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Quantitative Analysis of Scanning Transmission X-ray Microscopy Images of Gas-Filled PVA-Based Microballoons

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We report on the quantitative analysis of scanning transmission X-ray microscopy (STXM) images of gas-filled, poly(vinyl alcohol) (PVA)-based microballoons (MB) in a water environment. A model for the transmitted intensity is proposed on the basis of a perfect spherical shell stabilizing the microballoon. An extension of this model to take into account the deformation of the MBs is also presented. Taking into consideration a density gradient of the shell and the STXM resolution, we were able to explain very precisely two types of experimental STXM profiles obtained on gas-filled MBs. This enables the detailed characterization of MB properties such as radius and wall thickness and the determination of their wall density with unprecedented high resolution.

1. Introduction

During the last few years, considerable research effort has been devoted to the development of gas-filled microballoon (MB) systems in mostly because of their potential applications as drug delivery systems and ultrasound contrast agents. Such applications are based on the MBs lower density and their ability to carry therapeutic gases where the MB wall plays a key role in determining essential properties such as stability, ultrasound scattering efficiency, adhesion, and permeability. Therefore, from an applied but also from a fundamental point of view, it is very important to characterize the MB wall in detail.

With the need for more detailed information about these new, more complex systems, it is crucial to employ novel, more powerful, well-suited techniques to characterize them. Scanning transmission X-ray microscopy (STXM) imposes itself as such a technique, providing chemically sensitive high-resolution (better than 40 nm) images of samples even in a aqueous environment.

In STXM, high-brilliance monochromatic synchrotron radiation is focused by a Fresnel zone plate, and the sample is then raster-scanned through the focal point while recording the intensity of transmitted X-rays in order to produce a 2D image. This image contains information about the sample that can be immediately and qualitatively appreciated. In the present case of MBs, quantitative analysis of the data is required in order to rigorously explore important sample parameters (e.g., external radius, wall thickness, and wall density).

In this article, we present a quantitative analysis of STXM images recorded for PVA-based MBs in aqueous surroundings. The discussed model is based on an ideal system of perfectly spherical MBs where the density gradient of the stabilizing shell and the finite beam width of the focused X-ray beam are taken into account. A simple extension of this ideal model is also presented to take into account the deformation caused by a possible squeezing of the MBs within the wet cell. By using these “spherical” and “deformed” models, we were able to explain very precisely two types of experimental STXM profiles obtained from poly(vinyl alcohol) (PVA) MBs. From the STXM 2D data, it was therefore possible to characterize the MBs’ 3D shape and to determine with very high resolution their radius and wall thickness as well as gain some important insight into the MB wall density.

2. Experimental Details

2.1. Sample Preparation. The preparation of stable (air-filled) PVA-coated microballoons was previously described in the literature. The MB average diameter and shell thickness characterized with confocal laser scanning microscopy (CLSM) were reported to be 5 μm (1 μm standard deviation) and 0.9 μm (0.25 μm standard deviation), respectively. To analyze the MBs with STXM,
approximately 1 µL of a microballoon water suspension (6 months old, homogenized by gentle shaking prior to experiment) was sandwiched between two 100-nm-thick Si₃N₄ membranes (Silson Ltd., Northampton, U.K.), which were then sealed with silicone high-vacuum grease to maintain the water environment during the STXM investigation (the sample started to dry out after approximately 5 h in the microscope chamber). To avoid almost complete absorption of the X-ray beam by the water environment, it is important to prepare thin samples (<10 µm). To model a more general situation in practical applications of deformed MBs, some of the samples were squeezed in between the wet-cell membranes. The number of squeezed MBs depends both on the thickness of the wet cell and the diameter of the microballoons. In the present studies, up to 14% deformed MBs could be observed.

2.2. STXM. During an STXM experiment (Figure 1) high-brilliance monochromatic synchrotron radiation is focused by a Fresnel zone plate, and the sample is then raster-scanned through the focal point while recording the intensity of transmitted X-rays in order to produce a 2D image.

The microballoons were imaged using the PolLux STXM microscope at the Swiss Light Source (SLS), Paul Scherrer Institut, Villigen, Switzerland. The SLS storage ring runs at 2.4 GeV in “top-up” operation mode, which guarantees a constant electron beam current of 400 ± 1.5 mA. The PolLux STXM uses polarized X-rays from a bending magnet in the photon energy range between 200 and 1200 eV focused by 25 nm zone plates that allow a lateral resolution of about 40 nm in routine operation, spectral resolving power (E/ΔE) better than 5000 (at the N K-edge, approximately 400 eV), and a detected flux in helium of more than 2 × 10⁶ photons/s. The transmitted photon flux was measured using a photomultiplier tube (Hamamatsu 647P) with an estimated efficiency of 36% at 520 eV. The images presented here were recorded at an energy of 520 eV below the oxygen K-edge. During acquisition, the detected intensities were below the detector’s saturation range. It was carefully checked that while scanning one image no significant radiation damage occurred. The position of the sample scanner was controlled by a laser interferometer with a resolution of 2.8 nm.

