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Preparation of polymeric microbubbles: formulation studies and product characterisation

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Abstract

Gas filled particles are efficient ultrasound reflectors and are therefore suitable contrast agents for investigations of vital organs such as heart, liver and kidney. In the current study, air filled polymeric microbubbles were made by freeze drying emulsions of a polymer/(−)-camphene solution in a human serum albumin/water phase. An emulsion with low viscosity and a droplet volume median diameter of typically 5–6 μm, and a relatively narrow droplet size distribution, was produced by high shear homogenisation of the two phases. The emulsion flocculated rapidly and at room temperature, the organic phase solidified. The emulsions were therefore quenched immediately after preparation. Upon freeze drying, (−)-camphene sublimed and the droplets shrunk slightly to form hollow, approximately spherical, microbubbles with volume median diameters of typically 4–5 μm and well-defined walls, typically 150–200 nm thick. The suspensions from the emulsions with 2.5% w/v polymer in (−)-camphene gave the highest yield of microbubbles when related to the polymer and the most efficient microbubbles. © 1997 Elsevier Science B.V.

Keywords: Microbubbles; Polymer; Ultrasound contrast agent; Formulation studies; Physico-chemical characterisation

1. Introduction

Ultrasonography is a useful medical imaging technique for examining different human organs such as heart, liver and kidney. By use of grey scale and Doppler techniques valuable information about the clinical state of these organs can be obtained (Schlief et al., 1993; Shapiro et al., 1993). The principle mechanism of conventional, ultrasound imaging the detection of backscattered ultrasound radiation from objects (i.e. tissue, bone and blood) in the incident sound beam. An effective ultrasound contrast agent should enhance this

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backscatter, an effect seen for example after injecting liquids as saline and indocyanine green (Gramiak and Shah, 1968; Feigenbaum et al., 1970) into the aorta and heart, due to the gas bubbles formed.

Free gas bubbles are the most efficient ultrasound reflectors due to their low density and high elasticity (Morse and Ingard, 1968). However, as free bubbles are unstable and their size is difficult to control, such systems are not applicable for clinical use due to safety and efficacy problems. In a more ideal system, the gas should be encapsulated to control the size of the microbubbles and increase their stability in vitro and in vivo. The size of such bubbles should preferably be large due to the increased efficacy of large bubbles (Morse and Ingard, 1968; de Jong and Hoff, 1993), but smaller than approximately 10 μm to ensure capillary passage and thereby avoiding safety problems (Meltzer et al., 1980; Nanda, 1993).

Micro encapsulation of air in a shell of albumin is achieved in the ultrasound contrast agent Albunex™ (Feinstein et al., 1988). These microbubbles are in size 1–15 μm with a shell thickness of only some 15 nm, and are prepared by sonication of a 5% human serum albumin solution (Christiansen et al., 1994). In spite of effective encapsulation of air and excellent in vitro stability, these microspheres are unstable in vivo, and do not permit efficient visualisation of the left heart and myocardium. More stable microbubbles can be prepared by dissolving a film-forming polymer in a core which can be sublimed and subsequently removing the core as vapour (Farnand and Puddington, 1979; Schneider et al., 1991; 1992). For parenteral uses the film forming polymer should be biodegradable to enable elimination of the microbubbles from the body.

In the present study, polymeric microbubbles were prepared by freeze drying emulsions of a polymer/(−)-camphene solution in a human serum albumin/water phase. The aim of these studies was to investigate the stability of the emulsion, determine the limits for some formulation parameters and to perform physico-chemical characterisation of the product.

2. Materials and methods

2.1. Materials

A biodegradable double-ester polymer with ethylidene units developed at the Exploratory Department at Nycomed Imaging AS, was used (Patent no.: WO 96/07434). The ethylidene units are highly degradable by common esterase enzymes, but are relatively stable in the absence of enzymes (Patent no.: WO 96/07434). The polymer was synthesised by the Department of Process Chemistry, Nycomed Imaging AS, Norway and had a molecular weight, M_w of approximately 19 000 Da and a polydispersity, M_w/M_n of 1.96. Human serum albumin (HSA) 5% solution was purchased from the Swiss Red Cross, Switzerland. (−)-Camphene (m.p. 52°C) was purchased from Fluka Chemie, Switzerland and the 0.9 mg/ml NaCl solution from Kabi Pharmacia, Sweden.

