Determination of Air Content in Protein Microspheres

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INTRODUCTION

Medical imaging agents based on suspended particles with a size distribution in the nanometer and micrometer range are now reaching the market. These products are manufactured from a large variety of starting materials and by many different production techniques including spray drying, emulsion polymerization, emulsion or suspension cross linking, ultrasound, rotorstator, mixers, microfluidizers and solvent evaporation. A suspension of albumin microspheres is now used as a contrast agent for ultrasonographic diagnostic imaging (1). The microspheres are prepared by sonication of a heated solution of 5% (weight/volume) human serum albumin (HSA). During the sonication process, microbubbles of air are formed which become encapsulated in a thin shell of aggregated albumin. The particle size distribution satisfies a Chi-square distribution with mean diameters of a few micrometers (4 μm) with a shell thickness about 15 nm. The physico-chemical and ultrasonic properties of the air-filled microspheres have been described in literature (1,2,3,4,5).

The production line of the albumin microspheres has been outlined previously (6). Among the essential process steps to be mentioned explicitly are the formation of microspheres in the sonication reactor and the selection of the biologically active microspheres in the size fractionation unit (7). In the study reported here the microsphere distribution in the final product has been correlated to product density which is proportional to the air content (active ingredient). The aim of the correlation study was to define the fraction of microspheres that are truly air-filled as a function of the microsphere size. Non-air filled particles will be non-efficacious whereas the size dependent encapsulated air (yield) will influence the diagnostic efficacy of the contrast agent (8,9). The applicability of Coulter counter as an in process control for air-content in the product has been evaluated.

MATERIALS AND METHODS

Three vials of albumin microspheres suspension from each of 13 different technical production batches, produced by Nycomed Imaging AS, Norway, were investigated. Each vial was analyzed in triplicate by Coulter counting to measure microsphere concentration and size distribution, and was analyzed by gravimetry for determination of total encapsulated air volume, by measuring the decrease in density of the suspension after generation of air-filled microspheres. The applied analytical methods will be described in brief in this paper. The performance of the Coulter counting has been described in detail by Sontum and Christiansen (10).

Air Content

The total volume concentration of air-filled microspheres was calculated as follows:

$$V_g = \left(1 - \frac{\rho_{Suspension}}{\rho_{5\%HSA}}\right)100 \quad (1)$$

where $\rho_{Suspension}$ is the density of the suspension (i.e. microspheres in human albumin solution (HSA) 5%) and $\rho_{5\%\ HSA}$ is the density of 5% HSA. The analysis was performed by weighing 500 μl of sample with a Mettler AT 261 balance (Mettler-Toledo A.G., Switzerland) and calculating the density of albumin microspheres (g/ml). Each analysis was performed in triplicate. The density of the human albumin solution 5% was set equal to 1.017 g/ml, as given by the manufacturer (Swiss Red Cross, Switzerland).

Microsphere Volume Distribution

The concentration and size distribution of microspheres in the test samples were analyzed by a Coulter Multisizer Mark II instrument (Coulter Electronics Ltd., Luton, UK.). The instrument was routinely fitted with an aperture of 50 μm employing 64 size channels, giving a nominal particle measuring range from 1 to 38 μm. Analysis was performed by adding 20 μl of a suspension of albumin microspheres in 200 ml Isoton II[4] in a measuring beaker and counting the resulting suspension immediately after mixing using a siphon volume of 500 μl. The volume distribution of the product was calculated from the measured number frequency distribution. By assuming that all measured particles are microspheres containing air at ambient pressure and assuming that the shell of protein has negligible volume (the shell thickness is 15 nm (2)), the volume fraction of air in the product can be calculated by equation 2:

$$V_c = 100 \sum_{i=1}^{i=64} \frac{\pi d_i^3 N_i}{6V_s} \quad (2)$$

where d is the mean diameter for a sub-region in the size distribution, N is the number of particles in this region and V_s the measuring volume used.