2.3. Data Treatment. Image and profile processing were carried out with homemade software developed with the Igor Pro development tool that directly reads the raw STXM data files. The STXM radial transmission profiles presented were obtained by angular averaging of the interpolated values of the MBs transmission image (with a step rotation of 1°). This is helpful in reducing the noise present when considering only a single line across the MB, and it is justified because the MB STXM images present circular symmetry. Two corrections were subtracted from the raw transmitted intensity data: the background noise (determined experimentally as the intensity detected with a water cell that was thick enough that the transmitted intensity was very close to zero) and the initial error in the detector (determined as the ordinate intercept of a linear fit of the average intensity of 50 pixel × 50 pixel images through an empty cell vs acquisition (dwell) time per pixel). Both corrections were very small. Finally, the intensity errors of the radial profiles were determined by taking into account the standard deviation of a single image pixel (determined experimentally) and the number of pixels over which the angular average is calculated (sufficiently close to the center averaging only over one pixel, corresponding to larger errors).

3. Theoretical Description

The transmission of monochromatic X-rays through matter follows the Lambert–Beer law

\[ T = \frac{I}{I_0} = e^{-\mu E D} \]

(1)

where \( I \) and \( I_0 \) are the transmitted and incident beam intensities, \( \mu (\rho, E) \) is the mass absorption coefficient depending on the sample chemical composition (\( \rho \)) and photon energy (\( E \)), \( \rho \) is the density, \( D \) is the thickness of traversed/absorbing material, and \( k \) is the absorption coefficient. Considering our three-component system of a gas-filled (AIR), PVA-based shell (WALL) in water (H₂O), a general expression for the transmitted intensity can be written as eq 2, where the energy dependence of \( \mu \) and \( k \) has been left out for simplicity. In the following text, we will base our analysis on eq 1 and determine expressions for the thicknesses \( D_j \) of the respective components \( j \) (\( j = \text{H}_2\text{O}, \text{WALL} \), or AIR).

\[ I = I_0 e^{-k_{\text{H}_2\text{O}}D_{\text{H}_2\text{O}} - k_{\text{WALL}}D_{\text{WALL}} - k_{\text{AIR}}D_{\text{AIR}}} \]

(2)

To develop the model further, it is necessary to consider the geometrical shape of the balloon. The models presented below consider a perfect spherical and a deformed (squeezed) spherical shell.

3.1. Spherical Microballoon Model. Here we assume that the MB consists of a perfect spherical shell defined by an external radius \( R_e \) and internal radius \( R_i \) (Figure 2).

Similar to previous considerations in refs 12 and 13 but taking into account our three-component system, we start by assuming a constant wall density (\( \rho_{\text{WALL}} = \text{constant} \)). In the corresponding transmission profile (Figure 2), three zones can be distinguished where the expressions for the component thicknesses \( D_j \) traversed by the photon beam are given by

The reference system is made to coincide with the balloon center.

To third order) absorption function given by eq 4, where

\[ \Psi_{WALL-III} = \Psi_{WALL-II} - (2k_0 + Rk_1)B \]

\[ -(2/3)(R_e^2 + x^2)k_2 + \frac{1}{2}R_e(R_e^2 + \frac{3}{2}x^2)k_3 \] \[ B \]

\[ -x^2(k_1 + \frac{3}{4}x^2k_3) \ln \left( \frac{R_e + B}{lxl} \right) \]

(6-III)

The thicknesses (\(D_i\) values) are given by eq 3 in the respective zones. Note that for \(k_1\), \(k_2\), and \(k_3\) equal to zero, transmitted intensity expressions (eq 6) become equivalent to the expressions with constant wall density obtained by merging eqs 2 and 3, as expected. Therefore, eqs 6-I–6-III constitute more general expressions for the transmitted intensity profiles.