2.2. Production methods

The investigated substances were produced by a process developed at the Exploratory Department at Nycomed Imaging AS. An oil in water emulsion was prepared by using 60 ml 5% HSA and 20 ml of a polymer/(−)-camphene solution. The concentration of the polymer in the (−)-camphene varied from 0.5 to 7.5% (w/v).

The 5% w/v HSA solution was heated to 60°C prior to emulsification. The polymer/(−)-camphene solution was prepared by addition of the polymer to the melted (−)-camphene followed by stirring at 60–70°C. Emulsions (80 ml) were prepared by high shear stirring with a Ystral Rotor Stator T1500 (Ystral, Germany) in round bottom flasks with heating jackets. The emulsions were homogenised at high speed for 1 min at 60°C.

The emulsion (5 ml) was immediately filled after homogenisation into 20 ml vials and quenched in dry ice/methanol for approximately 10 min, before transferring to a Virtis Genesis Freeze Drier (Virtis, NY) with a shelf temperature at −40°C. The nominal freeze drying cycle is described in Table 1.

To investigate the stability some emulsion aliquots were transferred to glass vials with screw-on stoppers and stored at 60°C.

Microbubble suspensions were prepared by additions of 10 ml 0.9 mg/ml NaCl to 100 mg of the freeze dried substance in 20 ml glass vials with rubber stoppers. The vials were shaken at 250 rpm over night prior to analysis.

2.3. Characterisation of the oil in water emulsion

2.3.1. Droplet concentration and size distribution by Coulter counting

The droplet concentration and size distribution of the emulsion were measured using a Coulter Multisizer Mrk II E-model (Coulter Electronics, UK) fitted with a 50 μm aperture with a measuring range of 1.0–33.1 μm. The analytical procedure and method performance is detailed elsewhere (Sontum et al., 1997). Isoton II (Coulter Electronics, UK) at 27.0 ± 0.5°C was used as electrolyte. The volume concentration and the volume median diameter were used as response parameters and the precision in these parameters was typically within $\pm 3\%$ (R.S.D.). All emulsion samples were investigated by Coulter counting when freshly prepared and after storage for 24 h at 60°C. Samples were carefully homogenised immediately prior to analysis by gentle manual agitation.

2.3.2. Viscosity

The viscosities of the emulsions were measured at Bohlin VOR Rheometer (Bohlin AB, Sweden) in a double gap system (DG 24/27) and with torque element 1.637 g cm at 60°C. The shear rates used were in the range 15–367 1/s, strain delay time 45 s, integration time 10 s and measurement interval 10 s. The samples were preheated in a water bath to 60°C and gently shaken before applied to the measuring system. Evaporation from the sample was reduced by use of an isolating humidity enclosure and the measurement was completed within 7 min.

Table 1
The nominal freeze drying cycle

Freezing	−40°C for 1.5 h
Primary drying	−30°C for 24 h. The temperature was then gradually raised to +10°C during 7.5 h
Secondary drying	+30°C for 10 h
Pressure	The pressure varied from 0.15 to 0.30 mbar

2.3.3. Light microscopy

The emulsion was gently shaken before one drop was applied to the microscope slide. A cover glass was carefully used to cover the sample before investigating the sample with a $\times 40$ objective in a Nikon Optiphot light microscope (Nikon, Japan).

2.4. Characterisation of the microbubble suspensions

2.4.1. Microbubble concentration and size distribution by Coulter counting

The particle concentration and size distribution of the microbubble suspension samples were measured with a Coulter Multisizer Mrk II E-model as described under Section 2.3. This method and its precision and accuracy when applied to other suspensions of air filled microbubbles is described in full detail elsewhere (Sontum and Christiansen, 1994).

2.4.2. Air content by densitometry

The content of air encapsulated within the microbubbles in the suspension samples was measured by oscillation U-tube densitometry with a DMA-58 (Anton Paar, Austria). The instrument was calibrated with air and purified water prior to use. The density of the suspension was measured before and after elimination of the encapsulated air. The complete removal of encapsulated of air was achieved by 5 min high powered sonication with an XL-2015 sonicator (Heat Systems, USA). The air content was calculated as

$$C_{\text{air}} = ((\rho_1 - \rho_2)/\rho_2) \cdot 100$$

where C_{air} is air content (% (v/v)) and ρ_1 (g/ml) and ρ_2 (g/ml) are the densities of the suspension before and after elimination of encapsulated air.