Preparation of Infranatants

To evaluate the possible content of solid particles (precipitated protein) in the product, the population of air filled microspheres was eliminated by selectively removing the top layer

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[4] Isoton is a particle-free phosphate-buffered saline with an added dispersant; Coulter Electronics Ltd., Luton, UK.

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of fully segregated samples. Samples from several batches were allowed to segregate by undisturbed flotation for several days. The vials were then opened and the creamy top layer of air filled microspheres was completely removed by careful vacuum suction through a Burker pipette. After removal of the air filled fraction, the remaining sample was homogenized and analyzed by Coulter Counting.

Statistical Calculations

The statistical calculations were performed using Unscrambler version 6.11 (Camo A/S, Trondheim, Norway).

RESULTS

A selection of the size distributions used in the statistical modeling is visualized in Fig. 1. The analytical precision of the Coulter counter method is expressed as a relative standard deviation (RSD) was 1–2% for number concentrations between 4 and 25 μm and 26% for microspheres above 25 μm (10). The relatively large RSD in the >25 μm range is related to the low number of microspheres measured.

The Coulter counter method and the gravimetric method were applied on samples originating from the same vials. A systematic correlation error occurred between the two methods described by the estimated slope derived from univariate linear least square regression, see Fig. 2 and Table 1. If all microspheres are air-filled, the air volume measured gravimetrically should be either equal to the air volume estimated by the Coulter method, or somewhat higher when considering the limited size range of the Coulter method.

The statistical treatment of size distributions is often made difficult by small variations in size and extensive size covariance due to the nature of how such products are manufactured. Typically, traditional correlation methods such as product moment correlation analysis and ordinary least square (OLS) regression often fail. To explain the observed discrepancy between the two analytical methods and to describe and minimize the systematic error apparent in the univariate regression, principal component regression (PCR) analysis was used. The size dependent air contribution is shown by the regression coefficients in Fig. 3. The correlation between predicted air volume by Colter Counter against gravimetric measuring method is shown in Fig. 4. The statistical parameters describing the performance of multivariate PCR and univariate OLS for Coulter counter versus gravimetric measuring method is given

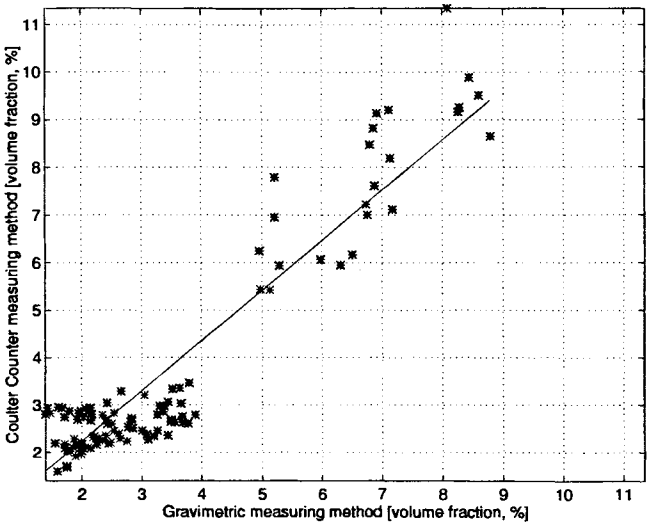

Fig. 2. The correlation between gravimetric and Coulter Counter measuring methods.

Table 1. Statistical Information About the Different Models

Model	Mean error	Regression slope	Regression intercept	Correlation coefficient
Univariate	0.61	1.06	0.00	0.92
Multivariate	0.65	1.00	0.00	0.96

