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To cite this article: Léon F van Heijkamp et al 2010 J. Phys.: Conf. Ser. 247 012016

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Spin-Echo Small Angle Neutron Scattering analysis of liposomes and bacteria

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Abstract. Two types of liposomes, commonly used in drug delivery studies, and \textit{E. coli} bacteria, all prepared in H\textsubscript{2}O, were resuspended in D\textsubscript{2}O and measured with Small Angle Spin-Echo Neutron Scattering (SESANS). Modeling was performed using correlation functions for solid spheres and hollow spheres. The signal strength and curve shape were more indicative of hollow particles, indicating that the H\textsubscript{2}O-D\textsubscript{2}O exchange occurred too fast to be observed with the available time resolution. Fitting the particle diameter and membrane thickness of the hollow sphere model to the data, gave results which were in good agreement with Dynamic Light Scattering (DLS) data and literature, showing as a proof-of-principle that SESANS is able to investigate such systems.

SESANS may become a good alternative to conventional tritium studies or a tool with which to study intracellular vesicle transport phenomena, with possible \textit{in vivo} applications. Calculations show that a substantial change in numbers of a mixed system of small and large biological particles should be observable. A possible application is the destruction by external means of great numbers of liposomes in the presence of tumor cells for triggered drug release in cancer treatment. Since SESANS is both non-invasive and non-destructive and can handle relatively thick samples, it could be a useful addition to more conventional techniques.

1. Introduction
The delivery of drugs or contrast agents to tumors and their uptake by tumor cells is of great interest to the medical community to aid the diagnosis and treatment of cancer \cite{1, 2, 3, 4, 5}. In order to develop improved methods to identify and destroy malignant tumor tissue, research has focused on the transport of substances across the bi-lipid layer using encapsulation by liposomes \cite{1, 6, 7, 8, 9, 10}. These lipid vesicles have a similar composition to cellular membranes and are therefore also used as a model in transport studies. The cellular transport of water is an important quantity for medical diagnostic tools using cellular contrast agents. Much work has been done studying two key processes: the internalization of small cells and particles by larger cells \cite{11, 12, 13, 14} and the rate at which water can diffuse in and out of cells \cite{15, 16, 17}.
For the development of cancer treatment it is desirable to be able to observe both liposomal uptake by tumor cells and cellular water exchange as directly as possible in living patients, using non-invasive methods. Many observations can be done down to the cellular level in a laboratory or inside living patients, using methods as Dynamic Light Scattering (DLS) and Magnetic Resonance Imaging (MRI). Water transport is often studied using tritiated water (T\textsubscript{2}O, HTO) as a tracer. Changing concentrations of this isotope produce local changes in levels of radioactivity. This technique is used to observe metabolic processes in laboratory animals [11], but not generally applied \textit{in vivo} to larger organisms because of its radioactivity, which increases the chance of cancer when applied internally. For water transport studies with liposomes, the tritiated liposomes have to be separated from the medium. Since it is a weak \(\beta\)-particle emitter, experiments require either large quantities or the very sensitive liquid scintillation detectors. All this makes it unsuitable for \textit{in vivo} application, whereas SESANS does not have these limitations. Furthermore, the exchange rate of this super-heavy water with normal, light water (H\textsubscript{2}O) provides little information on cellular structure.

Small Angle Neutron Scattering has been employed in the past to study cellular components, such as ribosomes [12], DNA (including helical structure) [13], protein [14] and lipid structures [18, 19] and protein hydration [20]. Using Spin-Echo Small Angle Neutron Scattering (SESANS) microstructures of 20 nm to 20 \(\mu\)m can be observed, corresponding to sub-cellular and cellular sizes. The sample thickness depends on the neutron transmission of the investigated substance. The sensitivity of neutron scattering experiments to the hydrogen-deuterium contrast is often exploited in watery samples. Typical sample thicknesses in SESANS are one centimeter when containing mostly light water, and up to several centimeters when using mostly heavy water, or deuterium oxide (D\textsubscript{2}O, HDO). Neutron scattering is non-invasive and a SESANS measurement exposes samples only to small doses of radiation, because it requires limited neutron intensities with moderate wavelengths. For stable biological samples, this technique can be easily applied \textit{in vitro} when containing either light or heavy water or both. SESANS can in principle also be applied to organisms \textit{in vivo} without deuterating, if there is enough contrast and no more than a few centimeters of water in the beam.

Water transport across cell membranes can be studied \textit{in vitro} with SESANS by observing changes in contrast, due to local changes in light and heavy water concentration. Although deuterium is a non-radioactive isotope, large amounts of this heavy water can still be harmful to organisms. Some normal reactions in cells, especially cell division, are disrupted by the stronger hydrogen bonds formed. Toxic levels of deuteration range from around 30\% to 50\% for rodents and large mammals respectively [21, 22]. Since it is not possible to replace large amounts of water with D\textsubscript{2}O without any harmful effects, an \textit{in vivo} SESANS study focussing on water exchange in human cells will be difficult.

SESANS experiments were performed on liposomes and bacteria, as prototypes for small and large cells. The aim was to observe both as whole cells and investigate water transport through the membranes. All samples were prepared or grown in H\textsubscript{2}O-based buffers or growth media and finally resuspended in D\textsubscript{2}O. The liposomal polydispersity was determined using DLS. Fitting the measurement data showed the average cell diameters, cell-wall thicknesses and the inner contrast with respect to the medium, which were in good agreement with expected values from other techniques and literature. To our knowledge, this was the first time observing entire cells using neutrons. The rate of hydrogen-deuterium exchange could only be observed if the timescale was within the temporal resolution of the technique, which was not the case for any of the measured liposomes. A neutron source with a higher flux and larger wavelength should significantly increase the temporal resolution of the SESANS setup. Anticipating future developments, time-dependent measurements may become possible so that water exchange may be observed as well.

Model calculations predicted that large-scale removal of liposomes from a sample, through
destruction by external means or biological process, should be observable with SESANS. A large drop in the liposome population of an investigated system would result in a change of curve shape and more noticeably, a change in the polarization saturation level, due to a change in the scattering power of the whole sample. SESANS measurements performed so far on small amounts of tumor cells exhibited too little scattering for a significant signal. Measurements on combined systems of liposomes with much higher concentrations of tumor cells are to be performed in the future.