2.4.3. Echogenic properties by ultra sound attenuation measurements

The echogenic properties of the suspension samples were characterised by using a slightly modified version of a previously described method (de Jong and Hoff, 1993). In the current set up the acoustic attenuation (dB/cm) of a sound beam going through a diluted suspension of the sample was measured. Two transducers with centre-frequencies of 3.5 and 5 MHz, with a combined measuring range of approximately 2–8 MHz were utilised. A sample volume of 20 μl was diluted and homogenised in 55 ml Isoton II, equilibrated to 27°C prior to measurement.

2.4.4. Transmission electron microscopy (TEM)

TEM was performed on a Jeol JEM 100CX microscope (Jeol, USA). The sample was casted in a gelatin/sucrose mix and cryo-sectioned at − 93°C in a microtome set at 90 nm section thickness. Sections were pictured at magnifications from 10 to 20.000 ×.

2.4.5. Light microscopy

The suspension was manually shaken before one drop was applied to the microscope slide. A cover glass was carefully used to cover the sample before investigating the sample in a × 40 objective in a Nikon Optiphot light microscope (Nikon, Japan). Pictures were taken with a Hitachii V4 150 E video printer (Hitachii, Japan).

3. Results and discussion

3.1. Emulsion

The viscosities of the emulsions are shown in Fig. 1. All the emulsions had low viscosities. The emulsions with 0.5% polymer showed Newtonian behaviour with viscosities of approximately 1.5 mPa·s. Rest of the emulsions were slightly shear thinning with viscosities in the range 1.6–3.2 mPa·s, depending on the shear rate used.

When investigated visually during storage, creaming of the droplets occurred after few minutes. The top layer consisted of a white creamy emulsion and the bottom layer was a greyish liquid. Upon gently shaking the emulsion was recaptured. When investigating different samples in a microscope, an increase in droplets flocculating and coalescing with time after preparation could be seen.

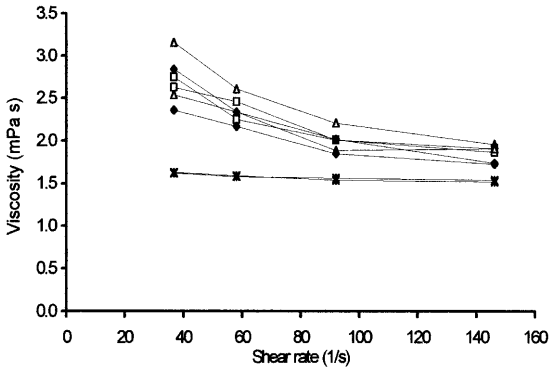

Fig. 1. Viscosity (mPa·s) of the emulsions with various polymer concentrations in (−)-camphene. 7.5%, (□); 5%, (△); 2.5%, (◇); 0.5%, (∗).

The volume median diameter of the droplets in freshly prepared emulsions varied between 5.1 and 5.8 μm, as seen from Fig. 2. After 24 h, the droplet size typically increased between 3 and 14%, typically as visualised in Fig. 3. Some big droplets and aggregates were seen in the emulsion

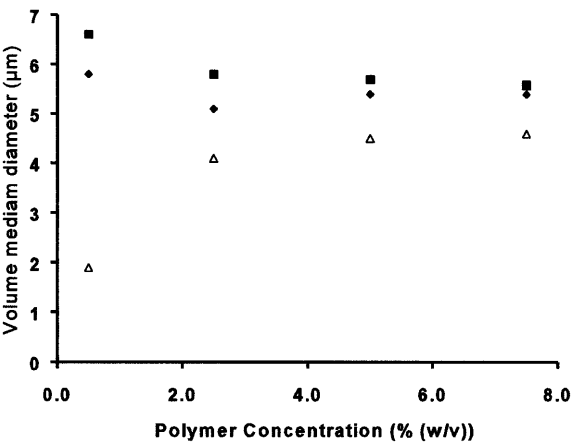

Fig. 2. Volume median diameters of droplets and microbubbles in emulsions with various polymer concentrations in (−)-camphene and corresponding suspensions. Freshly prepared emulsion, (◆); emulsion after 24 h at 60°C, (■); suspension, (△).