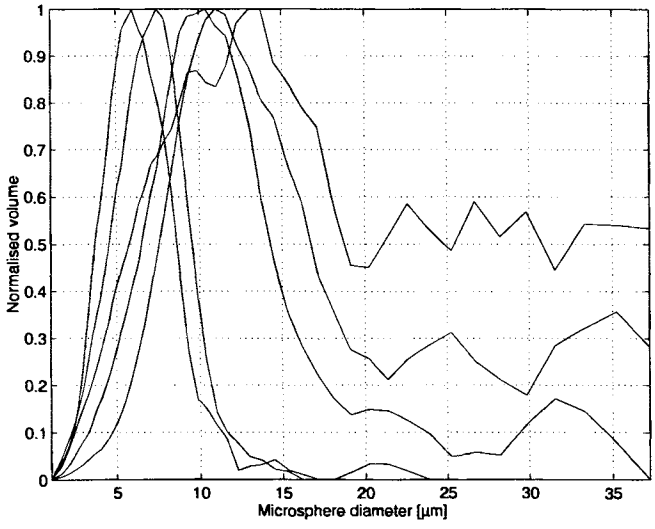

Fig. 1. A selection of volume distributions (normalized) measured by Coulter counter.

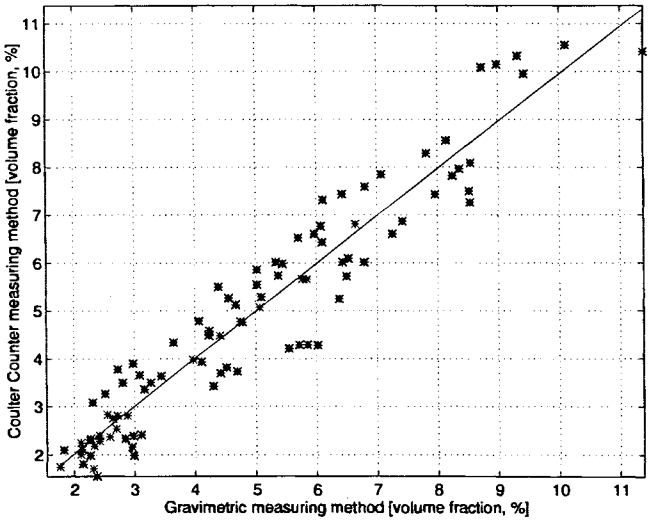

Fig. 3. The regression coefficient from the correlation between gravimetric and Coulter measuring method using a PCR model with three principal components.

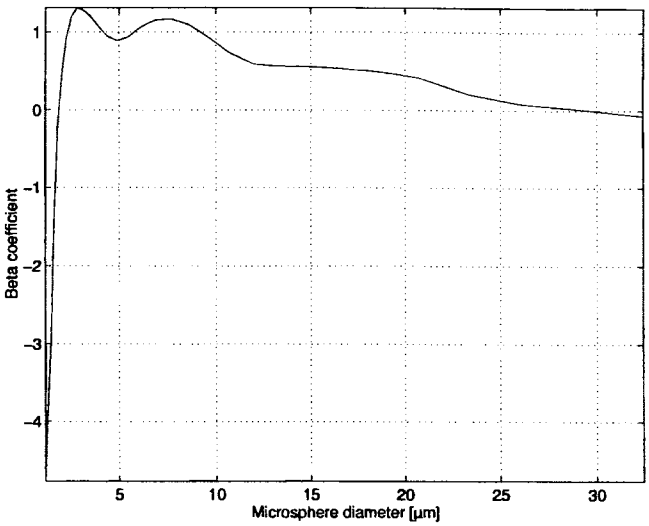

Fig. 4. The correlation between gravimetric and Coulter measuring method using a PCR model with three principal components.

in Table 1. Three principal components were included in the multivariate regression model, as suggested by full cross validation, i.e., leaving-one-sample-out (8,11). The volume fraction was calculated by using equation 3:

$$V_{PCR} = 100 \sum_{i=1}^{i=32} \frac{\pi \bar{d}_i^3 \bar{N}_i}{6V_s} \beta_i \qquad (3)$$

where d is the mean diameter for a sub-region in the size distribution, N is the number of particles in this region, V_s the measuring volume used and β_i. is the corresponding regression coefficients for the given size channel. If equation 1 and 2 are assumed to be equal (i.e., $V_g = V_c$) then the regression coefficients in equation 3 should be equal to unity for all sizes. The regression coefficients explain the size dependent deviation between the two measuring methods represented by equation 1 and 3. As apparent from Fig. 3, the regression coefficients are not equal to unity and the assumptions stated above must thus be wrong. The encapsulated content of air was found to be ≈50%, ≈100%, ≈50%, ≈25% and ≈0% for microspheres between 1–2 µm, 2–10 µm, 10–20 µm, 20–25 µm and 25–38 µm, respectively. With reference to these results one may conclude that the Coulter counter method overestimate the encapsulated air content of particles below 2 µm and above 10 µm. The variation in the regression coefficients between 2 to 10 µm is probably caused by noise and poor size resolution.

