutrients is commonly achieved by either direct addition of micronutrients to the culture water or by adding micronutrients to lipid emulsions fed to *Artemia* cultures. The current enrichment methods are not optimal due to low uptake efficiencies and a failure to increase micronutrient concentrations to desired levels (Hamre, unpublished results). Enrichment of *Artemia* with liposomes has been successful (Tonheim et al., 2000; Monroig et al., 2003, 2006), but production of large quantities of liposomes is both expensive and technically difficult.

Incorporation of nutrients in a lipid matrix can be accomplished by a method referred to as spray chilling. Molten lipid, containing the active ingredients, is sprayed into a cooled environment where the lipid beads harden. The lipid beads consist of active ingredients randomly distributed in the lipid matrix with some possibly protruding through the surface of the beads. This is in contrast to truly encapsulated material where ingredients are surrounded with an intact wall (see review by Gouin, 2004).

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Spray chilling is a commonly used technology in pharmacy and food technology where it has been used for sustained release (Akiyama et al., 1993), taste masking (Yajima et al., 1999), improving stability (Schwendeman et al., 1998) and encapsulation of vitamins and minerals (Gibbs et al., 1999). The development and use of LSB to deliver water-soluble nutrients to marine fish larvae has been successfully accomplished using triacylglyceride (TAG) LSB that were themselves incorporated in a zein-bound feed particle (Önal and Langdon, 2005a,b). Encapsulation of water-soluble nutrients in LSB significantly decreased leaching (Önal and Langdon, 2005a).

The method to produce LSB by spray chilling requires that the lipid is solid at low (<20 °C) temperatures; therefore, it is not possible to deliver significant proportions of unsaturated fatty acids as TAG because they typically have low melting points. In contrast, phospholipids, such as soy lecithin, often have high proportions of unsaturated fatty acids (58% linoleic acid and 8% linolenic acid; Wettstein et al., 2000), but melting temperatures are elevated compared to those of free fatty acids or triacylglycerols due to the presence of the phosphorus group. This characteristic of phospholipids makes them good candidates for making LSB that are solid at room temperature, but have a high content of unsaturated fatty acids. In addition, several studies have shown that marine fish larvae benefit from inclusion of phospholipids as a dietary lipid source (Kanazawa et al., 1983a,b; Cahu et al., 2003).

The goal of this study was to evaluate the effects of additions of soy lecithin on the characteristics of LSB designed to deliver micronutrients to enrich *Artemia*, a common live feed for rearing marine fish larvae.

2. Materials and methods

2.1. Production of LSB with different concentrations of soy lecithin

Menhaden stearine was used as the major lipid source in experiments to determine the effects of additions of soy lecithin on the properties of LSB. Menhaden stearine is a by-product of menhaden oil refining and has the consistency of peanut butter and a melting point of 43°C. LSB were prepared using a modification of the method described by Önal and Langdon (2000, 2004a). Briefly, this method involved mixing core materials with molten lipid and then spraying the mixture into a chilled chamber to harden the lipid to form beads. Menhaden stearine was melted in a water bath at 60°C and soy lecithin was dissolved in the melted menhaden stearine with sonication. Glycine has a low molecular weight (75.05Da) and is highly water-soluble and was, therefore, chosen as the core material to investigate if additions of soy lecithin affected LSB properties (Önal and Langdon, 2004a). LSB were prepared containing glycine as either dry crystalline particles or as a saturated solution containing a slurry of suspended particles. The finely powdered glycine used for all LSB was prepared by spray-drying (Mini Spray Drier B-191, Büchi, Switzerland) an aqueous glycine solution according to method described by Clack (2006).

2.2. Lipid spray beads with a core of dry particulate glycine

Beads were prepared containing 16% w/w dry glycine particles. Six diets containing different concentrations of soy lecithin were produced (Table 1). Each batch of LSB was prepared in triplicate with a mixture of 2.88g glycine and 15.12g lipid. The lipid/glycine mixture was sonicated to obtain complete dispersion of particles and the mixture was then sprayed immediately to avoid particle separation.

2.3. Lipid spray beads prepared with an aqueous slurry of glycine

The volume ratio of aqueous glycine slurry to lipid was chosen based on the ratio that was shown to result in the best retention and delivery efficiencies for glycine incorporated in menhaden stearine LSB (Clack, 2006). Each batch of LSB, consisting of 4g glycine, 7.2g of distilled water and 13.8g of lipid (Table 2), was prepared in triplicate with different proportions of soy lecithin. The glycine slurry was prepared and sonicated before it was mixed with the molten lipid and again sonicated. The mixture was sprayed immediately to avoid separation of the emulsion. The concentration of glycine (ww) is the same in the LSB containing particulate glycine and an aqueous slurry of glycine. However, it should be emphasized that there is a difference in glycine to lipid ratio between the two respective types of LSB (Tables 1 and 2) due to the water content of the LSB containing an aqueous slurry.

