Relationship Between Size of Injected Microspheres and Ultrasound Imaging of Heart by PLSR

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Purpose. To investigate the enhancement in ultrasound signal (US) in heart, for diagnostic purposes, as a function of size and number of injected air-filled microspheres by multivariate statistical methods.
Methods. The US enhancement in left ventricle was measured after injection of 31 suspensions of air-filled albumin microspheres divided on and injected in six dogs. The relationship between the size of microspheres between 1–38 μm and the US enhancement was explored by Pearson's product moment correlation analysis, ordinary least-squares regression, principal component regression (PCR) and partial least-squares regression (PLS).
Results. Relative advanced algorithms such as PCR and PLS were required to achieve accurate *in vivo/in vitro* correlation. The most effective microsphere sizes contributing to US enhancement in left ventricle in dogs were estimated to be in the 7–15 μm range.
Conclusions. In general, the effective *in vivo* sizes are dependent on the type of formulation due to the surprisingly large active *in vivo* sizes found for the tested concept. PCR and PLS are suitable methods for *in vivo/in vitro* correlation, especially for covariated and noisy data.

KEY WORDS: ultrasound imaging; heart; microspheres; multivariate analysis.

INTRODUCTION

At present, medical imaging of heart based on ultrasound (VS) diagnostic contrast agents is in the development phase at several pharmaceutical companies (Alliance, Bracco, ImaRx Pharmaceutical, Molecular Biosystems Inc, Nycomed Imaging ASA, Schering AG, and other). A typical concept for US imaging of heart is gas filled microspheres. The microspheres have to be stable enough to pass through the right ventricle and the lung capillaries to give US enhancement in the left ventricle too. Partly dependent on the frequency of the US beam, relative larger microspheres will increase the medical effectiveness. On the other hand, microspheres have to be small enough to avoid the unwanted biologic effect of being trapped in the lung capillaries. The first standardized ultrasound contrast agent, approved for sale, was based on a suspension of relative unstable microbubbles that were unable to give contrast in the left ventricle of the heart (1). Currently, several pharmaceutical companies are making the effort to develop a biologically compatible contrast agent with acceptable contrast effect after passing the lung circulation. The importance of microsphere size, stability, surface properties, elasticity, and shape together with biocompatibility aspects present a challenge in making products with proper efficacy.

Albunex^R (Molecular Biosystems Inc., USA) is a contrast agent for medical imaging with US, which consists of air-filled albumin. Albunex^R consists of sufficiently stable microspheres of adequate size to pass the lung circulation and to enhance the US signal intensity in left ventricle (2,3) after intravenous injection. The size of active microspheres *in vitro*, has been reported by De Jong *et al.* (2). The range from 3 to 10 μm was found to be the most useful *in vitro* sizes for the Albunex^R when using a transducer frequency from 2 to 7.5 MHz. Based on simulations of the lung capillaries, the active left ventricle range for enhanced signal intensity was considered to be 5 to 8 μm. A later article from De Jong *et al.* (3) concludes that the air-filled albumin in the range 5 to 12 μm delivered the most significant contribution to the *in vitro* scatter. Recently, Hoff *et al.* (4) suggested that the main contributor to *in vivo* acoustic backscatter is caused by microspheres with diameters between 4 and 10 μm. The author emphasizes, however, that these calculations were based on passing a simulated lung filter, and that other effects might influence results in an actual *in vivo* model.

The typical extensive variations in data from biologic systems signalize a need for advanced mathematic methods. Often minor variations in biologically tested drugs are available for interpretation during the drug development phases. By applying principal component related methods, the trends and relationships in covariated and noisy animal and human data will be more consistently described and thus will lead to faster drug development.

The present paper evaluates results from a study of the *in vivo* efficacy of various preparations of air-filled albumin microspheres in a dog model. To describe the relationship between microsphere size and the *in vivo* signal intensity in the left ventricle is challenging due to the multivariate influence mentioned above. Therefore, the relationship between size distribution in Albunex^R suspensions and measured backscatter enhancement in dogs will be evaluated by multivariate mathematic methods in this paper. The aim of the present study has been to test and discuss the suitability of different types of mathematic algorithms in order to define the active *in vivo* left ventricle microsphere sizes for a given contrast agent. The developed model was later used to optimize the facilities for production of air-filled albumin microspheres as described by Kvåle *et al.* (5).