DISCUSSION

The result shows that the assumptions that all microspheres detected by Coulter counter are air filled is not entirely correct. Pressure changes larger than approximately 27 kPa will cause a fraction of the relatively fragile microspheres to disintegrate (1) and generate shell fractions. Some of the measured microspheres will probably be solid particles originating from precipitated protein formed by the high temperature in the production process (12). This explains the negative contribution of small particles determined by the fractionation procedure. The negative regression coefficients in Fig. 3 are in correspondence to the infranatants in Fig. 5. Precipitated protein has a higher density than the albumin solution (forms sediment) and precipitation will lead to an increase in the matrix density used in the densitometric calculations. The results also point to an improvement in the gravimetric measuring method for determination of encapsulated air contents, by using the actual matrix density instead of a fixed value from the native HSA. This improvement has been implemented for analysis of later contrast agents for ultrasound imaging (13).

Air-filled microspheres in the range 2–25 µm support previous results from in vivo active microspheres in the size range 7–15 µm (8). However, a fraction of the particles in the size range 10–25 µm may contain microspheres filled with albumin solution instead of air or may contain aggregates of solid protein. This was observed for some batches which had a particle size distribution of infranatants encompassing this region. Microspheres larger than ≈25 µm where not found to encapsulate air.

The deviation between measured and model predicted air content is visualized in Fig. 4 and expressed by the fixed mean error in Table 1. The reduced precision is mainly caused by batch to batch variation, related to the variation in air content among the larger microspheres (RSD > 25%) (10). The relative standard deviation (RSD) varies from 30% for samples with low concentration to 5% for samples with high concentration indicating the latter as best suited for Coulter measurement. To reduce the analytical uncertainty and the sample to sample variation, triplicate measurement were performed.

The Coulter counter method was found suitable for estimating encapsulated air. However, for size distributions containing a relative large volume fraction of microspheres above 10 µm the PCR correction model was applied. The Coulter counter method together with PCR model is used as off-line process control tool (6), for predicting the air content in the product and thereby reducing the number of off-line process analyses.

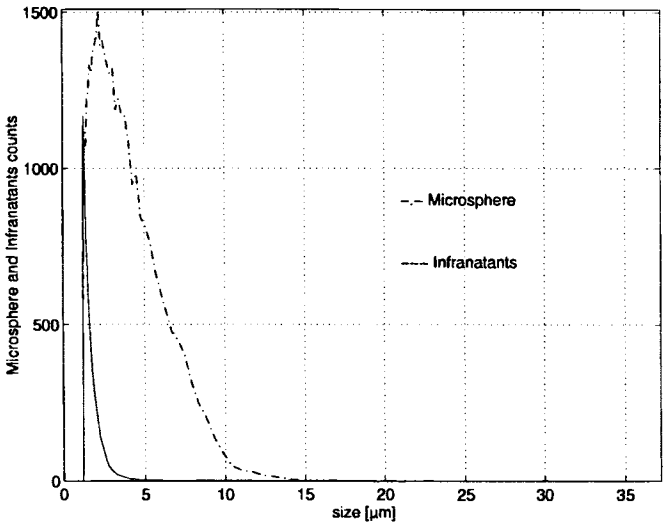

Fig. 5. Microsphere size distribution together with infranatants size distribution from the same production batch.

NOTATION

F Fraction, [dim]
N Number of microspheres, [dim]
d Microsphere diameter, [μm]
V Volume, [μl]

Greek Letters

ρ Fluid density, [g/ml]

Subscripts

c Coulter Counter
g Gravimetric
i Microsphere size class
s Sample

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