2.4. Replacement of menhaden stearine with paraffin wax

A series of preliminary experiments showed that LSB containing paraffin wax were less sticky at room temperature

Table 1
Composition of LSB containing particulate glycine

Lecithin content	Menhaden stearine [a]	Soy lecithin [b]	Glycine [c]
% (w/w)	g kg^{-1}	g kg^{-1}	g kg^{-1}
0%	840	0	160
1%	831.6	8.4	160
5%	798	42	160
10%	756	84	160
20%	672	168	160
40%	504	336	160

Each bead type was prepared in triplicate.
[a] Crude menhaden stearine (Omega protein, USA).
[b] Soy lecithin, refined (M.W = 327.27; Cat no. 102147 Biomedicals).
[c] Glycine (minimum 99%; Sigma).

Table 2
Composition of LSB containing an aqueous slurry of glycine

Lecithin content	Menhaden stearine [a]	Soy lecithin [b]	Glycine [c]	Water
% (w/w)	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
0%	552	0	160	288
1%	546	6	160	288
5%	522	30	160	288

Each bead type was prepared in triplicate.
[a] Crude menhaden stearine, Omega protein, USA.
[b] Soy lecithin, refined (M.W = 327.27; Cat no. 102147 Biomedicals).
[c] Glycine (minimum 99%; Sigma).

than LSB produced with menhaden stearine, and were also more stable in salt water. Therefore, LSB prepared with paraffin wax were chosen for further investigation of their use for *Artemia* enrichment. Cod liver oil was added to the paraffin to improve LSB nutritional quality and to potentially improve their digestion by *Artemia*.

To investigate the properties of LSB prepared with paraffin wax, beads were prepared with cores of ground oxytetracycline (OTC > 95% HPLC 05875, Sigma). OTC was used instead of glycine as an alternative example of a water-soluble material. OTC has earlier been successfully incorporated in LSB (Buchal and Langdon, 1998; Langdon and Buchal, 1998). Based on preliminary experiments, LSB consisting of 545kg^{-1} soy lecithin, 227g kg^{-1} paraffin wax, 164g kg^{-1} cod liver oil and 64g kg^{-1} OTC were chosen for further investigation (Table 3). The lipid/OTC mixture was sonicated to obtain complete dispersion of particles and the mixture was then sprayed immediately to avoid particle separation.

To investigate the use of LSB to deliver water-soluble nutrients to *Artemia*, paraffin wax LSB were also prepared containing finely ground (< 10μm; McCrone micronizing mill; McCrone, Westmont, IL) riboflavin (≥98%, R-4500, Sigma) as the core material (Table 3). Riboflavin is a fluorescent water-soluble vitamin and has proved to be a good compound to visually investigate digestion and release of core materials from LSB ingested by fish and bivalve larvae (Langdon et al., 2000; Önal and Langdon, 2004b, 2005b).

2.5. Encapsulation efficiency (EE)

EE represents the retained core material in percentage of included core material in LSB after production. The concentration of core material (glycine; % w/w) was analyzed according to methods described by Önal and Langdon (2004b). First, duplicate samples of 0.1g of each LSB production batch were weighed into 20ml glass tubes, 10ml of chloroform was added and the tubes were shaken until the LSB were completely dissolved. Ten ml of distilled water containing 1.5 mg ml^{-1} SDS was added and the tubes were placed on a rotary mixer for 30min. The tubes were then centrifuged and the water fraction was removed and collected. This extraction was repeated three times. The collected supernatants from each extraction were pooled and analyzed for glycine by the ninhydrin method, as described in the analytical methods section (below).

2.6. Quantifying core material on the surface of LSB

When producing LSB, some of the core material may be situated partly or wholly on the surface of the beads. An attempt was made to measure this surface-associated, non-incorporated glycine by carrying out a rapid rinse of the beads and measuring glycine concentrations in the filtrate. However, glycine in the filtrate also likely contained some glycine leached from the beads; therefore, measurements of surface-located core material were likely to be overestimates of true values. Two replicate 0.1g samples of each LSB production batch were weighed onto filters (25mm, 0.45μm, Metrical membrane filter; Pall Life Science). LSB on the filters were rinsed under vacuum with 10ml of water. The rinse water was collected and glycine concentrations determined using the ninhydrin method (see below).

2.7. Dispersion of LSB

Dispersion in both seawater and distilled water was investigated for all produced LSB (Tables 1, 2, and 3). LSB were taken directly from a −80°C freezer and added to cold (1°C) seawater or distilled water in 20ml screw-capped test-tubes. The suspensions were hand-shaken and allowed to settle for 30s before being visually examined under a microscope. All the tubes were kept in an ice bath during the investigation. The temperature of the suspensions with dispersed LSB was increased to room temperature (22°C) to investigate the stability of LSB suspensions.