The aim of this paper is to show as a proof of principle that SESANS has the potential to contribute to studies on biological bulk-phenomena, without disturbing the investigated systems. It may therefore become a useful addition to the broad range of techniques already available, with possible applications in the field of cancer research.

2. Biological background

2.1. Liposomes

A liposome is a spherical vesicle that consists of a bilayered phospholipid membrane, entrapping an aqueous phase. In this study the lipid vesicles only contain H₂O or D₂O or both. The membrane is made of natural substances: lipids and cholesterol that confer biocompatibility, non-toxicity, and biodegradable properties. The phospholipids are amphiphilic, consisting of a hydrophilic phosphate head group and two hydrophobic tails, which causes them to self-organize into lipid bilayers in an aqueous environment. Over the last decade, liposomes have largely been used in the medical field as a tool for drug delivery for therapeutic purposes and, more recently, for molecular imaging in disease diagnoses.

Liposomes are classified as Large Multilamellar Vesicles (LMV) or as Large and Small Unilamellar Vesicles (LUV and SUV). The SUV is the main interest of the present study, because it is the most used type of liposome in the medical field. The optimum liposome diameter for an efficient extravasation out of the blood into a tumor is known to be around 100 nm. This is an important property for passive drug-targeting of the diseased area, especially for tumor-targeting of multiple types of cancers. Stealth® liposomes are grafted with PolyEthylene Glycol (PEG)-chains and offer additional advantages, such as avoiding self-aggregation of the particles and enhancing the in vivo half-life by reducing the interaction with the blood plasma proteins. For this study, DSPC and DPPC SUV PEGylated liposomes have been synthesized with a diameter \( D \) of about 100 nm and a membrane thickness \( T \) of about 5 nm \[23\] and characterized using DLS and SESANS. DSPC and DPPC liposomes possess different membrane rigidity and therefore different water exchange properties.

2.2. Escherichia coli

The microorganism Escherichia coli (E. coli) is a well known species of bacterium, which was discovered by German pediatrician and bacteriologist Theodor Escherich in 1885. E. coli is placed on the bacteria branch in the phylogenetic tree of life, as a gram negative bacterium. E. coli cells can grow easily under both aerobic and anaerobic conditions and by fermentation. Optimal growth occurs at 37°C, but in some cases E. coli can also multiply at temperatures below 25°C and up to 49°C. The cells may have flagella and are about 2 µm long and 0.8 µm in diameter \[24\], with a volume of 0.6-0.7 µm³. Strains that possess flagella can swim and are motile. There are many different types of E. coli based on the antigen type: the somatic (O) antigen, the capsular (K) antigens and the flagellar (H) antigen. There are over 170 O antigens, over 100 K antigens and over 50 H antigens.

Bacteria do not possess a cell nucleus or any other large structures, but have their components scattered throughout the cytoplasm. Since none of these are observable with the techniques used in this study, E. coli will also be considered as empty vesicles. The strain K12 was selected, since it is safe to handle and easy to grow, with cell division occurring every 20 minutes under
Table 1. Coherent neutron scattering lengths, component volumes and scattering length densities for DPPC, its head and tails, and cholesterol [25]. By comparison, DSPC consists of C₇H₁₆NO₄P (head group) and C₃₇H₇₂O₄ (tails), giving C₄₄H₈₈NO₈P.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Formula</th>
<th>b [fm]</th>
<th>Vₚ [Å³]</th>
<th>ρₛ [nm⁻²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>C₄₀H₈₀NO₈P</td>
<td>27.63</td>
<td>1.232</td>
<td>2.2·10⁻⁵</td>
</tr>
<tr>
<td>head</td>
<td>C₁₅H₁₈NO₄P</td>
<td>60.1</td>
<td>326</td>
<td>18.4·10⁻⁵</td>
</tr>
<tr>
<td>tails</td>
<td>C₃₀H₆₂</td>
<td>32.4</td>
<td>891</td>
<td>-3.6·10⁻⁵</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>C₂₇H₄₆O</td>
<td>13.25</td>
<td>629</td>
<td>2.1·10⁻⁵</td>
</tr>
</tbody>
</table>

Table 2. Absolute scattering length density differences of phases under consideration.

<table>
<thead>
<tr>
<th>Phases</th>
<th>∆ρₛ [nm⁻²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O – D₂O</td>
<td>6.89·10⁻⁴</td>
</tr>
<tr>
<td>lipid – H₂O</td>
<td>0.76·10⁻⁴</td>
</tr>
<tr>
<td>lipid – D₂O</td>
<td>6.13·10⁻⁴</td>
</tr>
</tbody>
</table>

absolutely optimal conditions. It is also easily-manipulated, making it one of the most-studied prokaryotic model organisms, and an important species in biotechnology.

2.3. Contrast of lipid bilayers

Coherent neutron scattering occurs in materials due to fluctuations in scattering length density (SLD), defined as the total bound coherent scattering length b divided by the occupied volume Vₚ. In this system, the largest SLD-difference arises between either H₂O inside and D₂O outside the liposomes and bacteria, or between their lipid bilayers and the D₂O medium. For the lipid bilayers the average SLD is taken, so that the SLD-profile over the membrane is represented by a constant. Using the chemical formulas and the mass or number densities of the compounds, the overall SLD of lipid bilayers can be calculated according to

\[
\rho_s = \sum_i n_i \frac{b_i}{V_{m,i}}.
\]