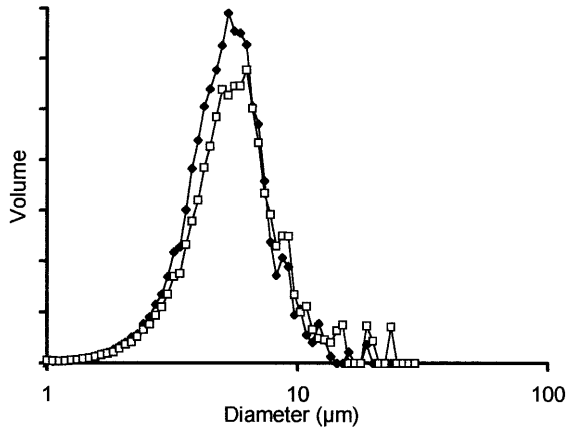

Fig. 3. Volume distribution of droplets in freshly prepared emulsion (◆) and emulsion after 24 h at 60°C (□) (5.0% polymer in (−)-camphene).

after 24 h, when investigated in the microscope. Most of these were >30 μm and thus outside the measuring range of the Coulter analysis. As visualised in Fig. 4, the measured droplet volume concentrations in freshly prepared emulsions were 80–88% of the theoretical value. This deviation is probably due to evaporation of the (−)-camphene during the production of the emulsions or to droplet populations outside the measuring range of the Coulter analysis. After 24 h the measured droplet concentrations decreased to 56–72% of the theoretical value. This drop in concentration is consistent with the visual observation of

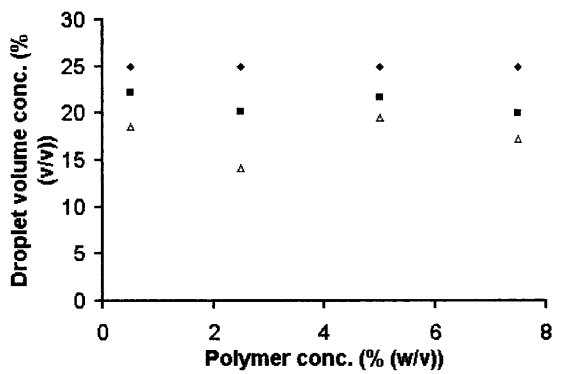

Fig. 4. Volume concentrations of droplets with various polymer concentration in (−)-camphene. Theoretical concentration (◆), freshly prepared emulsion (■) and emulsion after 24 h at 60°C (△).

an increase in the amount of large droplets.

When the emulsions were stored at room temperature the (−)-camphene solidified and the dispersions were converted into suspensions, which were quickly destroyed when the vials were placed on a roller table at room temperature.

3.2. Microbubble suspensions

The possible mechanisms behind the formation of the final microbubble are non trivial. If the entire droplet volume survived the freeze drying process encapsulated by polymer, the theoretical microbubble volume in the suspension samples would be approximately 10% (v/v). If, on the other hand, the 'frozen droplets' were able to shrink as the (−)-camphene sublimated, the final volume concentration would decrease from this value. The size of the microbubbles would then be smaller than the size of the corresponding droplets. It is also possible that some of the microbubbles collapse to form non air filled material. In addition, part of the oil phase may evaporate during the emulsification process and may never reach the freeze drying step.

The results from the characterisation of the suspension samples are summarised in Table 2. The volume distributions of the four suspensions are shown in Fig. 5 and their volume median diameters are compared with the respective droplet diameters in Fig. 2. As seen from these results, the microbubble sizes in all suspensions have decreased from the initial droplet size and the maximum detected volume concentration is 2.1%. The microbubbles in the suspension from the lowest polymer concentration appear to have collapsed completely with almost no encapsulated air and negligible acoustic efficacy. The reduction in size for the remaining samples was in the range 20–30% and the shift in the volume distribution is visualised for the 2.5% polymer sample in Fig. 6. From the cubic dependency of the particle volume on the diameter, such a decrease would correspond to a 50–60% decrease in volume and thus explain most of the deviation from the theoretical concentration. The remaining loss is probably attributed to evaporation during emulsification and collapse of microbubbles during freeze drying.

Table 2
Characteristics of the microbubble suspensions

Polymer in (−)-camphene (% w/v)	Particle volume concentration (% v/v)	Particle volume median diameter (μm)	Attenuation 3.5 Hz (dB/cm)	Encapsulated air (% v/v)
0.5	0.1	1.9	0.04	0.005
2.5	1.6	4.1	8.6	1.14
5.0	2.1	4.5	8.2	1.50
7.5	2.1	4.6	4.7	1.47

The suspension investigated in the light microscope contained globular and raisin-shaped particles in the range 0.5–10 μm, as shown in Fig. 7. In addition some aggregates in the range 10–100 μm could be seen in the samples.

