Lipids
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COMMUNICATION

Enhanced Bioavailability of Eicosapentaenoic Acid from Fish Oil After Encapsulation Within Plant Spore Exines as Microcapsules

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Abstract Benefits of eicosapentaenoic acid (EPA) can be enhanced by raising their bioavailability through microencapsulation. Pollen can be emptied to form hollow shells, known as exines, and then used to encapsulate material, such as oils in a dry powder form. Six healthy volunteers ingested 4.6 g of fish oil containing 20% EPA in the form of ethyl ester first alone and then as 1:1 microencapsulated powder of exines and fish oil. Serum bioavailability of EPA was measured by area under curve ($AUC_{0–24}$). The mean $AUC_{0–24}$ of EPA from ethyl ester with exine ($M = 19.7$, $SD = 4.3$) was significantly higher than ethyl ester without exines ($M = 2$, $SD = 1.4$, $p < 0.01$).The bioavailability of EPA is enhanced by encapsulation by pollen exines.

Keywords Exines · Microencapsulation · Eicosapentaenoic acid · Bioavailability

Abbreviations

Ar	Argon laser
$AUC_{(0–24)}$	Area under the curve between time 0–24 h
BHT	Butylated hydroxytoluene
C/M	Chloroform methanol
EPA	Eicosapentaeoic acid
FAME	Fatty acid methyl esters
GLC	Gas liquid chromatography
HeNe	Helium neon
LCPUFA	Long chain poly unsaturated fatty acids
M	Mean
SD	Standard Deviation
SEM	Scanning electron microscopy
SPSS	Statistical Package for the Social Sciences

Introduction

Eicosapentaenoic acid (EPA) and docosahexaenoic acid, the main long chain polyunsaturated fatty acids (LCPUFA), can only be obtained from a fish and shellfish rich diet. Recent trials have shown that EPA in the form of ethyl ester added to statins in hypercholesterolaemic Japanese resulted in 19% relative risk reduction in major cardiovascular events [1]. Instead of being taken to prevent nutritional deficiency they are now being taken to prevent diseases with an inflammatory pathology, including cardiovascular diseases [2]. One strategy to raise plasma concentration of LCPUFA is to optimise their absorption and bioavailability.

Microencapsulation has been used to mask unpleasant taste in food sciences as well as to protect against light and airborne oxidation [3, 4]. Pollen and plant spores, from mosses and ferns have an outer layer skeleton known as the exine that is composed of sporopollenin [5, 6]. Exine microencapsulation technology has been shown to provide excellent taste masking for fish oils [7], they have been investigated for use as a contrast agent [8] and attempts have been made to introduce them as a novel method of

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oral delivery of substances into the blood stream as opposed to the parenteral route [9].

In this study we have investigated whether encapsulating the ethyl ester form of fish oil with exine microcapsules extracted from readily available and renewable *Lycopodium clavatum* spores, can enhance the bioavailability, measured by area under the curve, of EPA delivered as ethyl ester alone.

Experimental Procedure

This was an open-labelled study. Six healthy volunteers without concomitant illnesses or medications were recruited from an advertisement for healthy volunteers in Hull University and Hull Royal Infirmary. The study protocol was approved by the Hull and East Riding Research Ethics Committee. All subjects received dietary counselling by an academic dietician to avoid fish or omega-3 fatty acid intake in their diet 2 weeks before and during the course of the trial. Coffee, flax seed and alcohol were avoided a day prior, during and a day after each visit. A run in period of 1 week was followed by two visits with a 3-week between-visits wash-out period. Each subject ingested 4.6 g of fish oil containing 20% of EPA in the form of the ethyl ester at each visit. In the first visit the fish oil was given in the form of a liquid immediately after defrosting. In the second visit the fish oil was encapsulated into exines and the subsequent powder was ingested. Blood samples were taken at baseline (prior to ingesting the fish oil preparations) for fatty acids and lipid analysis and again at 2, 4, 6, 8 and 24 h from ingesting the fish oil for fatty acids analysis. Serum was instantly separated by centrifugation at 2,000*g*, and stored at −80 °C before batch analysis of total serum fatty acid compositions by the Nutrition Group, Institute of Aquaculture, University of Stirling, Stirling UK, as described before [10]. 0.5 mL serum was extracted by the Folch et al. method [11], using chloroform/methanol (C/M; 2:1 vol/vol). The extracted lipid was dissolved in 0.8 mL of C/M, 2:1 vol/vol and dried under nitrogen in a pre-weighed glass vial, and desiccated for 16 h. Final lipid extracts were re-suspended in C/M (2:1 vol/vol) + 0.01% (wt/vol) butylated hydroxytoluene (BHT), at a concentration of 10 mg/mL and stored at −70 °C.

Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of 0.5 mg of total lipid and 50 µg of 17:0 internal standard in 2 mL of 1% (vol/vol) H_2SO_4 in methanol at 50 °C overnight [12]. Samples were neutralised with 2% $KHCO_3$ and extracted twice with 5 mL isohexane/diethyl ether (1:1 vol/vol) + BHT and finally dissolved in 0.3 mL of isohexane prior to FAME analysis.

Measurement of Serum Fatty Acids

FAME were separated and quantified by GLC (Fisons 8160, Carlo Erba, Milan, Italy) using a 60 m × 0.32 mm × 0.25 µm film thickness capillary column (ZB Wax, Phenomenex, Macclesfield, England). Hydrogen was used as carrier gas (flow rate of 4.0 mL/min) and the temperature programme was from 50 to 150 °C at 40 °C/min then to 195 °C at 2 °C/min and finally to 215 °C at 0.5 °C/min. FAME were identified using well characterised in house standards and commercial FAME mixtures (Supelco™ 37 FAME mix, Sigma-Aldrich Ltd., Gillingham, England). Blood was withdrawn after 30 min and examined under a confocal microscope to investigate for the presence of exines, which are naturally fluorescent.

Fish oil supplements were provided by Croda Europe, Goole, UK. Each vial had 4.6 g of fish oil containing 20% EPA in the form of its ethyl ester. They were shipped in dark containers and kept in a −20 °C freezer until ready for defrosting at visit 1. At visit 2, the defrosted oil was encapsulated with 4.6 g of exines no more than 24 h prior to ingestion and the dark container was filled with nitrogen to prevent oxidation. Exines extracted from *Lycopodium clavatum* spores were supplied by Sporomex Ltd, UK, and were prepared as detailed previously [7]. Microencapsulation was performed by mixing exines with oil (1:1 weight for weight) by gently stirring to form a homogeneous paste that was then subjected to a vacuum (ca. 10 hPa) for 2 h to facilitate passive loading of oil into the particles through the nano-porous sporopollenin walls.

Area under the curve ($AUC_{0-24\ h}$) was used to determine the bioavailability of EPA from the different supplements. The mean $AUC_{0-24\ h}$ for EPA was calculated using the linear trapezoid method and baseline levels were normalised to zero. We also observed visually the time of the maximum concentration (T_{max}). Comparisons of mean AUCs and T_{max} with and without exines were made using paired sample *t* test via SPSS version 15.

Results

The two male and four females' demographics are summarised in Table 1. The mean baseline of EPA percentage to total fatty acids in the six subjects was comparable to that reported in another study with healthy volunteers; $M = 0.69$, SEM = 0.04% versus $M = 0.64$, SEM = 0.08%, respectively [13]. There was no significant difference between the baseline concentration of EPA (mg/100 mL) in the first visit ($M = 2.15$, SD = 0.6) and the second visit ($M = 2.0$, SD = 0.6, $p = 0.49$). The mean AUC of EPA from ethyl ester with exine ($M = 19.7$, SD = 4.3) was significantly higher than that obtained from ethyl ester

Table 1 Subjects' demographics

Demographics	Mean (SD)
Systolic blood pressure (mm Hg)	131 (7)
Diastolic blood pressure (mm Hg)	78 (4)
Total cholesterol (mmol/L)	4.7 (0.45)
Triglyceride (mmol/L)	1.06 (0.24)
High density lipoprotein (mmol/L)	1.25 (0.35)
Low density lipoprotein (mmol/L)	2.7 (0.84)
Total cholesterol/high density lipoprotein	4 (1.21)
Body mass index (Kg/m^2)	23.5 (2.2)