Estimating the mass densities of the organic compounds to be between 0.8 and 1.0 kg/m³ and using tabulated scattering lengths [26], yields values around ρₛ=2·10⁻⁵ nm⁻² for both liposomes, ranging from 1.5·10⁻⁵ to 1.9·10⁻⁵ nm⁻² for DSPC and from 1.8·10⁻⁵ to 2.3·10⁻⁵ nm⁻² for DPPC. This result is irrespective of the ¹/₄ molar cholesterol, since it has a similar SLD. The few molar % contributions of the PEG chains are neglected. Literature values [25] of molecular volumes for DPPC and cholesterol support these assertions, giving ρₛ=2.2·10⁻⁵ nm⁻² and ρₛ=2.1·10⁻⁵ nm⁻² using eq. 1, as summarized in table 1. Lacking the necessary data to calculate the SLD for the membrane of E. coli, the liposome values have been used under the assumption that both membranes have an SLD of the same order of magnitude. H₂O and D₂O have similar mass densities of ρ=1.0 and 1.1 kg/m³ respectively, but massively different scattering length densities of ρₛ=−5.6·10⁻⁵ and +6.33·10⁻⁴ nm⁻². The resulting SLD-differences between the lipid membrane, H₂O and D₂O are listed in table 2.
3. Experimental

3.1. Sample preparation

3.1.1. Liposomes. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, C_{44}H_{88}NO_8P), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, C_{40}H_{80}NO_8P) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (DSPE-PEG2000-OMe) were supplied by Lipoid AG, Cham, Switzerland. The used cholesterol (C_{27}H_{46}O) was purchased from Avanti polar Inc., Alabaster, AL, USA.

DSPC and DPPC liposome batches were prepared independently. For the first batch a total compound amount of 200 µmol was used with DSPC:cholesterol:DSPE-PEG2000-OMe ratios of 1.75:1:0.25 dissolved 1:1 in chloroform (CHCl_3) and methanol (MeOH). The second batch consisted of 100 µmol with DPPC:cholesterol:DSPE-PEG2000-OMe ratios of 1.85:1:0.15, which were dissolved 1:1 in dichloromethane (CH_2Cl_2) and MeOH. Both mixtures were vacuum-dried to obtain thin lipidic films, which were hydrated and resuspended in MillQ. These mixtures were subsequently extruded through polycarbonate membrane filters with a pore size of 200 nm (ten times) and 100 nm (ten times), using Lipofast extruder (Avestin, Canada). The temperature during the extrusion was set around 50°C. The extruded solution was ultra-centrifuged for 60 minutes at 4°C at 45 krpm (0.30 Mg) and at 40 krpm (0.23 Mg) respectively for both batches, using an ultracentrifuge Beckman L7, rotor 50 Ti. The pellets were separated from the supernatant and injected into cuvettes with 5 ml D_2O.

3.1.2. E. coli. Wild-type E. coli K12 strains were grown at 37°C overnight in 200 ml Luria Bertani medium in 500 ml Erlenmeyer flasks under shaking conditions until a standard optical density was reached (O.D. = 2 using a wavelength of 600 nm). The cells were spun down using a Jouan CR 4.11 centrifuge at 3.5 krpm at 4°C for 60 minutes. Wet pellets were re-suspended in D_2O after washing with 10 ml D_2O to eliminate the excess of water in between the cells. Centrifugation was repeated and the obtained new pellets were re-suspended in 2 ml D_2O each after vortexing with a home-made bead beater at 2.5 krpm at 4°C for 30 minutes. Four homogeneous pellets totaling 0.4 g were injected into a cuvet with 4 ml D_2O.

3.2. The SESANS Technique

3.2.1. Principles. Spin-Echo Small Angle Neutron Scattering (SESANS) is a non-destructive technique that probes density correlations in real space directly from 20 nm up to 20 µm. SESANS uses spin-echo to encode small scattering angles of neutrons when traversing a sample and it measures the effective loss of polarization of a neutron beam as a function of spin-echo length. This method is used to study the structure of suspensions of mesoscopic particles and its range of accessible length scales makes it suitable for a variety of materials, including large colloids [27] and granular matter [28]. The used SESANS setup consists of a monochromatic, polarized neutron beam traveling in the horizontal x-direction through two magnetic field regions, in between which a sample is placed. Experiments are performed by varying the magnetic field strength to probe correlations along the vertical spin-echo length z. The technique is not sensitive to the y-direction and can be described classically, as the net Larmor precession of neutrons of a polarized neutron beam [29], or quantum-mechanically, as the vertical splitting and recombination of two neutron eigenstates, due to their different interaction with a magnetic field [30, 31]. The polarization P of the beam, normalized with the empty beam polarization P_0, depends on the scattered fraction of the beam Σ η and on the normalized, dimensionless function G(z):

\[ \frac{P}{P_0}(z) = e^{\Sigma \ell [G(z) - 1]} \] (2)

where ℓ is the neutron path length through the sample, i.e., the sample thickness, Σ is the average number of times a neutron scatters per unit length and G(z) is a measure of the microstructure.
A solid sphere of diameter $D$ has a homogeneous SLD-distribution $\rho_s(r)$ inside the sphere, where the average difference $\Delta \rho_s$ is with respect to the SLD of the medium outside the sphere.

Examples of the projected correlation functions for solid, hollow and filled spheres, using $T = \frac{1}{10} D$ for the shell thickness and $s = 0.1$ for the core contrast. See the text for further details.

It is the projection along the neutron beam of the density auto-correlation function, which correlates SLD-fluctuations throughout the sample and is denoted $\gamma(r)$ for isotropic systems, such as here. Long-range correlations disappear beyond distances $z_{\text{sat}}$, so that $G(z \geq z_{\text{sat}}) = 0$, causing the polarization signal to saturate. The amount of scattering $\Sigma$ depends on beam and sample properties:

$$\Sigma = \lambda^2 \langle \Delta \rho_s^2 \rangle \xi \text{ binary} = \lambda^2 \phi_V (1-\phi_V) \Delta \rho_s^2 \xi$$

with neutron wavelength $\lambda$, SLD-variance $\langle \Delta \rho_s^2 \rangle$ and correlation length $\xi$. For a two-phase system the mean-squared SLD-difference can be expressed as the squared average difference $\overline{\Delta \rho_s^2}$ (contrast) between the two phases, multiplied with the volume fraction $\phi_V$ of the suspended phase and the medium. The correlation length $\xi$ is defined as the integral over $\gamma(r)$ along $x$ at $z=0$ and can be interpreted as the average length that neutrons travel through one phase before encountering another phase, making $\xi$ a measure of typical length scale.

