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Characterization of Restricted Diffusion in Uni- and Multi-lamellar Vesicles Using Short Distance iMQCs

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Abstract

Improved understanding of the entrapment, transport, and release of drugs and small molecules within vesicles is important for drug delivery. Most methods rely on contrast agents or probe molecules; here, we propose a new MRI method to detect signal from water spins with restricted diffusion. This method is based on intermolecular double quantum coherences (iDQCs), which can probe the restricted diffusion characteristics at well-defined and tunable microscopic distance scales. By using an exceedingly short (and previously inaccessible) distance, the iDQC signal arises only from restricted diffusion spins and thereby provides a mechanism to directly image vesicle entrapment, transport, and release. Using uni- and multi-lamellar liposomes and polymersomes, we show how the composition, lamellar structure, vesicle size, and concentration affects the iDQC signal between coupled water spins at very short separation distances. The iDQC signal correlates well with conventional diffusion MRI and a proposed biexponential (multicompartmental) diffusion model. Finally, the iDQC signal was used to monitor dynamic changes in the lamellar structure as temperature-sensitive liposomes released their contents. These short distance iDQCs can probe the amount and diffusion of water entrapped in vesicles, which may be useful to further understand vesicle properties in materials science and drug delivery applications.

Keywords

Intermolecular multiple quantum coherences; liposomes; restricted diffusion

1. Introduction

Vesicles such as liposomes and polymersomes have widespread applications in medicine, for example to entrap and release contrast agents (e.g., gadolinium) [1; 2; 3; 4] or drugs (e.g., chemotherapeutic doxorubicin) [5; 6; 7; 8; 9; 10; 11]. There is significant interest in monitoring dynamic changes in the vesicle structure, which permits entrapment and transport of small molecules and drugs in vivo. The ability to initiate release of the vesicle contents only at the pathological sites is one of the most important aspects for drug delivery. More specifically, the vesicles can be specially formulated to release their contents via a controllable trigger mechanism, which may be temperature [9; 12; 13; 14; 15], pH [16; 17; 18], sound [19], or light [20; 21].

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Current approaches to measuring entrapment and release have several limitations. The entrapment and release can be monitored with fluorescence recovery after photobleaching [22; 23; 24], but this can interfere with the release mechanism (in the case of light-triggered release) [25; 26; 27] and requires entrapment of labeled fluorophores. Magnetic resonance imaging (MRI) can be used to non-invasively study changes in the structure of porous media, particularly when the release mechanism precludes optical detection methods [28]. Vesicle release can be monitored using entrapped MRI contrast agents that affect the signal intensity; for example, gadolinium and manganese would shorten the T_1 relaxation time of water after release [29; 30]. Another method based on diffusion MRI [31], which maps the diffusion of molecules, was recently proposed to monitor entrapment, transport, and release; in that case, the diffusion coefficient of a probe molecule was monitored [28]. All of these currently available methods require that an exogenous agent, such as labeled fluorophores, contrast agents, or probe molecules, be entrapped in the vesicles.

Here we demonstrate a novel magnetic resonance approach based on the detection of intermolecular multiple quantum coherences (iMQCs) between water spins separated by only a few microns, which does not require any exogenous contrast agents. Such iMQCs are normally unobservable in water because diffusion modulates the dipolar interaction more quickly than the characteristic time needed to produce a signal (tens of milliseconds). However, iMQCs are observable under conditions of restricted diffusion, such as in vesicles. We show that this approach can dramatically image restricted diffusion, generating signal either between pairs of water molecules entrapped in the same vesicle or entrapped in different vesicles. This signal can be used to monitor the encapsulation and release of molecules inside liposomes and other vesicles. Moreover, this technique has further implications in materials science for the study of restricted diffusion in highly structured materials, which may yield insights into permeability, fluidity, and percolation.

II. Vesicles: Liposomes and Polymersomes

Vesicles are ideal as contrast and carrier agents because of their size, easily tunable properties, and enhanced pharmacological characteristics [32; 33]. Liposomes and polymersomes are self-assembling bilayer vesicles that encapsulate an aqueous core. Liposomes are composed of phospholipids, while polymersomes are composed of block copolymers. Polymersomes are stronger and more stable than liposomes, with increased hydrophobic and hydrophilic encapsulation volumes and decreased water permeability. When the vesicles are initially formed, they have multiple layers and are very large with a wide size distribution, which are called unextruded or multilamellar liposomes (Figure 1, right). Forcing the vesicles through nanoporous extruders produces single-layered vesicles of a given size and narrow size distribution, which are called extruded or unilamellar vesicles (Figure 1, left). Extrusion can form large (>300 nm diameter) or small vesicles (100 nm diameter).