To characterise the microscopic structure of the produced microbubbles, a cryo-TEM technique was applied on suspension samples from the 5% polymer formulation. As seen from Fig. 8, these microbubbles were hollow with well-defined walls, typically 150–200 nm thick, surrounding a void. Calculations of the theoretical average shell thickness, based on the particle size and the measured particle volume concentration and encapsulated air, yields 200 nm. Thus, these measurements are in good agreement with the TEM results.

The yield, measured as particle volume or encapsulated air volume per polymer amount is visualised in Fig. 9. As seen from this figure, the yield increases strongly with decreasing polymer concentration in the oil phase until the concentration becomes too low and the microbubbles collapse. Comparing the 7.5% polymer sample to the 2.5% polymer sample, the increase in encapsulated air per polymer amount is 133%. These observations would indicate that the average shell thickness of the microbubbles decreases with decreasing polymer concentration, until a critical thickness is reached which no longer supports the structure, resulting in the collapse of the microbubble.

A thinner wall structure should increase the average elasticity of the microbubble and lead to an increase in the attenuation efficacy. This parameter, measured as attenuation per polymer amount at 3.5 MHz, is plotted in Fig. 10. As seen from this figure, the efficacy increases as much as 3.3 times, when comparing the 7.5% polymer sam-

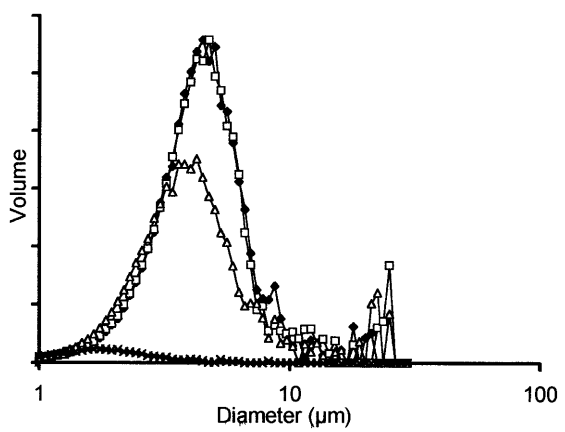

Fig. 5. Volume distributions of microbubbles in suspension prepared from emulsions with various polymer concentrations in (−)-camphene. 7.5%, (◆); 5.0%, (□); 2.5%, (△); 0.5%, (×).

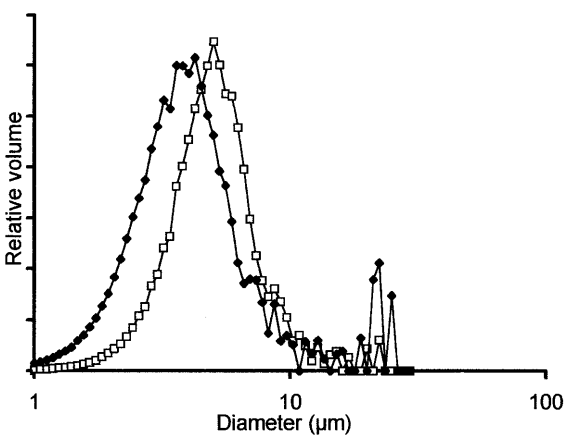

Fig. 6. Volume distributions of droplets and microbubbles in emulsion (□) and corresponding suspension (◆) (2.5% polymer in (−)-camphene).

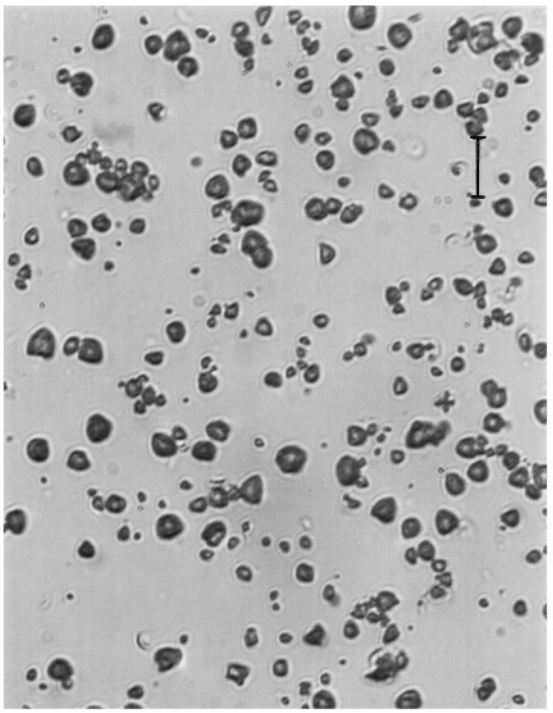

Fig. 7. Suspension visualised by light microscopy (5% polymer in (−)-camphene). Size bar equals 10 μm.

ple with the 2.5% polymer sample. Thus, the attenuation efficacy per particle volume increases strongly with decreasing polymer concentration.