### 3.2.2. Correlation Functions.

For dilute systems, the auto-correlation function $\gamma(r)$ gives the shared volume fraction of an object with an overlapping copy of itself. Analytical expressions for $\gamma(r)$ and its projection $G(z)$ have been derived for several dilute, monodisperse [32] and polydisperse [33] model systems such as solid and multi-layered spheres, using geometry and calculus.

A solid sphere of diameter $D$ has a constant density distribution $\rho_s(r)$ inside volume $V_{\text{sphere}} = \frac{1}{6} \pi D^3$. The density correlation function is equal to the shared volume fraction of two identical spheres when separated by a distance $0 \leq r \leq D$ from center to center. This lens shaped volume consists of two equal sphere caps, so that $\gamma_{\text{ss}}(r) = 1 - \frac{1}{2} \frac{r}{D} + \frac{1}{4} \left( \frac{r}{D} \right)^3$ is readily derived. The analytical expression for the projected correlation function is

$$G_{\text{ss}}(z) = \Re \left\{ \frac{1}{\sqrt{1 - \left( \frac{z}{D} \right)^2}} \left[ 1 + \frac{1}{2} \left( \frac{z}{D} \right)^2 \right] + 2 \left( \frac{z}{D} \right)^2 \left[ 1 - \frac{1}{4} \left( \frac{z}{D} \right)^2 \right] \ln \frac{\frac{z}{D}}{1 + \sqrt{1 - \left( \frac{z}{D} \right)^2}} \right\},$$

where $\Re$ denotes the real part. The correlation length of a solid sphere is

$$\xi_{\text{ss}} = \frac{3}{4} D.$$
Figure 3. A hollow sphere of diameter $D$ and shell thickness $T$ has a constant SLD-difference $\Delta \rho_s$ distributed over a spherical shell, with respect to the medium on the inside and the outside.

Figure 4. The hollow sphere correlation length $\xi_{HS}$ of diameter $D$ as a function of shell thickness $T$, between the limits of an infinitely thin shell ($T \downarrow 0$) and a solid sphere ($T = \frac{1}{2}D$).

A hollow sphere of outer diameter $D$ and shell thickness $T$ has its density homogeneously distributed in a spherical shell of volume $V_{\text{shell}} = \pi D^2 T - 2\pi DT^2 + \frac{4}{3}\pi T^3$ and is obtained by subtracting the density distribution of a sphere from that of a larger copy. The elaborate correlation function has been derived analytically by auto-convolution of the density distribution of the hollow sphere [32]. The projected correlation function is

$$G_{HS}(z) = \frac{2}{f\left(\frac{D-2T}{D}\right)} F\left(\frac{2z}{D}, \frac{D-2T}{D}\right).$$

(6)

The normalization of $G_{HS}(z)$ is performed by the smooth dimensionless function

$$f(\sigma) = (1 - \sigma) \left(1 - \sigma^3\right) + \frac{1}{2} \left(1 - \sigma^2\right)^2 \ln \frac{1 + \sigma}{1 - \sigma},$$

(7)

with argument $0 \leq \sigma < 1$, which is the ratio between inner and outer diameter $\frac{D-2T}{D}$. When considering a fixed diameter $D$, then for an infinitely thin shell $T \downarrow 0$ gives $\lim f(\sigma \uparrow 1) = 0$, and for $T = \frac{1}{2}D$ the shell fills the sphere, so that $f(\sigma = 0) = 1$. From this function the correlation length has been derived, which is given by

$$\xi_{HS} = \frac{2T}{D} \left[1 + \frac{4}{3} \frac{D^2 - 2DT + T^2}{D^2 - 2DT + \frac{4}{3}T^2} \ln \frac{D - T}{T}\right]^\frac{3}{4}D.$$

(8)

For the limiting case of a shell of half the diameter $\xi_{HS}$ becomes equal to $\xi_{SS}$ in nearly constant fashion, and for a hollow sphere with an infinitely thin membrane $\xi_{HS}$ can be shown to approach zero nearly linearly. Figure 4 shows the dependency of the correlation length of a hollow sphere on the relative shell thickness. $F(\zeta, \sigma)$ expresses the correlations between different parts of the geometry of a hollow sphere in a dimensionless function

$$F(\zeta, \sigma) = H_0(2, \zeta) - H_0(1+\sigma, \zeta) + H_0(1-\sigma, \zeta) + \sigma^4 H_0\left(2, \frac{\zeta}{\sigma}\right)$$

$$- \sigma^4 H_0\left(\frac{1+\sigma}{\sigma}, \frac{\zeta}{\sigma}\right) + \sigma^4 H_0\left(\frac{1-\sigma}{\sigma}, \frac{\zeta}{\sigma}\right)$$

$$- 2\sigma^3 h_1(1-\sigma, \zeta) + \frac{3}{8} \left(1-\sigma^2\right)^2 \left[h_0(1+\sigma, \zeta) - h_0(1-\sigma, \zeta)\right].$$

(9)
Figure 5. A filled sphere has separate SLD-differences $\Delta \rho_s(r)$ for its core and its shell, characterized by the parameter $s$. It is equal to a hollow sphere for $s = 0$ and to a solid sphere for $s = 1$.

which is constructed from

$$h_1(\alpha, \zeta) = 2\Re \left\{ \sqrt{\alpha^2 - \zeta^2} \right\}$$ (10)

$$h_0(\alpha, \zeta) = \Re \left\{ \ln \frac{2\alpha + h_1(\alpha, \zeta)}{2\alpha - h_1(\alpha, \zeta)} \right\}$$ (11)

$$H_0(\alpha, \zeta) = -\frac{3}{8} \zeta^2 (1 - \frac{1}{16} \zeta^2) h_0(\alpha, \zeta) + (1 - \frac{3}{8} \alpha + \frac{1}{64} \alpha^3 + \frac{3}{128} \alpha \zeta^2) h_1(\alpha, \zeta).$$ (12)