Greater understanding of the properties of vesicles has important applications for materials science and drug delivery. For example, the amount of water entrapped in vesicles varies with the size and concentration of the vesicles, which in turn affects the diffusion of water inside and outside the vesicles and relates to drug encapsulation efficiency and drug delivery *in vivo*. The total volume of water entrapped by uni-lamellar liposomes (V_E , in liters) varies with the size of the liposomes according to the following equation [34; 35]:

$$V_E = \frac{500}{3} R_L m_{lipid} A_{lipid} N_A = \frac{500 R_L M_{lipid} A_{lipid} N_A}{3 MW_{lipid}} \quad (1)$$

where R_L is the radius of the liposomes (in m, as measured by dynamic light scattering), m_{lipid} is the moles of lipid (= lipid mass (M_{lipid})/molecular weight (MW_{lipid})), A_{lipid} is the area occupied by 1 lipid (in m², typically between 0.5 and 0.8 nm²) [34; 36], and N_A is Avogadro's number. For small liposomes, less water is entrapped in the liposomes compared to larger liposomes. The entrapped volume of polymersomes can be similarly calculated, taking into account the higher molecular weight and larger area occupied for the polymers. The iDQC signal is based on two coupled water spins, and thus a crucial parameter is the percent of water entrapped in the vesicles. For a given vesicle formulation, the percent of water entrapped depends on the vesicle size and lipid (or polymer) concentration:

$$\text{Percent Entrapped (\%)} = \frac{V_E}{V_{total}} = \frac{500R_L C_{lipid} A_{lipid} N_A}{3MW_{lipid}} \quad (2)$$

Where V_{total} is the total volume of water and C_{lipid} is the lipid (or polymer) concentration (= M_{lipid}/V_{total}).

The liposomes we have studied here have diameters of 100 nm, 350 nm, and 430 nm, and we assume that the area occupied by 1 lipid is 0.6 nm² [32] and that there are 125 μmol of lipids (corresponding to 100 mg of various lipids with an average molecular weight of 800 g/mol). The volume of water entrapped in the liposomes varies with the radius of the liposomes (Equation 1). For a diameter of 100 nm, 350 nm, and 430 nm, the volume entrapped by the liposomes is 380 μL, 1300 μL, and 1600 μL, respectively (Table 1). The percent entrapped depends on the lipid concentration in water (Equation 2). When the concentration is extremely high, e.g., 100 mg lipid per 1 mL of water, almost all of the water is entrapped. For polymersomes, the encapsulation volume is changed by both the increased molecular weight of the polymers and the larger area occupied by each polymer. The dominant factor, however, is the increased molecular weight, which decreases the number of moles for a given weight; as a result, compared to the liposomes, less water is encapsulated for a similar amount of polymer and similarly sized polymersomes (Table 1). The diameter of the polymersomes studied here is 400 nm, the area occupied by each polymer [37] is assumed to be 1 nm², and there are 22 μmol of polymers (corresponding to 100 mg of various polymers with an average molecular weight of 4500 g/mol). For these polymersomes, the aqueous encapsulation volume is only 440 μL (although polymersomes also have the advantage of a large hydrophobic encapsulation area [38]). These polymersomes would encapsulate approximately 13% of the water if formed in 3.3 mL water (corresponding to 30 mg polymer per 1 mL water).

These calculations are valid for a homogeneous population of vesicles, such as the single-layered vesicles with a narrow, well-defined size distribution produced after extrusion. The pre-extrusion vesicles are inhomogeneous, with many lamellae and variable radii. As a result, the encapsulation volume (and thus percent encapsulated) is not easily calculable. Qualitatively, the encapsulation volume for larger vesicles will be increased, while the volume occupied by the multilamellar structure must be subtracted from the total volume and will reduce the encapsulated volume compared to a large unilamellar vesicle. In any case, the unextruded vesicles present a unique case of restricted diffusion, in that entrapped water is not only present inside the vesicles, but is also trapped between the lipid or polymer layers.

III. Intermolecular Multiple-quantum Coherence Imaging

The detection of intermolecular multiple quantum coherences has been previously shown to enhance contrast [39], probe tissue microstructure [40; 41; 42; 43] and anisotropy [44; 45], and image functional characteristics (including brain activation [46; 47] and temperature [48]). In all of these cases, the tunable distance between the two coupled spins (called the correlation distance d_c) has been longer than what we will use here; it ranges from tens of microns to a few millimeters. These distances were chosen because larger distances quickly approach sample size limitations, while smaller distances are unobservable due to diffusion effects.