EXPERIMENTAL

Analytical and Biological Methods

The manufacturing procedures, analytical characterization, biological testing, and ultrasonic equipment used during this study are described in detail elsewhere by Sontum *et al.* (6). In brief, air-filled microspheres with a thin shell of heat aggregated human albumin was produced by sonication of human albumin solution as described in (7). To increase the variation span investigated, the size distribution of some initial suspensions was altered through fractionation, temperature cycling, or impact stress. The mathematic models were developed from 31 injectable suspensions, prepared and distributed in six dogs.

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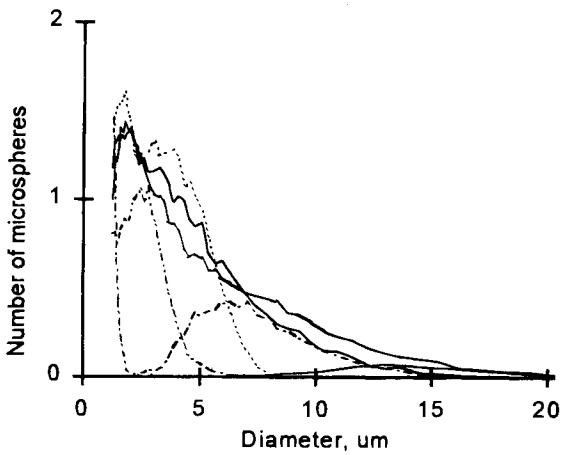

Fig. 1. Example of the extreme microsphere distributions used in modeling.

The 31 suspensions originated from 17 various size distributions (products) with a mean diameter from 1.8 to 12.2 μm. Some products were injected in two or three dogs. Each suspension was injected from three to six times in each dog. The concentration and the size distributions of microspheres were analyzed in triplicate by a Coulter Multisizer Mark II (Coulter Electronics Ltd., Luton, UK) (8). The instrument was fitted with a 50 μm aperture, employing 64 logarithmic spaced channels, giving a nominal particle measuring range from 1 to 38 μm, Figure 1. The *in vivo* efficacy of the produced suspensions was tested in a closed chest model in six mongrel dogs. The ultrasound scattering from left ventricle was measured by an ultrasound imaging scanner (Vingmed CFM-750, Vingmed Sound A/S, Horten, Norway) at a frequency of 3.25 and 5 MHz. The efficacy was measured as the maximum gray level enhancement in the left ventricle in the late diastolic phase.

To obtain a linear measure for *in vivo* efficacy, the increase in backscatter intensity relative to background level (I/I_0)was calculated from the measured gray level enhancement (GL) using the experimentally established relation (6):

$$I/I_0 = 10^{GL/46}$$

The animal experiments were approved by the national Experimental Animal Board in Norway.

Correlation and Regression Methods

The correlation between number of microspheres at each size and the first gray level enhancement (I/I_0) from each of the 31 injection series was investigated by Pearson's product moment correlation analysis (CA) (9). To estimate the effect of size on I/I_0, ordinary least-squares regression analysis (OLS), principal component regression (PCR) analysis, and partial least-squares regression (PLS) analysis were tested (10). The gray levels obtained from 28 suspensions injected in five initial dog studies were used to develop the regression models. Three injected suspensions were tested in the sixth dog study to verify high gray levels resulting from large microspheres. The mathematical calculations were performed by The Unscrambler version 6.11 (Camo A/S, Trondheim, Norway).

RESULTS

Results for the microsphere number distributions measured by Coulter counting for 6 of the 17 distributions products are shown in figure 1. The precision of parameters from the Coulter analysis has earlier been reported to be 1–2% relative standard deviation (RSD) for the number concentrations between 4 and 25 μm, and 26% for microspheres ≥ 25 μm (8). The size of the RSD is dependent upon the number of microspheres in the different size ranges. As the number of microspheres above 20 μm was low in the investigated suspensions, the relative noise was large and, consequently, this size range was omitted from the correlation and regression analysis. The initial gray level enhancement measured in each injection series varied from 2 to 78 and was expressed in exponential values described above.