An intermediate model is that of the filled sphere, which has a solid spherical core with a shell around it. A filled sphere has the density distribution of a hollow sphere filled with a third phase. The corresponding correlation function is constructed from the hollow sphere model by addition of a dimensionless parameter $s$, defined as the difference in SLD between core and medium normalized with the $\Delta \rho$ between shell and medium:

$$s = \frac{\rho_{\text{core}} - \rho_{\text{medium}}}{\rho_{\text{shell}} - \rho_{\text{medium}}} = \frac{\Delta \rho_{\text{core}}}{\Delta \rho_{\text{shell}}}. \quad (13)$$

The projected correlation function for a filled sphere then becomes

$$G_{FS}(z) = F\left(\frac{2z}{D}, \frac{D-2T}{D}\right) + s \frac{h_0(2, \frac{2z}{D}) - s \left(\frac{D-2T}{D}\right)^4 H_0\left(2, \frac{2z}{D}, \frac{2z}{D}, \frac{D-2T}{D}\right)}{\frac{3}{2} \left[f\left(\frac{D-2T}{D}\right) + \frac{s}{1-s} - s \left(\frac{D-2T}{D}\right)^4 \right]} \quad (14)$$

The behaviour of this correlation function is very close to that of hollow spheres and solid spheres, when $s$ is close to 0 and 1 respectively. How quickly it deviates with $s$ from these two cases, depends on the value of $T$. The difference between a filled sphere and a hollow sphere is negligible for $0 \leq s \lesssim \frac{2T}{D}$. The correlation length of a filled sphere is

$$\xi_{FS} = \frac{1-s}{f\left(\frac{D-2T}{D}\right)} + s - s(1-s) \left(\frac{D-2T}{D}\right)^4 \frac{3}{2} D. \quad (15)$$

Figure 6. The correlation length $\xi_{FS}$ of a filled sphere of diameter $D$ as a function of shell thickness $T$, for $s = 0$ (hollow sphere), $s = 1$ (solid sphere) and six intermediate values. At $T = 0$ a filled sphere only has a core, which is also a solid sphere, except for an empty core ($s = 0$).
An example of the projected correlation functions for dilute, monodisperse solid, hollow and filled spheres is shown in figure 2, using a shell thickness $T = \frac{1}{10}D$ for the hollow and filled spheres and a value of $s = \frac{1}{10}$ for the filled spheres, giving their core 10% of the $\Delta \rho_s$ between shell and medium.

### 3.3. SESANS Experiments

#### 3.3.1. Experiments & Sample stability.

SESANS measurements were performed with a neutron wavelength $\lambda = 2.09 \text{ Å}$ on the DSPC and DPPC batches of liposomes and on *E. coli*. All samples were measured in quartz cuvettes of thickness $\ell = 1.00 \text{ cm}$, with volume fractions of respectively $\phi_V = 0.02$ and $\phi_V = 0.09$ for the liposome and *E. coli* samples. The DSPC liposome concentration was found by phosphate determination according to Rouser; the *E. coli* concentration was calculated from the pellet mass, assuming weight densities equal to $\text{H}_2\text{O}$ and assuming no losses during the final suspension.

Repeat measurements and DLS showed the liposome samples to remain stable for several days. An onset of sedimentation was observed for the *E. coli* after one day, most likely because of cell death as the $\text{D}_2\text{O}$ environment contained no nutrients. Measurements with lower *E. coli* concentrations provided similar data with regards to particle dimensions.

#### 3.3.2. Modeling.

Two cases were considered for curve fitting: no $\text{H}_2\text{O}$-$\text{D}_2\text{O}$ exchange versus full exchange. All fits were performed using non-linear least-squares regression with 95% confidence bounds. The first case could be represented by droplets of $\text{H}_2\text{O}$ in a $\text{D}_2\text{O}$ environment, since the effect of the cell membrane on the neutron signal would then be negligible. This model consisted of the projected correlation function $G(z)$ for dilute, monodisperse solid spheres and curve fitting was performed for the amount of scattering and particle size, i.e., the saturation level $P_{\text{sat}}$ and diameter $D$. In the second case, the $\text{H}_2\text{O}$ would be extremely diluted throughout the $\text{D}_2\text{O}$ inside and outside the cells, making the cell membrane contribute significantly to the signal. This system was modeled as hollow spheres filled with $\text{D}_2\text{O}$ using the $G(z)$ for dilute, monodisperse spherical shells. Fitting was performed for the saturation level $P_{\text{sat}}$, the outer diameter $D$ and membrane thickness $T$.

#### 3.3.3. Assumptions.

All samples were treated as isotropic systems and modeled with analytical expressions for monodisperse spherical particles. Since the concentrations used were dilute to semi-dilute, excluded volume effects were insignificant. Samples without sedimentation were isotropic suspensions, since all particles had rotational freedom. Particle size distributions were not considered to have a substantial effect and not included in the modeling, based on the very small degree of liposomal polydispersity as observed with DLS and the fact that *E. coli* multiply through cell-division, causing a uniform and highly monodisperse population under standard conditions.

In order to avoid over-parameterization certain assumptions were made. The liposomes were assumed to be spherical since there was no cause for deformation. As a first approximation, the aspect ratio of the *E. coli* cells was disregarded. However, *E. coli* are more rod-like than spherical with an aspect ratio of 2.5. The correlation function of a rod differs from that of a sphere, although arguably less for a hollow body than for a solid one.

### 4. Results

#### 4.1. Characterization of liposomes with DLS

To confirm the consistency of the two liposome formulations, dynamic light scattering was used to establish their size distributions in order to characterize them by their average diameter and polydispersity. Due to faster degradation, there was not enough *E. coli* material available
Figure 7. Distribution of DSPC and DPPC liposome diameters acquired by DLS. The inset shows approximately normal size distributions on a log-scale. Curves were fit using a lognormal distribution of arbitrary amplitude. Mean liposomal diameters are shown for comparison.