3.2. Deformed Microballoon Model. Generally, in practical applications the MBs will be subjected to stresses that can deform them so that the perfect spherical model no longer applies. We propose a simple extension of the spherical model to take into account some deformation of the samples. The so-called “deformed model” considers two half-spheres (discussed in the spherical model) separated by a flat middle section (see Figure 3). Besides the external (\(R_e\)) and internal (\(R_i\)) radii also present in the spherical model a third, “corner” radius (\(R_c\)) is necessary to characterize the balloon. Indeed, for zones I–III one can use the same expressions from the spherical model (eq 6) by replacing \(R_e\) with \(R_c\), \(x\) with \(lxl - \Delta\), and \(R_i\) with \(R_c - h\) (\(\Delta = R_e - R_i\)). The zone ranges are the same except for zone III, which stops when zone IV, described by eq 7, starts (Figure 3). Inversely, one can imagine the spherical model to be a particular case (when \(R_c = R_i\)) of the more general deformed model.

Note we have only to consider a sample thickness equal to the diameter of the balloon even if, for this model, the wet-cell thickness might be larger (case where the balloon adheres to one membrane only). This is because the “extra” water in the wet-cell as well as the two Si3N4 membranes will absorb a constant fraction of the transmitted intensity and are taken into account in \(I_0\) (which is also a fitting parameter).

By relacing eqs 3-I–3-III in eq 2, we obtain the transmission expressions for each of the three zones for a spherical shell with constant wall density. Previous experiments, however, suggest that a constant wall density may not correctly describe the sample.

The presence of a wall density gradient will translate into an absorption coefficient along the traversed thickness.

In this case, because the wall composition varies within the thickness of material traversed by the X-ray beam, it is necessary to replace the product \(k_{wall}D_{wall}\) in eq 2 by the integral (eq 5) of the wall absorption coefficient along the traversed thickness.

\[ \Psi_{WALL} = \int_{D_{WALL}} k_{WALL(z)} \, dz \]

(5)

In zone I, nothing changes (there is no PVA-based wall), but in zones II and III, the integral (eq 5) has to be calculated, after which it is straightforward to obtain the expressions for the transmission in the presence of a radial density gradient of the wall material for the three zones (eq 6):

Zone I

\[ I_1 = I_0 e^{-2R_e} \]

(6-I)

Zone II

\[ I_{III} = I_0 e^{-2R_e} \Psi_{WALL-II} \]

where

\[ \Psi_{WALL-II} = (2k_0 + Rk_1)A \]

\[ + \left( \frac{2}{3} (R_e^2 + x^2) k_2 + \frac{1}{2} R_e (R_e^2 + \frac{3}{2} x^2) k_3 \right) A \]

\[ + x^2 (k_1 + \frac{3}{4} x^2 k_3) \ln \left( \frac{R_e + A}{lxl} \right) \]

(6-II)
We emphasize that the flat deformed model was proposed for simplicity. In addition, other more complex models could have been considered.

3.3. Beam Size. It was assumed that the X-ray beam is infinitesimally small in diameter (infinite resolution). However, the beam size is given by the zone plate parameters, which hold only for a certain depth of focus. To take the finite dimension of the beam into account, the complete (all zones) modeled intensity profiles (eqs 6 and 7) were discretely convoluted (eq 8) with a Gaussian distribution (eq 9) (chosen for simplicity) with an fwhm given by \( \sim 2.35 \sigma \).

\[
I_{R(\chi)} = (IG)_{(\chi)} = \sum_n I_{(n)}G_{(n-x)}
\]

(8)

\[
G_{(n)} = \frac{1}{\sqrt{2\pi}\sigma^2}e^{-x^2/2\sigma^2}
\]

(9)

With the developed expressions (eqs 6–8), we are ready to analyze the STXM transmitted intensity images. A minimization procedure will be employed to find the wall radius, thickness, and absorption coefficient, \( k_{(n)} \), that produces the best correlation with the experimental data. From \( k_{(n)} \), it is possible to determine the PVA-based wall water/air content. In the next section, the necessary expressions will be presented.

3.4. Wall Content Characterization. Because the PVA-based microbubbles have air inside and water outside, it is reasonable to assume that the PVA-based wall also contains one or both. The absorption coefficient obtained from the fitting procedure mentioned above can be used to characterize the wall content. Here we will introduce the expressions needed for this characterization. The absorption coefficient of the wall \( k \), assuming that the absorption of the wall is equivalent to the combined absorption of its separated components, can be written as the average of its component absorptions weighted by the relative wall volume (\( C_{H_2O}, \text{PVA or AIR} \), varying from 0 to 1) (eq 10). The energy and space dependence are represented by \( E \) and \( x \) respectively. Assuming the wall contains only H\(_2\)O, PVA and AIR expression (eq 11) must be verified.