4. Conclusion

An oil/water emulsion, with a volume median diameter of typically 5–6 μm and a relatively narrow droplet size distribution, has been successfully produced by high shear homogenisation of polymer/(−)-camphene in HSA/water. The emulsions had low viscosities of <4 mPa·s at 60°C, and creamed rapidly at standstill. Upon gentle rehomogenisation the original emulsion was essentially recaptured, even after 24 h. The (−)-camphene solidified and the emulsion degraded rapidly due to amalgamation of the semisolid particles, when the emulsions were kept at room temperature. Therefore, the emulsion should be kept above the melting point of (−)-camphene

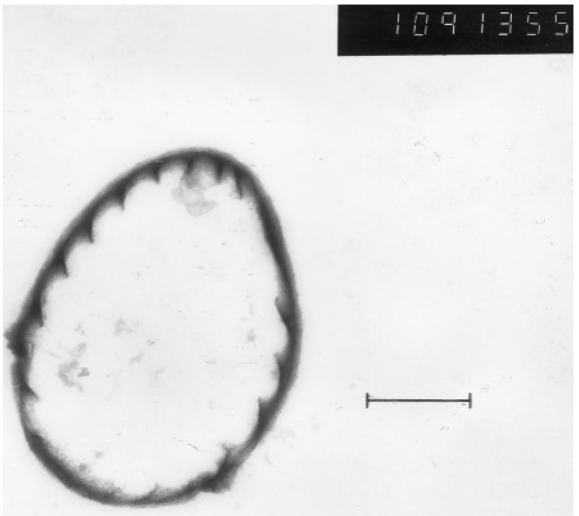

Fig. 8. Micrograph from cryo-TEM on microbubbles (5% polymer in (−)-camphene) in suspension. Particle cross section. Size bar equals 1 μm.

and be quenched immediately after preparation, without precooling.

Upon freeze drying the (−)-camphene sublimed and the droplets shrunk slightly to form hollow, approximately spherical microbubbles with a volume median diameter of typically 4–7 μm and well-defined walls, typically 150–200 nm thick. By varying the concentration of the polymer in the oil phase, both the yield and the efficacy of the produced substance changed significantly. Notably, going from 7.5 to 2.5% poly-

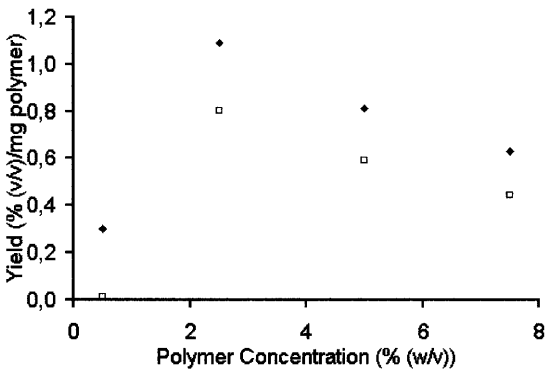

Fig. 9. Yield: microbubble volume (◆) and encapsulated air (□) per mg polymer versus polymer concentration in emulsion.

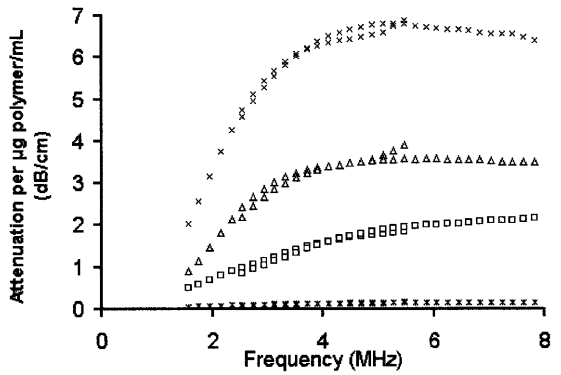

Fig. 10. Efficacy: attenuation efficacy per mg polymer versus frequency. Polymer concentration in (−)-camphene. 7.5%, (□); 5.0%, (△); 2.5%, (×); 0.5%, (∗).

mer, the attenuation efficacy/mg polymer increased more than 3.3 times. However, 0.5% polymer in the oil phase was too low to produce stable structures and gave a product with negligible echogenicity.

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