Table 3. Fit results of the size distribution acquired by DLS of DSPC and DPPC liposomes, with a lognormal distribution of sizes. Fit parameters $\mu$ and $\sigma$ were converted to a mean diameter and polydispersity index.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Meaning</th>
<th>DSPC liposomes</th>
<th>DPPC liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$</td>
<td>fit value</td>
<td>4.854 ± 0.004</td>
<td>4.905 ± 0.006</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>fit value</td>
<td>0.174 ± 0.004</td>
<td>0.251 ± 0.005</td>
</tr>
<tr>
<td>$D_{\text{mode}}$ [nm]</td>
<td>peak</td>
<td>124.4 ± 0.5</td>
<td>126.7 ± 0.8</td>
</tr>
<tr>
<td>$D_{\text{median}}$ [nm]</td>
<td>middle</td>
<td>128.3 ± 0.5</td>
<td>135.0 ± 0.8</td>
</tr>
<tr>
<td>$D_{\text{mean}}$ [nm]</td>
<td>average</td>
<td>130.2 ± 0.5</td>
<td>139.3 ± 0.9</td>
</tr>
<tr>
<td>PDI [-]</td>
<td>width</td>
<td>0.094 ± 0.002</td>
<td>0.138 ± 0.003</td>
</tr>
</tbody>
</table>

to use DLS to confirm their monodispersity. Both lipidosome size distributions had modes (peak-positions) around 125 nm with an approximately normal distribution of diameters on a logarithmic scale, as shown in the inset of figure 7.

The average diameter and distribution width were acquired by fitting a lognormal distribution of diameters to the DLS data, using arbitrary amplitude and obtaining parameters $\mu$ and $\sigma$, see Table 3. The distribution width was expressed by the polydispersity index (PDI), where a large PDI would cause the mean and median (the diameter halving the population) to shift away from the mode. The median and mean diameters of the DSPC liposomes were close to the mode and with a PDI just below 0.1, the DSPC liposomes were effectively monodisperse particles with a diameter of 130 nm. The DPPC liposomes had a slightly wider distribution with a PDI of 0.14, resulting in an average size of 140 nm.

4.2. SESANS Results

4.2.1. Liposomes. Less than 4% scattering was observed for the liposome suspensions in D$_2$O, requiring more than half a day of measurement time to reach an acceptable signal quality such as shown in figures 8 and 9. For both the DSPC and the DPPC batch, the polarization saturated at spin-echo lengths $z_{\text{sat}}$ above 150 nm and at a saturation level $P_{\text{sat}}=0.965$, where both quantities contained length scale information. Since the liposomes were approximately monodisperse, $z_{\text{sat}}$ corresponded to the diameter of the liposomes, which also determined the saturation level through the correlation length (see eq. 3 and eq. 2 for $G(z)=0$). The amount of scattering provided a constant for the product of contrast and correlation length $\Delta \rho_s^2 \xi$, where $\xi_{SS}$ and $\xi_{HS}$ were calculated according to eq. 5 and eq. 8 for the solid and hollow sphere models.
The difference in SLD between particle and medium was retrieved as

$$|\Delta \rho_s| = \sqrt{-\ln P_{\text{sat}}} \ell \lambda^2 \phi_v (1 - \phi_v) \xi$$  \hspace{1cm} (16)$$

For solid spherical particles of $\approx 150 \text{ nm}$ the correlation length was $\xi_{ss} = 110 \text{ nm}$, yielding $|\Delta \rho_s| = 1.9 \times 10^{-4} \text{ nm}^{-2}$, which was a factor 3.6 smaller than the $\text{H}_2\text{O}-\text{D}_2\text{O}$ SLD-difference. For hollow spheres of outer diameter $\approx 150 \text{ nm}$ and shell thickness $\approx 5 \text{ nm}$, the correlation length $\xi_{hs} \approx 25 \text{ nm}$, which gave $|\Delta \rho_s| = 4.1 \times 10^{-4} \text{ nm}^{-2}$, a factor 1.5 smaller than calculated between membrane and $\text{D}_2\text{O}$. The scattered intensity therefore supported the notion that liposomes were better described as hollow than as solid spheres.

4.2.2. E. coli. About four times more concentrated than the liposome samples, the E. coli suspension in $\text{D}_2\text{O}$ exhibited an equally larger scattering power. In spite of only six hours measurement time, an adequate SESANS signal quality could therefore be reached. The polarization saturated around 88% at spin-echo lengths above 700 to 800 nm, as shown in figure 10. Considering the E. coli as solid spherical particles of $\approx 750 \text{ nm}$ gave a correlation length $\xi_{ss} = 560 \text{ nm}$, yielding $|\Delta \rho_s| = 8.0 \times 10^{-4} \text{ nm}^{-2}$, which was a factor 8.6 smaller than the $\text{H}_2\text{O}-\text{D}_2\text{O}$ SLD-difference. For hollow spheres of outer diameter $\approx 750 \text{ nm}$ and shell thickness $\approx 5 \text{ nm}$, the correlation length $\xi_{hs} \approx 35 \text{ nm}$, which gave $|\Delta \rho_s| = 3.3 \times 10^{-4} \text{ nm}^{-2}$, a factor 1.8 smaller than calculated between membrane and $\text{D}_2\text{O}$. The amount of scattering therefore indicated that modeling E. coli as hollow spheres was more appropriate than as solid spheres.

4.2.3. Curve fitting. Describing the SESANS liposome data with the solid sphere model gave reasonable fits, yielding an effective size of 120 nm for both batches with a relative error of 8%, see table 4. Fitting the hollow sphere model to the data produced an apparently better fit for DSPC and worse for DPPC liposomes, with diameters of 150 and 170 nm and shell thicknesses around 10 nm, as shown in table 4. The relative errors were 7% and 15% for the diameters and 40% and 90% for the shell thicknesses, showing that the data were not very sensitive for $T$. This was supported by the small correlations of $T$ with the other parameters, as seen in table 5, which also shows that cross correlations between parameters were acceptably low for both models. The largest interdependency was observed between the saturation level $P_{\text{sat}}$ and the particle diameter $D$, which were connected through the correlation length.