\[
k_{WALL(x,E)} = k_{H_2O(x)} + k_{PVA(x)} + k_{AIR(x)}
\]

(10)

\[
k_{H_2O(x)} + k_{PVA(x)} + k_{AIR(x)} = 1
\]

(11)

There are three unknown variables (\( C_{H_2O}, C_{PVA}, \text{and } C_{AIR} \)); all other parameters are either given from the fitting procedure or are known for the pure elements (Table 1\(^{15} \), and two equations (eqs 10 and 11). By solving the equations, one can obtain a relation between two of the unknown variables. This suggests that it is possible, in principle, to determine without ambiguity the composition of a three-component wall by imaging at two different energies (carefully chosen to maximize the sensitivity of the measurement).

4. Results and Discussion

4.1. Spherical and Deformed Microbubbles. Two representative STXM transmission images of PVA-based gas-filled MBs are presented in Figure 4 and were collected at 520 eV by raster scanning an area of \( 10 \times 10 \mu m^2 \) (200 pixels \( \times \) 200 pixels, Figure 4, left; 150 pixels \( \times \) 150 pixels, Figure 4, right) with a detection (dwell) time per pixel of 2 ms.

The radial transmitted intensity profiles, presented in Figure 5 (circles), were obtained by angular averaging of the STXM 2-dimensional images of MB A (Figure 5, left) and MB B (Figure 5, right) from 0 to 360° (to obtain symmetrical profiles around zero). Both profiles, from left to center, are characterized by a medium transmitted intensity (from the water environment) followed by a decrease in transmission (from the MB wall) until it reaches a minimum and a subsequent increase until it reaches a maximum above the water medium transmission level (as a result of the less absorptive gas interior of the MB, rendering these profiles incompatible with water-filled balloons). This typical gas-filled shell profile is qualitatively explained by the relation between the absorption coefficients of the three MB components (\( k_{AIR} < k_{H_2O} < k_{PVA} \)) at the working energy (Table 1\(^{15} \)) and was already reported\(^{14} \).

One qualitative difference between the two profiles is the flat intensity region of MB B (Figure 5, right). By using the models developed above, we were able to obtain fitting curves that follow the experimental data quite closely. The radial profile of MB A was explained by the spherical model (Figure 5, left) whereas the radial profile of MB B was explained (Figure 5, right) only by introducing the deformed model. Although the results supported by higher-resolution images are more reliable, we believe that the results presented here are not significantly influenced by the different pixel resolution (set by experimental conditions) at which the two images were acquired.

4.2. Radial Absorption Function. Different radial wall absorption functions (constant, linear, quadratic, and cubic) were taken into account to fit the experimental data. On the scale of Figure 5, it is difficult to distinguish between the best-fit curves to these functions. However, it makes sense to enlarge the area close to the MB wall visually (Figure 6) because we want to characterize the walls properties. We can see that with the cubic radial absorption function a much better correlation with the data was obtained (solid line, Figure 6, left) when compared with the constant and linear functions (dotted and dashed lines, respectively, Figure 6, left). The absorption profiles for the constant, linear, and cubic functions are presented in Figure 6 (right). (For clarity, the quadratic function is not shown.) To compare the four functions further, some parameters are presented in Table 2, where one can see that the standard deviation from the experimental data is significantly smaller for the cubic function. The estimated radius and wall thickness of the balloon increase with the polynomial order of the absorption function whereas the wall absorption average decreases (to maintain the total absorption). The use of a fourth-order polynomial wall absorption function produced no significant change in the absorption profile, balloon dimensions, or deviation from the experimental data. Therefore, in the rest of this work, we will focus on the results obtained with a cubic wall absorption function.

<table>
<thead>
<tr>
<th>( \rho ) (g/cm(^3))</th>
<th>( \mu ) (cm(^2)/g/( \mu m ))</th>
<th>( k ) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)O</td>
<td>1.000</td>
<td>0.110</td>
</tr>
<tr>
<td>PVA</td>
<td>1.269</td>
<td>0.721</td>
</tr>
<tr>
<td>air</td>
<td>0.0012</td>
<td>1.384</td>
</tr>
</tbody>
</table>

| Table 1. Density, Mass Absorption Coefficient (\( \mu \)), and Absorption (\( k \)) of the Pure Components at 520 eV\(^{15} \) |


4.3. Fit Parameters. The fit parameters derived from the profile analysis of MB A and MB B (Figure 5) are summarized in Table 3.