The standard CRAZED sequence [39; 49; 50; 51; 52] used for double quantum coherence (iDQC) detection is shown in Figure 2. The correlation distance is set by the gradient strength (G) and gradient time (T):

$$d_c = \frac{\pi}{\gamma GT} \quad (3)$$

where γ is the gyromagnetic ratio. At very short correlation distances, Brownian motion causes the spins to diffuse over distances comparable to the separation distance, averaging the dipolar field interaction to zero. The root mean square (rms) distance over which a molecule diffuses in a given time t is $\sqrt{2Dt}$. Using the diffusion coefficient of water at room temperature ($D = 2.3 \times 10^{-9} \text{ m}^2/\text{s}$), the rms distance is $9.59 \mu\text{m}$ for $t = 20 \text{ ms}$ and $40.7 \mu\text{m}$ for $t = 360 \text{ ms}$ (typical range of experimental timescales). As the correlation distance decreases below the diffusion limit, the signal decreases to zero.

The self-diffusion coefficient (D_s) of the vesicles can be estimated using the Stokes-Einstein equation, approximating the vesicles as rigid spheres [34]:

$$D_s = \frac{k_B T}{6\pi\eta R_L} \quad (4)$$

where k_B is the Boltzmann constant, T is the temperature, and η is the solvent viscosity (for water, $\eta = 8.94 \times 10^{-4} \text{ Pa s}$). For 100 nm vesicles, the vesicle diffusion coefficient is $4.8 \times 10^{-12} \text{ m}^2/\text{s}$. Thus, the rms distance is $0.438 \mu\text{m}$ for $t = 20 \text{ ms}$ and $1.86 \mu\text{m}$ for $t = 360 \text{ ms}$ (typical range of experimental timescales). Due to these small distances, vesicle diffusion is negligible.

In cases of extremely restricted diffusion, Brownian motion and thus the diffusion coefficient would be greatly reduced. Previous results have shown diffusion coefficients that are 2 orders of magnitude lower for molecules entrapped in liposomes, as monitored by diffusion MRI [28]. In that case, N-methyl-4-trifluoromethylpyridium was used as a probe molecule to monitor light-induced release from liposomes; the free and entrapped molecule diffusion coefficients were 5.3×10^{-10} and $4.3 \times 10^{-12} \text{ m}^2/\text{s}$, respectively. Here, we utilize the iDQC signal of water to directly image restricted diffusion associated with vesicles; this approach can be used to monitor the encapsulation and release of molecules inside liposomes and other vesicles. These results will also be compared to standard diffusion data. Although diffusion-dependent variations of the CRAZED sequence have been proposed [53; 54; 55], we use the conventional CRAZED sequence with short correlation distances to directly image and characterize the restricted diffusion of vesicular water.

There are several competing factors that affect the observed iDQC signal: the number of lipid layers (1 for extruded vesicles; many for unextruded vesicles) and the size and concentration of the vesicles. Ultimately, these all affect the amount of water in each

possible environment: inside and outside the vesicles, as well as in between the lipid (or polymer) layers for unextruded vesicles. The DQ signal comes from two water spins separated by the correlation distance; for the extruded vesicles, each water spin could be either inside or outside the vesicles. As a result, the signal could come from two spins inside vesicles (inter-vesicle water signal), from one spin inside and one spin outside vesicles (cross-vesicle water signal), or from two spins outside the vesicles (non-vesicle water signal). For the unextruded vesicles, water can also be trapped in between the many layers of the vesicles. Because the iDQC signal depends on the dipolar field interaction not averaging to zero via diffusion, there is likely no signal obtained from non-vesicle water spins at the short correlation distance. The diffusion will be affected by how much water is present in each of these environments, which depends on the volume of water entrapped and the concentration of the vesicles.

IV. Materials and Methods

Vesicle and Sample Preparation

The liposomes are temperature-sensitive and were prepared by the Needham group at Duke University as previously described [30; 56]. Unextruded liposomes were prepared by lipid film hydration, while extruded liposomes of the required size (100 nm, 350 nm and 430 nm) were prepared subsequently by extrusion [57]. The concentrations of the 100 nm and 430 nm liposomes were 40 mg/mL, while the 350 nm liposomes were prepared at an initial concentration of 100 mg/mL. Unextruded liposomes at the same concentration were also prepared concurrently. The concentrated (100 mg/mL) liposomes were serially diluted six times, for final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/mL. The polymersomes were prepared by the Therien group at Duke University as previously described [58]. Unextruded and 400 nm extruded polymersomes were prepared at a final concentration of 30 mg/mL. All sizes were determined by dynamic light scattering. For each batch of vesicles, the extruded and unextruded vesicles were placed in 5 mm NMR tubes, which were then placed in a water-filled 50 mL tube (3 cm diameter). Microscopy images were acquired on a Zeiss Axio Observer widefield microscope (for unextruded vesicles only). A 63 $\times$ objective was used.