Modeling the E. coli data as monodisperse, solid spheres produced a diameter of 720 nm with a relative error of 15%, but did not capture the initial decay of the measured data, see figure 10.
Figure 10. SESANS measurement of *E. coli* and fit curves for solid and hollow spheres. The inset shows the same data on a log-scale.

Table 4. Fit results of SESANS measurements of liposomes and *E. coli*, modeled as solid, hollow and filled spheres. Parameters are the polarization saturation level (scattering power) and particle dimensions. Filled sphere *E. coli* values were obtained in the hollow sphere limit, with *s* approaching zero. Goodness-of-fit indications are given by $\chi^2_k$, where *k* equals the number of measured points minus the number of fitted parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DSPC liposomes</th>
<th>DPPC liposomes</th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solid sphere</td>
<td>Hollow sphere</td>
<td>Solid sphere</td>
</tr>
<tr>
<td>$P_{sat}$ [-]</td>
<td>0.965±0.001</td>
<td>0.964±0.001</td>
<td>0.966±0.001</td>
</tr>
<tr>
<td>$D$ [nm]</td>
<td>122±9</td>
<td>152±11</td>
<td>121±11</td>
</tr>
<tr>
<td>$T$ [nm]</td>
<td>−</td>
<td>13±5</td>
<td>−</td>
</tr>
<tr>
<td>$s$ [-]</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

The hollow sphere model seemed to produce better fits, with a sharper initial decay. Minimizing $\chi^2_{38}$ to 1.6 produced a saturation level $P_{sat}=0.885±0.007$, an outer diameter $D=815±133$ nm, but an unphysical shell thickness of $T=120±30$ nm. These hollow sphere values are not shown in table 4. Using the filled sphere model helped to avoid this pitfall, producing the same saturation level and outer diameter, as well as a realistic shell thickness. The extra degree of freedom in this model was parameter $s$, the ratio between the $\Delta\rho_s$ values of the core and the shell, both with respect to the medium. A jump in the value for $T$ to the unphysical range appeared when $s<0.0735$, which was established by fitting with $s$ fixed increasingly closer to zero.

As shown in table 4, fitting with filled spheres without constraints produced a physical but imprecise value of $T=7.7±43.5$ nm. The value $s=0.064$ was small enough to effectively represent a hollow sphere, as the core-medium contrast had a negligible contribution to the overall 3-phase contrast, corresponding to a mere 0.36% of the shell-medium contrast. Since the hollow and filled sphere models gave identical curves at these parameter values, these *E. coli* dimensions were taken as an appropriate fit. Relative errors of the diameter and shell thickness were 12% and 565%, with small correlations between $T$ and the rest of the model. The very large uncertainty for $T$ meant, that even though it could be established to be small compared to $D$, it could not be quantified accurately from this data. The cross correlations between all parameters were similar to those of the liposome fits, see table 5.
Table 5. Correlations between parameters of solid and hollow sphere models fitted to SESANS data of DSPC and DPPC liposomes and E. coli bacteria. $P_{\text{sat}}$ is the polarisation saturation level, $D$ is the particle diameter and $T$ the shell thickness for hollow spheres.

<table>
<thead>
<tr>
<th></th>
<th>DSPC liposomes</th>
<th>DPPC liposomes</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solid sphere</td>
<td>Hollow sphere</td>
<td>Solid sphere</td>
</tr>
<tr>
<td>$P_{\text{sat}}$</td>
<td>1</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>$D$</td>
<td>0.4</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$T$</td>
<td>–</td>
<td>0.2</td>
<td>0.0</td>
</tr>
</tbody>
</table>

5. Discussion

5.1. Measurement results

The DSPC and DPPC liposomes gave close to identical fit results using the solid sphere model and similar, but less accurate fit results for the hollow sphere model. The hollow sphere model exhibited a larger sensitivity to the shorter spin-echo lengths within the resolvable range, than the solid sphere model. Therefore the differences between the hollow sphere results of DSPC and DPPC were attributed to the number of sampled spin-echo points below 150 nm. The statistical quality of the measurements did not allow for any extension of the modeling to include concentration effects, anisotropy, aspect ratios or polydispersity. The amounts of scattering from the DSPC and DPPC liposomes and from the E. coli cells suggested that these samples with D$_2$O medium were better described by hollow spheres than by solid spheres. The SESANS observations were in better agreement with the contrast between lipid membranes and D$_2$O than with the contrast between H$_2$O and D$_2$O. The shells with mostly hydrogen-atoms were therefore more likely enclosing D$_2$O-cores than H$_2$O-cores.

Results of curve fitting of the liposome data were ambiguous, finding a better hollow sphere fit for DSPC and a better solid sphere fit for DPPC, despite the strong similarities between the raw data of both samples. Comparison with DLS results showed best agreement between solid spheres, with diameters of 120 nm, and the modes of the size distribution at 125 nm. The hollow sphere models produced 15% and 20% larger particle sizes of 150 and 170 nm for DSPC and DPPC than the mean diameters of 130 and 140 nm as observed by DLS. However, since the DLS characterisations were performed after the SESANS measurements and liposome samples are only semi-stable, it is possible that the size distributions had shifted to somewhat smaller averages. The shell thicknesses of the hollow spheres were determined to be between 0 and 20 nm by fitting, where the data did not allow for a more accurate assessment. The findings were nevertheless in reasonable agreement with the expected value of 5 nm, which is commonly reported for the thickness of a lipid bilayer membrane without PEGylation, and with the expected value of about 10 nm for a membrane with PEG-brushes grafted to each side.

E. coli cells are known to have a cell length of 2 $\mu$m, a diameter of 0.8 $\mu$m and a cell wall thickness of about 5 nm. The filled sphere model agreed best with these expected values close to the limit of a hollow sphere, i.e., near zero contrast between inside and outside, finding a diameter of 820±100 nm and a thickness of 8±40 nm. The large uncertainties found in the diameter and thickness were due to the aspect ratio of the rod-like bacteria and because the cell wall is sandwiched between a thin plasma membrane and a thick outer membrane capsule. Since E. coli multiply through cell-division, a high degree of monodispersity was expected, but this could not be confirmed with DLS due to lacking supplies of the fast degrading bacteria and their shape, which complicated analysis.
5.2. Outlook
When a change in SESANS curve shape and/or polarization saturation level is observed in experiments, a change in structure, concentration or contrast can in principle be deduced. It is therefore worthwhile to investigate if SESANS could be applied to study such a change in a biological system.