MBs dimensions such as the radius and wall thickness are characterized with unprecedented resolution. These values are compatible with previous confocal laser scanning microscopy (CLSM) measurements, although with much better resolution. We remark that the different wall thickness obtained stems from the fact that two different balloons subjected to different stresses were analyzed. The deformation of MB B, in accordance with the proposed model, was characterized. From these values, the balloons volume could be determined to be 61 (39 shell volume) and 20 (10 shell volume) fL for MB A and MB B, respectively.

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were determined to be <1% in both cases. The incident beam intensity \( I_0 \) corresponds, after taking into account the Si\(_3\)N\(_4\) membranes (200 nm, approximately 42% transmission at 520 eV), to an incident beam of around 2.7 Mcps, which is compatible with experimental measurements. The water environment absorption \( k_{\text{water}} \) resulting from the fitting procedure is 8% higher than that of pure water. This is most probably due to PVA contamination (corresponding to 1.1% volume PVA in solution, which was qualitatively confirmed with energy scans). In an attempt to take into account all of the possible sources of uncertainty, the parameter errors presented in Table 3 were calculated as the maxima of either the errors given by the minimization algorithm, 5% or 50 nm (for length parameters only). To gain insight into the relative parameter contribution to the intensity curves, the models sensitivity to each parameter was measured as the best-fit curve average deviation from two curves obtained by adding ±5% to the respective parameter. Most parameters presented similarly high sensitivity except for the air absorption coefficient and the beam fwhm. In the first case, this is because air absorbs very little compared to water or PVA and consequently has almost no influence on the transmitted intensity. The models’ insensitivity to the beam resolution is due to the low gradient function used to describe the wall absorption; with a sharper wall absorption function, the sensitivity of the model to this parameter would increase. To avoid algorithm-induced errors due to this lack of sensitivity, the two parameters were fixed during the minimization: the air absorption coefficient was fixed to that of pure air (Table 1) and the beam width was set at 40 nm. All other parameters presented in Table 3 were determined by the minimization algorithm.

4.4. Wall Water/Air Content Characterization. The fitting transmission profiles of Figures 5 and 6 were obtained with the radial wall absorption profiles represented with solid lines in Figure 7 for MB A (left) and MB B (right). Both balloons present similar absorption profiles, evolving continuously from an absorption close to that of air on the inner wall interface to an absorption close to that of water on the outer interface, presenting a maximum somewhere in the middle. From these absorption profiles and by using the expressions derived before (eqs 10 and 11), it is possible to gain more insight into the wall composition. Because there are only two equations and three unknowns (percentages of water, PVA, and air), it is not possible to characterize it completely; however, we can analyze extreme cases where besides PVA there is only water \( (C_{\text{AIR}} = 0, \text{Figure 7, dashed line}) \) or air \( (C_{\text{H}_2\text{O}} = 0, \text{Figure 7, dotted line}) \).

The high water/air content in the wall is a consequence of the low average wall absorption coefficient obtained in the minimization procedure (much lower than that of dry PVA, see Table 1). For both graphs in Figure 7, close to the inner balloon interface the water content line (in the absence of air) goes above 100% (not plotted). This is because the absorption coefficient of the wall in this zone is smaller than that of water, suggesting that there is a very important presence of air close to this interface, as expected. It is probable that the PVA-based MB wall contains pores, smaller than the experimental resolution, with water or air. For a complete characterization of the wall composition, detailed experiments are planned to be performed at different energies on the same MB.

It is interesting that the wall from MB B (squeezed balloon) presents more than a 15% higher absorption average and consequently lower water/air content. This might suggest stretching of the wall with water/air expulsion when the balloon is squeezed. The higher absorption value close to the inner wall could also be due to the increased internal pressure. However, to make such claims deformation experiments would need to be performed on the same microballoon. STXM would indeed be an excellent tool for characterizing the deformation behavior of microcapsule/balloon systems.

5. Conclusions

Two models of X-ray transmission profiles of gas-filled shells (spherical and deformed) were considered to quantitatively analyze the experimental STXM data of air-filled microballoons in water surroundings. The X-ray beam resolution and a third-order polynomial radial wall absorption function were taken into account in the models.

This work shows that the STXM 2D images of PVA-based MBs are compatible with either 3D perfect or squeezed, gas-filled spherical shells. From the models, the MBs’ physical parameters such as radius, wall thickness, and wall absorption can be determined with unprecedented high resolution. The proposed analytical models open new applications for the quantitative characterization of multicomponent microcapsule materials by means of STXM.

From the wall absorption profiles, the water (in the absence of air) and air (in the absence of water) volume contents in the wall were determined. Experiments at different energies should allow for the complete (unconditional) characterization of the wall composition.

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