2.8. Retention efficiency

Retention efficiency is the percentage of initial incorporated core material lost after suspension of LSB in water (Önal and Langdon, 2004b). Leaching experiments were carried out based on a modified method described by Önal and Langdon (2004b). Preliminary studies indicated that clumping of LSB

Table 3
Composition of LSB containing paraffin wax

LSB type	Paraffin wax [a]	Soy lecithin [b]	Cod liver oil [c]	OTC [d]	Riboflavin [e]
	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
1	227	545	164		64
2	227	545	164	64	–
3	240	580	180	–	–

[a] Paraffin wax mp 56–60 °C (76231 Fluke chemica).
[b] Soy lecithin, refined (M.W = 327.27; Cat no. 102147 Biomedicals).
[c] Cod liver oil (Twinlab, USA).
[d] Oxytetracycline hydrochloride (minimum 95%; Sigma).
[e] Riboflavin (Sigma).

significantly reduced leakage rates. To reduce clumping, leakage experiments were carried out in an aqueous solution of 0.1% sodium dodecyl sulfate (SDS; Sigma). To investigate the effect of SDS on leaching, LSB containing 0% phospholipid (not dispersible without SDS) and 40% phospholipid (dispersible without SDS) were suspended in distilled water with and without 0.1% SDS for 10min and leakage rates compared. To avoid initial clumping and facilitate dispersion, it was important to disperse the LSB in chilled water.

To investigate leaching, 60 mg of LSB was added to pre-cooled glass tubes and 10ml of cold (1°C) 0.1% SDS (No. L-4509 Sigma) solution added. It was necessary to use chilled SDS solution to maintain hardened, non-sticky beads to ensure their dispersal. The tubes were stirred vigorously (Vortex-Genie, Fisher Scientific) for a few seconds to disperse the LSB and then placed on a rotary mixer (20RPM; tissue culture rotator: 099A RD4512; Glas-Col, Terre Haute, IN) for 20min at room temperature (22°C). There was a temperature increase during the 20min leaching experiment from 1 to 22°C, but this temperature increase was similar for all samples. The contents of the tubes were then filtered onto 0.45μm filters (Metrical membrane filter, Pall Life Sciences). The filtered supernatant was collected and glycine concentrations determined using the ninhydrin method (see below).

LSB prepared with paraffin wax and soy lecithin with a payload of OTC (Table 3) were leached in distilled water (without SDS) at room temperature (22°C) for various periods of time. Thirty mg of LSB was weighed into glass tubes and 10ml of distilled water was added and the tubes were then placed on a rotary mixer (20RPM) for 2, 10, 30 and 60min. The contents of the tubes were then filtered onto 0.45μm filters (Metrical membrane filter, Pall Life Sciences). The filtered supernatant was collected and OTC concentrations determined using a spectrophotometer (Beckman DU 530; absorbance 358nm).

2.9. Enrichment of Artemia

Decapsulated *Artemia* cysts (*Artemia salina*; Salt Creek Inc) were hydrated in fresh water for 1h before being hatched at room temperature (22°C) under continuous aeration and light in 10-micron filtered seawater. Three days after incubation, the unfed *Artemia* were washed onto a 250μm sieve and rinsed with clean seawater. Nine 2-l conical, aerated bottles were filled with 1.5L seawater and 363±13 *Artemia* L^{-1} were added. The *Artemia* were acclimated for approximately 1h before enrichment. In one treatment ($n=3$), *Artemia* were fed 133 mg l^{-1} LSB that were partially prepared with paraffin wax and contained 6.0% w/w riboflavin (Table 3). In the second treatment ($n=3$), *Artemia* were fed 133 mg l^{-1} empty LSB (Table 3) and pre-dissolved riboflavin at the same concentration as that delivered with additions of riboflavin-containing LSB (8 mg l^{-1}). The third treatment ($n=3$) consisted of unfed *Artemia*. During a 1h period of enrichment, samples of riboflavin LSB-fed *Artemia* were examined using an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a UV light source and compared with samples from the two control treatments.

After enrichment, the *Artemia* were washed thoroughly with filtered seawater on a 250μm mesh sieve. A small sub-sample of washed *Artemia* fed on riboflavin-containing LSB was examined under the microscope to ensure that no LSB were attached to their surfaces. *Artemia* from all treatments were then freeze-dried for riboflavin analysis. No attempt was made to purge the guts of the LSB-fed *Artemia*.

2.10. Analytical methods

Glycine concentrations were analyzed based on the method described by Doi et al. (1981). Each one ml sample was acidified by addition of 10μl of 1% acetic acid and one ml ninhydrin reagent (2% solution, Sigma) was added. The samples were placed on a heating block at 100°C for 10min and then transferred to an ice bath before 5ml ethanol (95%) were added to stabilize the color. Absorbance was determined spectrophotometrically at 570nm (Beckman DU 530) and converted to glycine concentration using regression equations derived from standard curves. The samples were analyzed in UV-grade polymethylmethacrylate cuvettes (VWR).

OTC concentration was determined spectrophotometrically (DU 530; Beckman Coulter, Fullerton, CA) by measuring absorbance at 358nm. The samples were analyzed in quartz cuvettes (VWR). The absorbance was converted to OTC concentration using regression equations derived from standard curves.

Riboflavin concentrations were determined by high-performance liquid chromatography (HPLC) with fluorometric detection according to a method described by Brønstad et al. (2002). Freeze-dried *Artemia* (0.25–0.30g) were added to 45ml 0.1M HCl. The samples were autoclaved at 121°C for 30min, cooled to room temperature and the pH adjusted to 4.0 with 2.5M sodium acetate. Sodium acetate trihydrate was added at a concentration of 100 mg per g sample and the samples were incubated at 37°C overnight. The samples were then diluted to 50ml and the solution was filtered before HPLC analysis (HP 1100, Agilent; exitation: 468nm and emission: 520nm). The concentration of riboflavin was calibrated with the use of external standards.