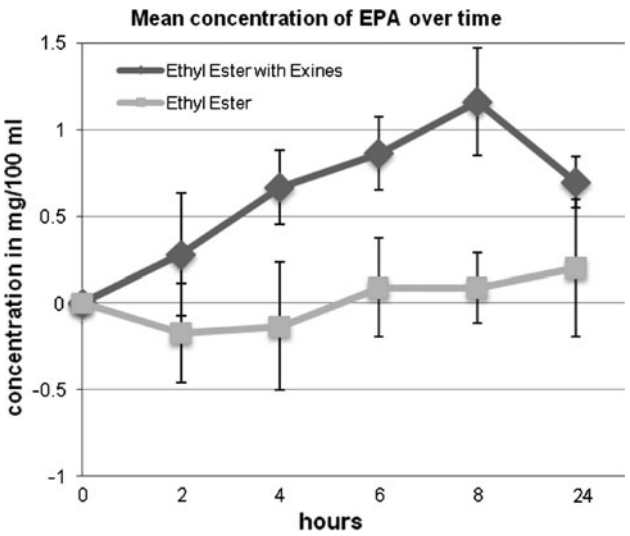

Fig. 1 The change in mean EPA serum level over time obtained from the ethyl ester of EPA with and without exines

without exines ($M = 2$, SD $= 1.4$, $p < 0.01$). When the mean concentration of EPA in serum over time was plotted, after subtracting the mean sera concentrations of the respective time from the mean baseline concentration, it was evident that microencapsulation in exines had significantly enhanced the EPA absorption as reflected by the serum concentration (Fig. 1). The mean time of maximum (T_{max}) concentration for EPA when fish oil was encapsulated with exines ($M = 7.6$ h) was not different from the maximum concentration without exines ($M = 6.8, p = 0.4$), results not shown. Confocal microscopy (Bio-Rad Radiance 2100 laser scanning microscope equipped with Ar (488 nm), Green HeNe (563 nm) and Red diode (637 nm) laser lines connected to a Nikon TE-2000E inverted microscope) showing an empty fluorescent exine before ingestion and an apparently intact exine in blood plasma after ingestion (Fig. 2). Micrographs of oil filled exines before ingestion and those recovered from blood, following ingestion, were also obtained using a Leica Cambridge Stereoscan 360 scanning electron microscope (SEM) operated by Tony Sinclair, Institute of Chemistry for Industry, University of Hull (Fig. 3).

Discussion

In this study, there was a significant rise in the bioavailability of EPA as measured by $AUC_{0-24\ h}$ when the ethyl ester form of fish oil was encapsulated into the novel exine microcapsules, which has not been reported before. Previous studies have focussed on the encapsulation of fish oil to preserve its qualities and prevent oxidation [4], rather than to enhance bioavailability. Although there are no previous studies on the effect of the bioavailability of EPA encapsulated into exines, encapsulation technology is commonly used in pharmaceutical preparations to improve bioavailability. For example, the use of a mixture of wax and fat has been used to achieve controlled drug release in the circulation [14] while the use of microspheres to produce mucoadhesive polymers can help maintain intimate contact with the mucosa of the gastrointestinal tract thereby achieving improved bioavailability [15]. Exines have been used as a natural substance to mask-taste but this is the first pilot study to investigate its potential use to improve bioavailability of orally ingested fish oil in the ethyl ester form

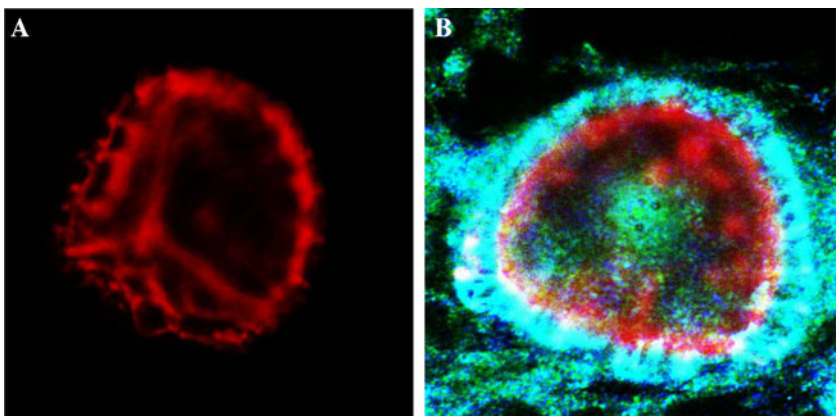

Fig. 2 **a** Confocal microscopy (×60) of an empty sporopollenin exine (*red*) showing its architecture and fluorescence. **b** An exine (*red*) previously filled with fish oil, found in the blood, 30 min after ingestion showing accumulation of material on the outside (*blue*)