Correlation and Regression Methods

The correlation coefficients calculated by CA gave the best linear correlation between I/I_0 and the number of microspheres at a size of approximately 8.0 to 12.0 μm. The correlation values are shown in Figure 2. The effect of different microsphere sizes on I/I_0 was evaluated by OLS, PCR, and PLS analysis. The microsphere distribution from 1 to 19 μm distributed on 50 Coulter channels and the corresponding I/I_0 were applied to multivariate regression analysis. To be able to perform OLS, 51 Coulter channels were reduced to 17. This was done by averaging three adjacent channels to one. However, any various averaging resulted in reasonable regression coefficients by OLS, Figure 3, indicating that the OLS was not a suitable regression method.

The different variation in number of microspheres at various sizes had to be accounted for before PCR and PLS regression were performed on the data. Number standardization with standard deviation (SD) was performed for each size, see SD in Figure 4. The following linear relationship between number of microspheres at different sizes and exponential gray level was established:

$$Y = \beta_0 + \beta_1 X_1 + \cdots + \beta_{51} X_{51}$$

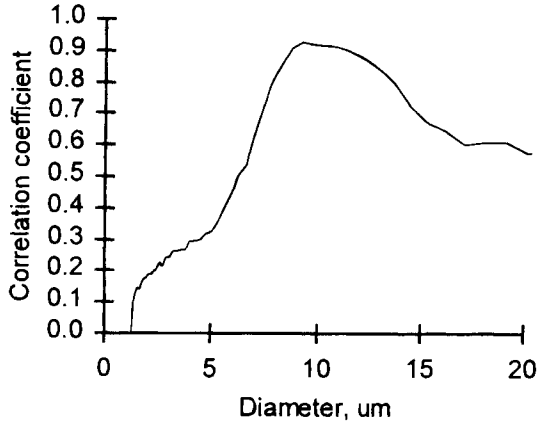

Fig. 2. The correlation between the number of microspheres at various sizes and the backscatter enhancement, I/I_0, by using Pearson's product moment correlation analysis.

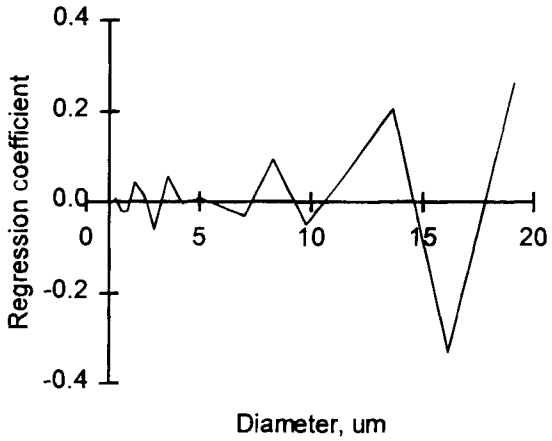

Fig. 3. The OLS regression coefficients estimated for the backscatter enhancement, I/I_0.

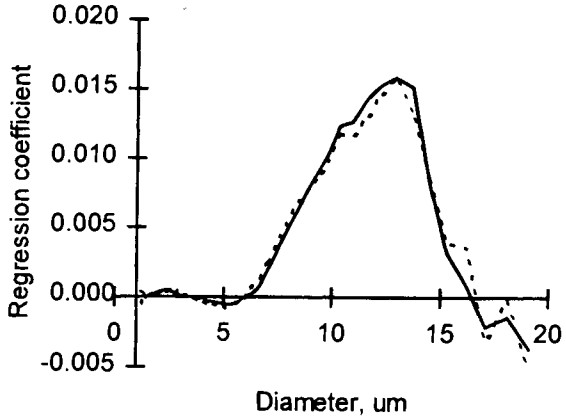

Fig. 5. The effect of microsphere size on the backscatter enhancement, I/I_0, in left ventricle estimated by PLS (solid line) and PCR (dotted line) analysis.

where $Y = I/I_0 = 10^{GL/46}$

GL = gray level
X_n = number of microspheres at the specific size
β_0 = constant
β_n = regression coefficient

The regression coefficients found by PLS and PCR analysis are shown in Figure 5. The number of principal components was chosen by full (leave-one-out) cross-validation. Two products, each injected in each dog, deviated from the two corresponding products injected in two other dogs and were excluded from the final model. Including these results did not change the regression coefficients but reduced the correlation coefficient from 0.93 to 0.88. The six initial PLS-components explained 76, 83, 87, 87, 87, and 86% of the validation Y-variance. Three components were chosen explaining 84% of the variance in X-matrix. Five principal components were included in the PCR analysis and 87% of the variance in Y was explained and 97% of X was then described. The relationship between the 26 Coulter triplicate predicted I/I_0 and the measured I/I_0 is illustrated in Figure 6.