2.11. Statistical analysis

Biological and analytical data are expressed as means±1 standard deviation. Regression analysis together with one-way analysis of variance (ANOVA; Sokal and Rohlf, 1969) and Tukey's HSD post-hoc multiple comparison test were used to investigate the effect of concentration of soy lecithin on encapsulation efficiency, the proportion of non-incorporated glycine and leaching rate. The effect of LSB enrichment on the concentration of riboflavin in *Artemia* fed LSB containing riboflavin and Artemia fed empty riboflavin dissolved in the culture water was analyzed by Student *t*-test with a significance level (*P* value) of 0.05. The unfed control group

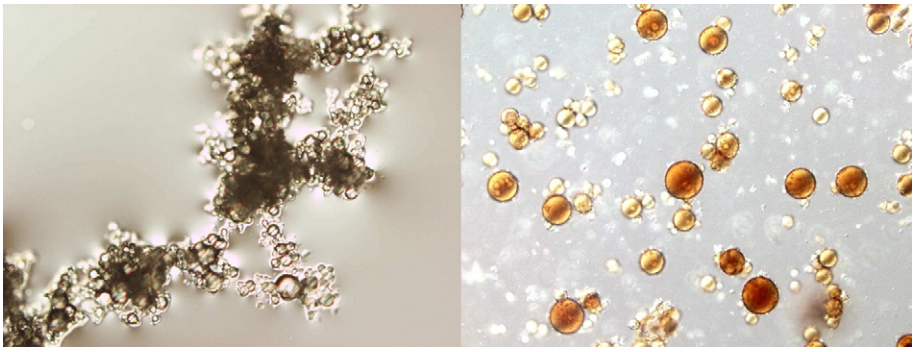

Fig. 1. Left: LSB containing 840 g kg^{-1} lipid (100% menhaden stearine) and 160 g kg^{-1} crystal glycine suspended in seawater. Magnification ×100. Right: LSB containing 840 g kg^{-1} lipid (60% menhaiden stearine and 40% soy lecithin) and 160 g kg^{-1} particulate glycine suspended in seawater. Magnification ×400. The pictures were taken using differential interference contrast (DIC) (Olympus BX 51).

was tested in duplicates and was therefore not included in the statistical analysis. All statistical analyses were performed using Statistica 7.1 (Statsoft, Inc, Tulsa, USA).

3. Results

3.1. Dispersion of LSB and effect on leakage rate

LSB containing aqueous slurry of glycine and 0%, 1% or 5% soy lecithin (Table 2), did not disperse in either salt or freshwater, but clumped immediately. In contrast, LSB containing dry particulate glycine and 40% soy lecithin (Table 1) dispersed in both fresh and salt water while LSB prepared with less than 40% lecithin (Table 1) did not disperse in either fresh or sea water (Fig. 1). LSB containing paraffin wax and 54.5% lecithin (Table 3) dispersed well in both fresh and saltwater.

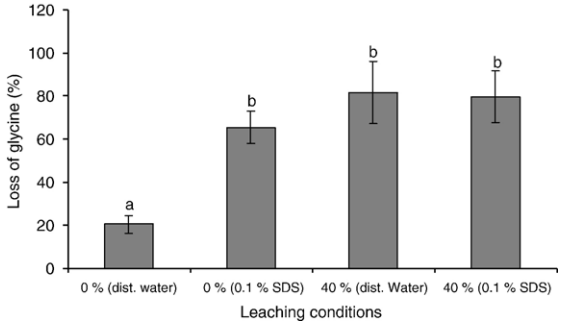

Fig. 2. Percent loss of glycine after 10 min leaching of LSB in either distilled water or water containing 0.1% SDS. LSB initially contained 16% particulate glycine and either 0% or 40% w/w soy lecithin. The LSB containing 0% lecithin are only dispersed in the 0.1% SDS solution. The LSB containing 40% lecithin are fully dispersed in both distilled water and the 0.1% SDS solution. Data are means of three replicates. Error bars represent standard deviations. LSB types sharing the same letter are not significantly different from each other (ANOVA, followed by Tukey HSD, $P<0.05$).

Addition of 0.1% SDS to suspensions of LSB containing 0% lecithin and crystal glycine (Table 1) led to improved dispersion and increased the leakage of glycine from 20% to 64% after 10min (Fig. 2). The leakage of glycine from already dispersed LSB containing 40% soy lecithin and particulate glycine (Table 1) was not significantly (ANOVA, $P \geq 0.05$) affected by addition of SDS to the incubation water (Fig. 5).

3.2. LSB containing a core of dry particulate glycine

There was an increase in the viscosity of the melted lipid-core mixture with increasing additions of soy lecithin, but all bead types could be sprayed. Small amounts of glycine were lost during the production process (Fig. 3) with encapsulation efficiencies ranging from 95±3 to 102±2% for the different bead types. There was a significant difference in incorporated concentrations of glycine (ANOVA, $P = 0.029$) between the LSB containing 1% and 20% soy lecithin (Fig. 3).