Liposomes can be destroyed by external factors, such as ultrasound, or taken up and broken down through endocytosis by much larger cells, such as tumor and white blood cells, or macrophages. Typical diameters of tumor cells are $5 \leq D \leq 7 \, \mu m$ in rats and $10 \leq D \leq 20 \, \mu m$ in humans, whereas macrophages are about $13 \, \mu m$ in rats and about $21 \, \mu m$ in humans. SESANS measurements have already been performed on tumor cells from the rat pancreatic CA20948 tumor cell line with an approximate diameter of $6 \, \mu m$, but only low concentrations were available, causing too little scattering for a significant signal to be discerned. However, we can still show through a simple calculation that a change in liposome population in situ may constitute a noticeable signal change.

Consider a dilute system of small liposomal vesicles and of much larger cells, so it can be considered a bimodal distribution of spherical particles, say types A and B. Take their size distributions as approximately monodisperse and let A and B have similar contrasts with respect to the medium. The shape and level of a SESANS curve then depend only on whether these particles are solid or hollow in nature and on the two scattering contributions. If these contributions are not comparable, then the curve will merely show the signal of the particles which scatter the most: the presence or absence of the other particles cannot be observed. If the amounts of scattering are comparable, then the measured signal is sensitive to the removal of smaller particles from the system. This can affect both the shape of the curve, through the disappearance of a kink at the diameter of the smaller particle, and the saturation level, as the total amount of scattering is altered.

The scattering contributions for both types A and B depend on the two correlation lengths and volume fractions, and will be comparable for similar products $\phi_V (1-\phi_V) \cdot \xi$. In the dilute regime cross-correlations can be neglected, which gives a simple description of the signal as

$$\frac{P}{P_0}(z) = \exp \left\{ \ell \lambda^2 \Delta \rho_s^2 \left[ \phi_{V,A} \xi_A (G_A(z)-1) + \phi_{V,B} \xi_B (G_B(z)-1) \right] \right\}$$

(17)

For solid spheres the correlation length scales with the diameter and is therefore much larger for the large particles. The correlation length of large hollow spheres is however much smaller than of small hollow spheres with the same shell thickness.

An example calculation is shown in figure 11 for a system of 50 mg liposomes, 25 mg rat tumor cells and 5.5 grams of $D_2O$, corresponding to volume fractions of 1.0% and 0.5% for the liposomes and tumor cells. This gave a total sample volume of 5.0 ml. A standard sample thickness of $\ell=1.00 \, cm$ was used for the calculation and a wavelength $\lambda=6 \, \AA$, was taken to increase signal strength. Liposomes and tumor cells were modeled as hollow spheres of 130 nm and 6 \, \mu m with a lipid bilayer membrane thickness of 5 nm, giving correlation lengths of 24 nm and 43 nm respectively. For the $\Delta \rho_s$ between membrane and $D_2O$ the value $6.13 \cdot 10^{-4} \, nm^{-2}$ from table 2 was used.

The situation afterwards was calculated by removal of 90% of the liposomes, corresponding to exactly $1.8 \cdot 10^5$ liposomes per tumor cell. The tumor cell and $D_2O$ fractions (mole, mass and volume), as well as the total sample volume were hardly affected with less than 1% difference and were therefore neglected. In the unlikely case of massive endocytosis by tumor cells, the tumor cell volume would only change significantly when internalizing more than $\sim 10,000$ liposomes. When modelling with solid spheres about 100 times less liposomal and tumor material was required for good signal strength, but the differences before and after were much less pronounced.
6. Conclusions

Liposomal vesicles and *E. coli* bacteria have been observed in their entirety by SESANS. Samples have been prepared in H$_2$O and resuspended in D$_2$O; a state of relative neutron invisibility for the membrane and other cellular components, leaving solid spherical droplets of H$_2$O in D$_2$O. However, the liposomes and *E. coli* data from SESANS are better described with the projected correlation function for a hollow sphere, as they exhibit little scattering and a sharp initial decay of the polarization curves. It is therefore evident that the H$_2$O/D$_2$O-exchange has occurred before or during the initial stages of the SESANS measurements, i.e., within at most half an hour. To observe H$_2$O/D$_2$O-exchange measurement times need to be reduced drastically, requiring higher neutron fluxes, preferably in combination with a larger wavelength than available for this study. Fulfilling these prerequisites should make SESANS a viable alternative to *in vitro* tritium studies, since tritiated water has more undesirable properties than deuterated water.

Fit results using the hollow sphere model are in reasonable agreement with DLS for liposomes and in good agreement with literature on *E. coli*, but better statistics are required for accurate quantitative fitting, especially in the low spin-echo range. Although no new insights have been obtained on liposomes and *E. coli* themselves, the results constitute a proof-of-principle that SESANS is able to non-invasively observe entire microscopic biological entities with typical sizes between 50 nm and 20 µm in bulk. Under the right conditions it may be possible to observe changes in a system composed of two different biological species, as has been shown for a mix of small particles (e.g., liposomes) and large cells (e.g., tumor cells). Considerable change in signal requires great numbers of small particles to disappear per large cell. Here SESANS may be an additional tool for drug delivery studies, where liposomes are destroyed by external means to release drugs at the tumor site.

Acknowledgments

The authors thank Chris Duif (NPM$^2$/R3$^2$, TU Delft, The Netherlands) for assisting with the SESANS measurements, Ulla Woroniecka (RIH/R3, TU Delft, The Netherlands) for synthesizing liposomes and Krishna Kowlgi (DCT, TU Delft, The Netherlands) for assisting with DLS measurements.

This work is part of the research programme of the *Stichting voor Fundamenteel Onderzoek der Materie* (FOM), which is financially supported by the *Nederlandse Organisatie voor Wetenschappelijk Onderzoek* (NWO).
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