DISCUSSION

The correlation analysis (CA) in Figure 2 illustrates strong correlation between the I/I_0 and the microsphere sizes in the range 8 to 12 μm. Two important unwished aspects affects CA. One, the collinearity between the number of microspheres at different sizes, especially above approximately 9 μm, see Figure 7. This resulted in too high correlation coefficients for non-active microspheres. Two, low number of larger microspheres lead to low signal to noise ratios, which influenced the analytical precision for the measurement of larger microspheres. This lead to apparent correlations between I/I_0 and the sizes above approximately 14 to 15 μm.

The PLS and PCR algorithms were found to be more adequate than the OLS, comparing Figure 3 with Figure 5. PLS and PCR are more suitable to collinear variables and low signal-to-noise ratios than OLS (10,11). The data was standardized with the standard deviations shown in Figure 3 to give each size equal possibility to influence on the response. The standardization is typically used when the data are of different ranges. Modeling without standardization lead to artificial effects of small microspheres and large number of principal components in PLS. However, the use of standardization might have exag-

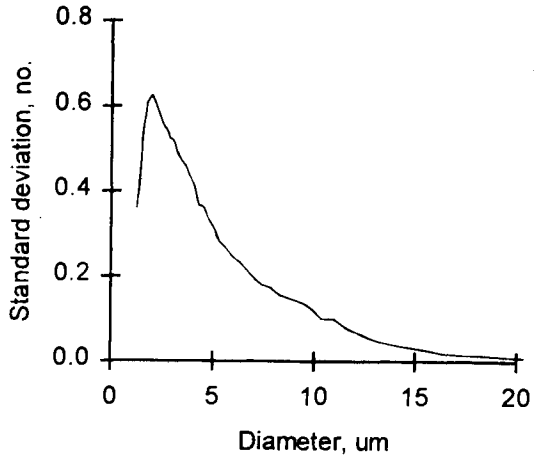

Fig. 4. The standard deviations for the microsphere distributions used to standardize the data before PLS and PCR modeling.

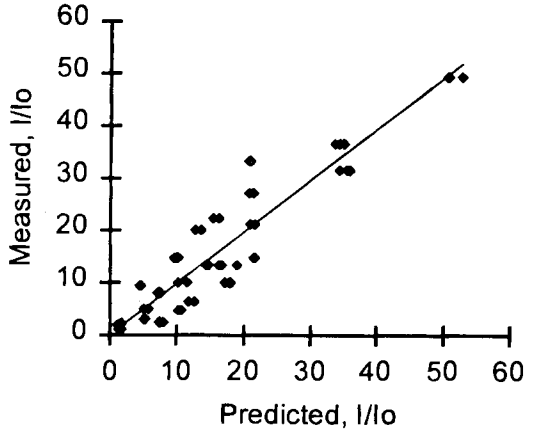

Fig. 6. The cross-validated relationship between predicted and measured I/I_0 is illustrated. The correlation coefficient, r, is 0.93, SE is 4.5, slope is 0.99 and offset is 0.15.

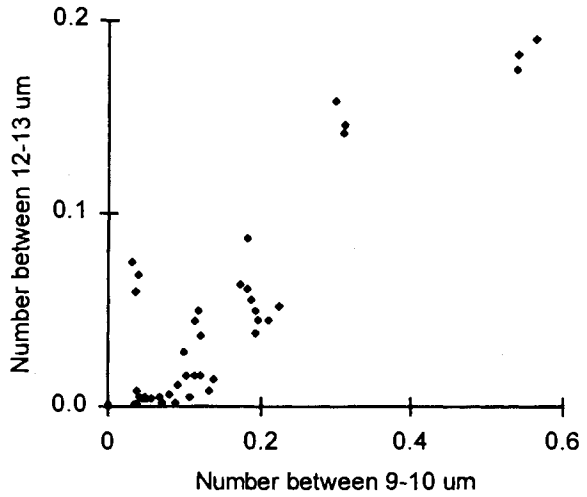

Fig. 7. The collinearity for the number of larger microsphere sizes is illustrated.

gerated the effect of larger microspheres (11). The number of microspheres above 20 μm was low and since these sizes were expected not to contribute to the biologic effect, as indicated in Figure 5, these were removed before modeling.