There was a positive correlation ($r^2 = 0.89$, $P < 0.00001$) between the amount of soy lecithin added to the beads and loss

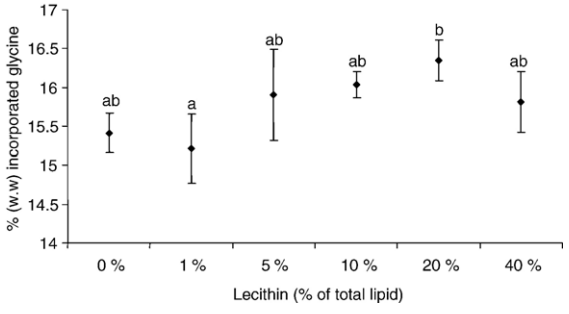

Fig. 3. Concentration of incorporated particulate glycine in LSB prepared with different concentrations of soy lecithin and menhaden stearine. Concentrations of glycine are given as % dry weight. Data are means of three replicates. Error bars represent standard deviations. LSB types sharing the same letter are not significantly different from each other (ANOVA, followed by Tukey HSD, $P<0.05$).

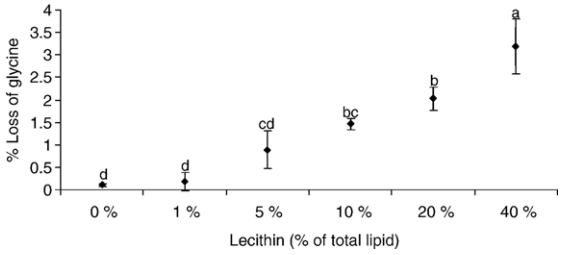

Fig. 4. Percent losses of glycine due to rinsing LSB with distilled water, for LSB containing particulate glycine and different concentrations of soy lecithin and menhaden stearine. Losses are given as % lost (dw) based on the initial glycine concentration of each LSB preparation. Data are means of three replicates. Error bars represent standard deviations. LSB types sharing the same letter are not significantly different from each other (ANOVA, followed by Tukey HSD, $P<0.05$). (% loss = 0.35 + 0.946 × Lecithin %, $R^2 = 0.89$, $P<0.00001$).

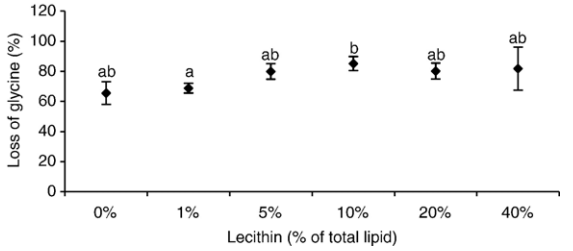

Fig. 5. Percent loss of glycine after 20 min leaching in distilled water for LSB containing particulate glycine and different concentrations of soy lecithin and menhaden stearine. Losses are given in terms of % of initially incorporated glycine (dw). Data are means of three replicates. Error bars represent standard deviations. LSB types sharing the same letter are not significantly different from each other (ANOVA, followed by Tukey HSD, $P<0.05$).

of glycine when LSB were rinsed with distilled water. The overall rinse loss of glycine ranged from only 0.1 ± 0.03% for LSB containing 0% soy lecithin to 3.2 ± 0.6% for LSB containing 40% soy lecithin (Fig. 4).

Loss of glycine after 20min of leaching (Fig. 5) ranged from 65 ± 7 to 85 ± 5%. There was no correlation ($P > 0.05$) between concentration of soy lecithin and glycine loss, but there was a significant difference ($P = 0.041$) in loss of glycine between LSB containing 1% and 10% lecithin (Fig. 5).

3.3. Lipid spray beads prepared with an aqueous slurry of glycine

It was impossible to prepare LSB with an aqueous core with additions of more than 5% lecithin because with higher lecithin concentrations LSB did not harden in the chilled chamber and they clumped on the chamber walls. There was no significant correlation ($P > 0.05$) between soy lecithin concentration and encapsulation efficiency of glycine and no significant differences in glycine encapsulation efficiencies among LSB types prepared with slurry of glycine (ANOVA, $P > 0.05$) (Table 4). The encapsulation efficiency of glycine ranged from 101 ± 1% to 108. ± 1%. There was no significant correlation ($P > 0.05$) between soy lecithin concentration and encapsulation efficiencies for water and no significant (ANOVA, $P > 0.05$) differences among the LSB types prepared with slurry of glycine (Table 4). The encapsulation efficiency of water ranged from 95 ± 3% to 102 ± 3%.

The loss of glycine due to rinsing ranged from 23 ± 4% to 27 ± 2% of the incorporated glycine (Table 4), but there was no significant ($P > 0.05$) correlation between soy lecithin concentration and loss. The leaching experiment showed no significant ($P > 0.05$) correlation between soy lecithin concentration and loss of glycine due to leaching and no significant (ANOVA, $P > 0.05$) differences in leaching among the bead types (Table 4). The overall loss in 20min was 98 ± 5% of incorporated glycine for all the slurry bead types combined.