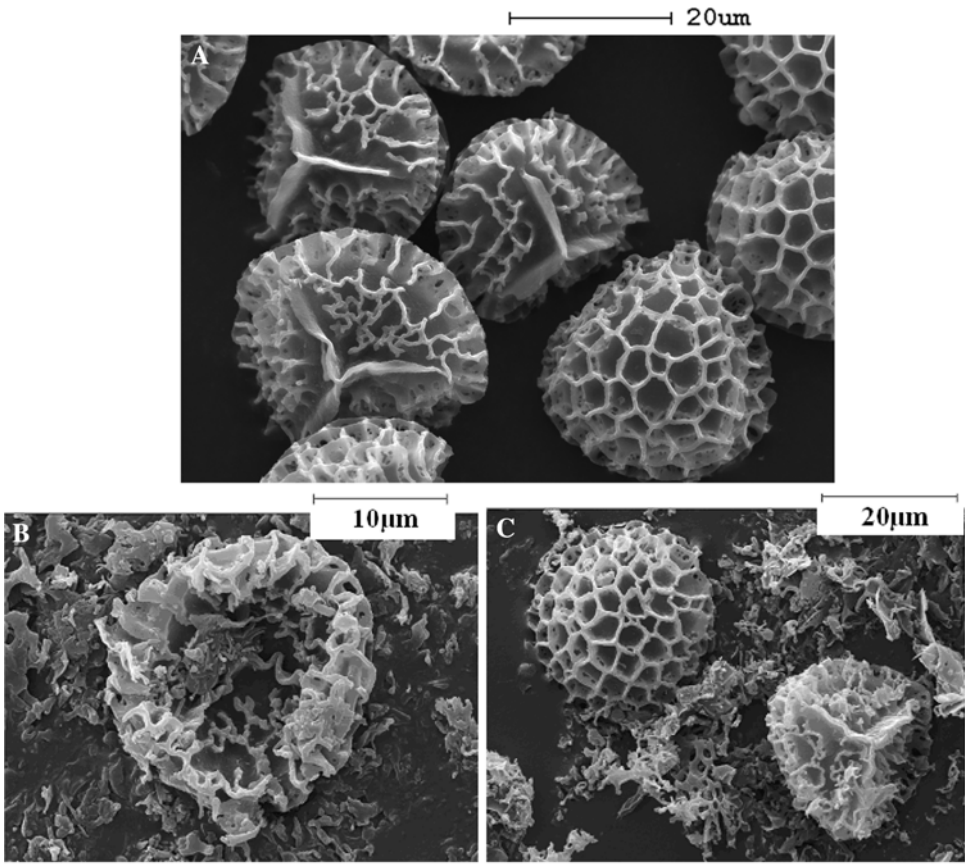

Fig. 3 SEM images of exines (25 µm) from *L. clavatum* spores prior to ingestion, filled with oil (**a**) and those recovered in vivo 30 min after ingestion (**b**, **c**)

[7]. The mechanism by which exine microencapsulation can enhance oil absorption is unclear, but might be due to the protective structure of fish oil-enriched exines whereby the whole unit could travel unhindered through the mucosal lining without releasing its inner core until it has entered into the circulation. This increase in bioavailability was independent of the T_{max} that is a measure of the time to achieve the maximum concentration, suggesting that exines may enhance the absorption at the early stages and continue to do so throughout the 24 h period, in contrast to a natural slower pace of absorption of EPA in the early period of supplementation.

Whilst it is difficult to cost this method, it is expected there would be no significant extra cost compared with other microencapsulation processes; however, no other technique has the advantage of anti-oxidant properties giving a long shelf-life, or has been shown to taste mask and also to have a relatively high loading level. The preparation of the exines is simple and inexpensive with the total cost of the microencapsulation within the exines being less with readily available pollens such as that for rye or maize.

The major limitation to this pilot study is the small number of participants. However, as a proof of the hypothesis, our results were highly significant and further in vitro and in vivo studies are warranted to explain this phenomenon.

In summary, this study showed that exines obtained from *Lycopodium clavatum* spores encapsulating fish oil in the ethyl ester are associated with an improvement in LCPUFA bioavailability as measured by the $AUC_{0-24\ h}$, that may be due to the oil being transported into the blood stream more efficiently by the intact exines.

Conflict of interest statement None.

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