Some minor deviations in regression coefficients between PCR and PLS were observed above 15 μm. Since the PLS analysis uses both X-matrix and Y-matrix to select the principal components, this gives a more optimal extraction of the relevant information in X for regression on Y compared with PCR. The number of principal components is more crucial in PLS. An aspect associated with the PCR is the use of a sharper threshold compared with the PLS analysis when defining its' shrinking solution from the size distributions (X-matrix) (11). The low S/N-ratio due to the low number of large microspheres may then lead to a low tolerance by PCR and can be the explanation to the minor differences in regression coefficients above 15 μm shown in Figure 5.

The residuals observed in figure 6 were caused mainly for two reasons. The major reason was the large biologic variation among dogs. However, the biologic variation only decreased the correlation coefficient calculated for measured versus predicted I/I_0 without changing the regression coefficients significantly. Each of the triplicate analyses of the size distributions gave some minor differences in the predicted gray levels. However, the prediction of gray levels indicated a reproducible quality of the microspheres of comparable size. The β_0 in the PLS and PCR model were -1.6 and -0.8, respectively, which indicated neglectable systematic errors. The predicted backscatter enhancement from average Coulter distributions in table I also indicate no large errors.

Table I. The Accuracy of Predicted Backscatter Enhancement from Averaged Microsphere Distributions in the Sixth Dog

Product	Meas. I/I_0	Pred. I/I_0	Meas.GL	Pred.GL	Recovery, %
A	25	22	64	62	97
B	41	42	74	74	100
C	37	40	72	73	101

Three procedures were chosen to test further the reliability of the calculated regression coefficients. One procedure was to average the triplicate Coulter distributions and perform full (leave-one-out) cross-validated regression on the 26 objects to indicate the optimum number of principal components. The second procedure was to perform PLSR also with the highest measured and the average GL from each injection series, respectively. The third test was to verify consistent regression coefficients by removing the highest gray levels shown in Figure 6 (all predicted I/I_0 above 30) and perform full cross-validation on the remaining 23 average Coulter distributions. All new constructed regression coefficients showed an effective size range from 7 up to approximately 15 μm. The effective sizes found by modeling of inter-individual data were in agreement with the effective sizes found by univariate intra-individual calculation by Sontum (6).

In conclusion, it was found that microsphere sizes between 7 and 15 μm were the main contributors to the increased acoustic backscatter in left ventricle. This is a shift in effective microsphere size compared with Hoff *et al.* (4) who found that the active size range was 4 to 10 μm based on *in vitro* studies using simulated lung filter models. The optimal *in vitro* range found by De Jong *et al.* (3) was from 4 to 12 μm when using transducer frequencies of 2.5 and 5 MHz. When increasing the frequency to 3.25 and 5 MHz as in this study, smaller microspheres than 12 μm would consequently be expected to be effective. Three possible phenomena could meet the earlier observations and explain the contribution from large microspheres. One, the microspheres are sufficiently elastic in nature to pass smaller capillaries. Second, the highly blood-soluble air inside the microspheres can be expected to diffuse into the blood stream gradually, causing the microsphere size to decrease slowly to an adequate size or form for passing the lung capillaries and give US enhancement. Third, the contribution of air from unstable larger microspheres may saturate the local blood stream and thereby stabilize the smaller ones causing an increased number of effective microspheres to reach the left ventricle. These possible phenomena indicate that the active microsphere sizes found, have validity only to the tested contrast agent.

The active microsphere size for backscatter enhancement in left ventricle in animal and human will depend on the type of contrast agent injected. To give accurate estimates of active microsphere sizes in the left ventricle, advanced algorithms to handle the biologic variation may be needed. The effect of the lung capillaries may be different in animal and human for equal contrast agents. To decide whether the effect of the capillary bed observed in dogs is representative for humans, is difficult. To achieve a true human transpulmonary passage for a given particle, standardized clinical testing procedures including standardized US equipment and measurements have to be used. Since this may be difficult to obtain, simulated models based on studies of animals may be the most appropriate alternative to achieve useful information.

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