3.4. Replacement of menhaden stearine with paraffin wax

Small amounts of OTC were lost during production of LSB containing paraffin wax and the encapsulation efficiency was 95 ± 2%. Large amounts of OTC were lost within the first min of suspension of the LSB in water (Fig. 6), but after 10min a 76 ± 5% loss stabilized and the remaining OTC (about 20%) was retained for 60min (Fig. 6). Riboflavin-containing LSB (Table 3) still had particulate riboflavin visible in the lipid matrix after dispersion in saltwater for more than 1h (Fig. 7). LSB containing paraffin wax were more stable than those with mennhaden stearine, since no signs of disintegration of the beads were detected when dispersed in either fresh or saltwater for 1h. LSB prepared with 40% lecithin and menhaden stearine started to disintegrate within this time span.

3.5. Artemia enrichment

Artemia ingested riboflavin-containing LSB and their guts became full after 30min of feeding (Fig. 8). The LSB seemed

Table 4
Encapsulation efficiency of glycine and water and losses of glycine by rinsing and leaching from LSB with 0%, 1% and 5% lecithin and a core of an aqueous slurry of glycine

	0% lecithin	1% lecithin	5% lecithin
Encapsulation efficiency of glycine (%)	101 ± 3[a]	108 ± 6[a]	106 ± 1[a]
Encapsulation efficiency of water (%)	102 ± 3[a]	95 ± 3[a]	99 ± 4[a]
Loss of glycine by rinsing (%)	25 ± 4[a]	23 ± 4[a]	27 ± 2[a]
Loss of glycine by leaching (20 min) (%)	98 ± 8[a]	101 ± 4[a]	97 ± 2[a]

The encapsulation efficiencies for both glycine and water are given as % of added concentrations. The losses of glycine by rinsing and leaching (20 min) are given as % of incorporated glycine. Data are means of three replicates. LSB types sharing the same letter are not significantly different from each other (ANOVA, followed by Tukey HSD, $P<0.05$).

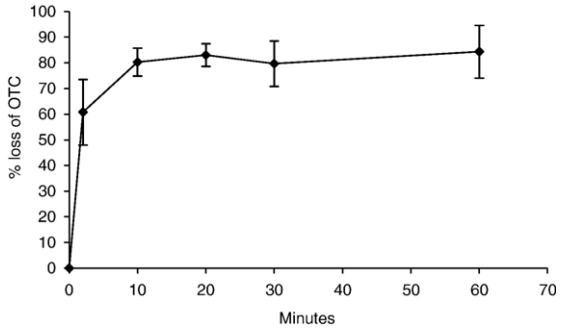

Fig. 6. Percent loss of oxytetracycline (OTC) from LSB prepared with 545 g kg^{-1} soy lecithin, 227 g kg^{-1} paraffin wax, 160 g kg^{-1} cod liver oil and 64 g kg^{-1} oxytetracycline. Leaching was carried out in distilled water and losses were expressed in terms of % loss of initially incorporated OTC. Data are means of three replicates. Error bars represent standard deviations.

to be broken down upon ingestion, because the digestive tract was filled with material with no whole beads visible (Fig. 8). No LSB were observed to be attached to the outside surface of washed *Artemia*. There was more than a six-fold increase in riboflavin concentration from 47.9±2.1 to 329±62 mg kg^{-1} (dw) in *Artemia* after 1h enrichment with riboflavin-containing LSB (Fig. 9). There were no differences in riboflavin concentration between the unfed control and the control fed a combination of empty LSB and riboflavin dissolved in the water (Fig. 9).

4. Discussion

Önal and Langdon (2005b) showed that LSB made of triacylglycerol were hydrophobic and that the LSB clumped and did not disperse in aqueous solution. By inclusion of high concentrations of phospholipids in the LSB, we were able to produce LSB that dispersed freely and were vehicles for delivering both lipid and water-soluble compounds to suspension feeders, such as *Artemia*. Addition of phospholipids can also increase the concentration of unsaturated fatty acids in LSB without causing a decrease in melting point.

There were no methodical difficulties producing LSB containing high concentrations of lecithin and particulate glycine or OTC. We did not succeed in melting pure lecithin and it was therefore necessary to dissolve the lecithin in either menhaden stearine or a mixture of wax and fish oil. Due to the high melting point of soy lecithin and other phospholipid sources, it is unlikely that it will be possible to produce LSB consisting of 100% phospholipid by using a spray technique. There was also a problem with cold-hardening LSB before they impacted the walls of the spray chamber for LSB made up of lecithin concentrations of > 5% w/w and containing an aqueous core. This problem might be solved by increasing the height of the spraying tower or by reducing the concentration of water in the beads.

All LSB types had high incorporation efficiencies in accordance with earlier studies (Önal and Langdon, 2004b, 2005b). Incorporation efficiencies may be increased further when lipid beads contain an emulsifier (Vilivalam and Adeyeye, 1994; Önal and Langdon, 2004b). The above 100% encapsulation efficiency of glycine for the LSB containing an aqueous slurry of glycine (Table 4), may be due to evaporation of water from the LSB during storage and thereby a small increase in glycine concentration. Not all the core material associated with the beads is necessarily fully incorporated. The results of this study showed that rinsing losses were only 1% for LSB containing particulate glycine but as high as 25% for LSB with aqueous cores.

Önal and Langdon (2004b) reported that LSB prepared with 5% of the surfactant sorbitan monoaplmitate or sorbitan sesquioleate showed improved dispersion but higher leaching rates than LSB prepared with triacylglycerol and no surfactant. To compare leaching among LSB with different dispersion properties, it was necessary to use 0.1% SDS to make the LSB types more equally dispersible. Dispersion of LSB containing 100% menhaden stearine with 0.1% SDS led to a three-fold increase in loss of glycine while there was no significant effect of SDS on leakage from more dispersible (less hydrophobic)

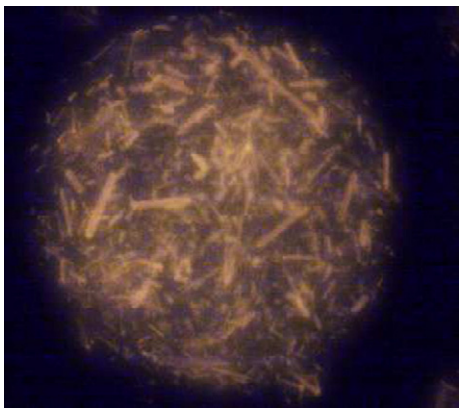

Fig. 7. LSB prepared with paraffin wax, soy lecithin and cod liver oil (227 g kg^{-1}, 545 g kg^{-1} and 164 g kg^{-1}) and 64 g kg^{-1} riboflavin (Table 3, LSB type 1). The picture was taken using an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a UV light source (excitation of 450–490 nm and emission of 515–528 nm). LSB were dispersed in seawater for 1 h and 55 min and riboflavin particles were still visible within the beads.

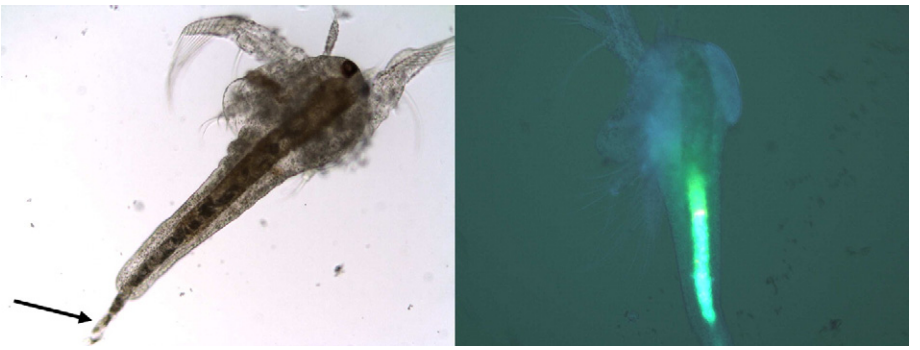

Fig. 8. Left: *Artemia* enriched with LSB containing riboflavin after 1 h enrichment; a string of faeces can be seen (Leica DM 1000). Right: Digestive tract of *Artemia* fed on riboflavin-containing LSB, filled with fluorescent riboflavin, shows that *Artemia* ingested LSB. Whole LSB are not visible, indicating that they were broken down. The picture was taken using an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a UV light source (excitation of 450–490 nm and emission of 515–528 nm).

LSB containing 40% lecithin. Leaching rates in this study were much higher for all investigated LSB than those reported in earlier studies (Buchal and Langdon, 1998; Önal and Langdon, 2004a,b; Clack, 2006) and this difference could have been due to the use of a dispersants in the leaching experiments.

Inclusion of soy lecithin did not affect leaching rates significantly for any of the investigated LSB types when suspended in 0.1% SDS, but there was an indication that LSB containing particulate glycine and low concentrations of soy lecithin showed reduced losses of glycine. This might have been due to clumping during the leaching trials since LSB prepared with low concentrations of lecithin did not completely disperse in 0.1% SDS.

Earlier studies have shown that LSB containing an aqueous core of both glycine and OTC had lower leakage rates compared to LSB with particulate cores (Buchal and Langdon, 1998; Önal and Langdon, 2004a); however, in this study we found that LSB with an aqueous glycine core had a nearly complete loss of glycine after 20min suspension. It is not clear if this higher loss was due to increased dispersion by the use of 0.1% SDS or due to other factors.

Leaching of OTC from LSB prepared with paraffin wax followed a classic burst release profile, with a rapid loss of 80% incorporated glycine during the first min followed by a slower rate of leaching over a period of an hour. Leakage rates of OTC from LSB prepared with paraffin wax were similar to losses from LSB containing particulate glycine. Loss of OTC was higher than previously reported by Buchal and Langdon (1998), but the leaching profile was similar to that described by Önal and Langdon (2005a). Retention efficiencies for riboflavin-containing LSB were not investigated, but riboflavin has a low aqueous solubility and high concentrations of particulate riboflavin were observed in LSB after dispersion in water for 1h (Fig. 7).

Dispersion in water (without SDS) of LSB containing particulate glycine occurred only with LSB containing 40% soy lecithin. LSB containing 22.7% wax, 16% fish oil and 55% lecithin dispersed well in water and were more stable than LSB containing menhaden stearine. This was probably due to the higher melting point of paraffin wax and high phospholipid concentration.

Beads that can withstand mechanical stress and ambient temperatures during handling and storage would be useful for large-scale production and storage of LSB. Increasing the melting point of the lipid mix of LSB without decreasing the proportion of unsaturated fatty acids by the addition of indigestible waxes needs further investigation. Use of waxes could reduce digestion and increase gut evacuation rates, for example.

Artemia ingested LSB and the digestive tract was completely filled within 30min. This was in accordance

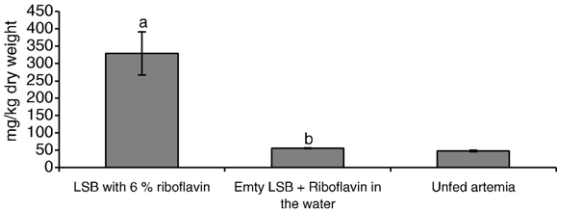

Fig. 9. Concentration of riboflavin (μg g^{-1} dry weight) in *Artemia* either enriched for 1 h with LSB containing riboflavin, fed on a combination of empty LSB and riboflavin dissolved in the culture water or unfed *Artemia*. Data are means of three replicates except for unfed *Artemia* that were tested in duplicate. Error bars represent standard deviations for the two enriched *treatments* and mean±range for the unfed *Artemia*. Treatment means sharing the same letter are not significantly different from each other (Student *t*-test, $P=0.002$).

with previous reports where *Artemia* were fed latex particles (Gelabert Fernandez, 2003). The short enrichment period necessary for complete gut filling, suggests that the commonly used incubation period when enriching *Artemia* may be significantly reduced. However, this may only be the case for nutrients such as vitamins that can be delivered in sufficient amounts through the availably gut volume. Microscopic observations indicated that there were no whole beads present in the digestive tract. Effects of particle size and concentration on ingestion rates of *Artemia* are described by several authors (Reeve, 1963a,b; Gelabert Fernandez, 2001, 2003; Han et al., 2005), but there is not found literature supporting *Artemia*'s capability to masticate particles. It is therefore more likely that the LSB have been ruptured by rapid enzymatic breakdown, but this needs further investigation. There was no significant increase in riboflavin concentration in *Artemia* exposed to riboflavin dissolved in culture water compared with that of unfed controls, indicating that drinking the culture medium was not sufficient to enrich *Artemia* with riboflavin. A concentration of ca 50 mg kg^{-1} riboflavin in unenriched *Artemia* is similar to that reported by Van der Meeren (2003). A seven-fold increase in riboflavin concentration was obtained after feeding *Artemia* on LSB containing particulate riboflavin.

Enrichment with LSB containing 6.4% riboflavin increased the concentration of riboflavin in *Artemia* with 281 mg kg^{-1} (48 to 329 mg kg^{-1}). This was equivalent to *Artemia* ingesting an LSB ration equivalent to 4.4% of total body dry weight, although the actual ingested ration might have been higher because losses of riboflavin from the beads and *Artemia* were not considered or investigated. The lipid content of newly hatched *Artemia* (*A. franciscana*) is 145 mg g^{-1} dry weight (Evjemo et al., 2001) while the lipid content of enriched *Artemia* is reported to be 220–250 mg g^{-1} dw (Evjemo et al., 2001; Van der Meeren, 2003). Therefore, lipid in LSB ingested to increase the riboflavin content with 281 mg kg^{-1} would represent 18 to 30% of the total lipid content of the *Artemia* (dw).

LSB appear to be a good tool for delivering riboflavin to *Artemia*. Even with high leaching losses, LSB retaining 20% of its core material should be sufficient to increase the concentration of most micronutrients in *Artemia*. Delivery of sufficient amounts of nutrients that are needed in larger quantities, such as free amino acids, would be more difficult using LSB types described in this study.

Liposomes have also proved to be a successful tool for enriching *Artemia* with lipid and water-soluble nutrients (Hontoria et al., 1994; Ozkizilcik and Chu, 1994; McEvoy et al., 1996; Tonheim et al., 2000; Monroig et al., 2003, 2006) and with good retention of incorporated water-soluble nutrients (Hontoria et al., 1994; Monroig et al., 2003). Improvement in the proportion of unsaturated fatty acids is possible by preparing liposomes with krill phospholipid extracts (McEvoy et al., 1996; Monroig et al., 2003, 2006); however, an increased proportion of phospholipids led to reduced retention of carboxyfluorescein (Monroig et al., 2003). In contrast to LSB, the core material of liposomes has to be delivered in aqueous solution and the volumetric proportion of liposomes made up of aqueous core solution is usually low (Touraki et al., 1995). The benefits of using LSB rather than liposomes include flexibility in choice of the physical nature of the core material, high core incorporation rates and choice of hydrophobic material (TAG, phospholipid, paraffin wax etc.) for the bead matrix.

In summary, this study demonstrates that LSB containing high concentrations of lecithin can be dispersed easily in water and that addition of lecithin does not affect retention of glycine compared to LSB prepared with triacylglycerol alone. After a high initial loss, 20% of initially incorporated particulate glycine and OTC was retained for up to an h. LSB containing paraffin wax in the hydrophobic matrix were rapidly ingested and digested by *Artemia* and the concentration of riboflavin increased seven-fold within one h of enrichment.

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