iDQC method

MRI data were acquired on a Bruker 8.4T (^1H : 360 MHz). The iDQC-CRAZED sequence [49; 50; 52] was used (Figure 2). The 90° and 120° pulses were BIR-4 adiabatic pulses, and the 180° Hermite pulse was slice selective. The repetition time for all images was 6 s, and the echo time was 360 ms for the 350 nm and 430 nm liposome images and 20 ms for the 100 nm liposome images. For the polymersomes, the echo time was 40 ms. The mixing time τ was 5 ms. The slice thickness was 2 mm with a 6 cm FOV. The matrix size was 128 $\times$ 128.

Diffusion method

Diffusion weighted images (21 total) were acquired using the standard method developed by Stejskal and Tanner [31]. The gradient strengths (G) were varied (with constant gradient duration ($\delta = 7$ ms) and inter-gradient spacing ($\Delta = 14$ ms)) to provide diffusion weighting with b -values ($= \gamma^2 \delta^2 G^2 (\Delta - \delta/3)$) between 0 and 25000 s/mm². We can also define q , which relates to the degree of diffusion weighting and whose inverse is analogous to the correlation distance in the iDQC sequence; here, $q (= \gamma G \delta / (2\pi))$ is between 0 and 2400 cm⁻¹, leading to a minimum $q^{-1} = 4.2$ μm .

Data processing

All images were processed using Matlab and ImageJ software (NIH, Bethesda, MD). The diffusion maps were created from fits of the natural log of the signal intensity vs. b -value using exponential-weighted linear least squares [59]. The diffusion signal was also fit to a model biexponential, describing the combined signals of unrestricted water outside the vesicle and restricted water inside the vesicle:

$$Y = A_1 e^{-bD_1} + A_2 e^{-bD_2} \quad (5)$$

where A_1 and D_1 are the amplitude and diffusion coefficient associated with the free (fast diffusing) component, and A_2 and D_2 are the amplitude and diffusion coefficient associated with the restricted (slow diffusing) component in the vesicle. Since biexponential fitting is sensitive to initial parameters and prone to local minima [60], we searched for globally optimal parameters with a simulated annealing approach. Initial guesses for the parameters were $D_{1,2} = 0.002 \text{ s/mm}^2$ and $A_{1,2} = 50$ (arbitrary units). During each annealing iteration, we used Matlab's robust nonlinear least squares algorithm (curve fitting toolbox) with the initial parameters. Then to generate an initial guess for the next iteration, the best-fitting parameters were perturbed with random numbers from a normal distribution with standard deviation σ (the amplitudes A are perturbed additively; the diffusion coefficients D are perturbed multiplicatively). Initially, $\sigma = 1.0$ and is reduced exponentially with each iteration at a cooling rate of 0.9. The algorithm is terminated when $\sigma = 0.01$. The final amplitudes $A_{1,2}$ are taken as $100 \times A_{1,2}/(A_1 + A_2)$.

V. Results/Discussion

Microscopy images show that the liposomes are initially multi-layered with a wide range of sizes (Figure 3, left). Similar images were acquired for the unextruded polymersomes (right). Extrusion through a nano-porous extruder forms single-layered liposomes or polymersomes of a specific size and narrow size distribution (ranging from 100 nm to 430 nm here). These nano-size vesicles cannot be seen using light microscopy (although they are visible using electron microscopy), and thus, extruded vesicle images are not shown here.

Figure 4 shows a conventional spin echo image and two iDQC images at a long and short correlation distance. The iDQC signal grows in and is highest at a TE approximately equal to T_2 . The T_2 relaxation times were measured (using a multi-echo sequence) to be 1.05, 0.375, and 0.107 s for water, unextruded, and extruded (100 nm) liposomes, respectively. Thus, the appropriate TE was selected for each set of vesicles. From the iDQC images, the long correlation distance image has signal from all three samples (water and unextruded and extruded liposomes). In general, the long correlation distance images can be used to detect the bulk signal, as 103 μm is beyond the rms diffusion limit of water. To highlight restricted diffusion spins, a short correlation distance should be used to remove any free diffusion spin signals. At 8 μm correlation distance, there is no signal from the water spins, while there is signal from the extruded and unextruded liposomes.

Figure 5 shows iDQC images for a range of correlation distances (from 60 μm to 8 μm), echo times (20 or 360 ms), concentrations (25 mg/mL or 40 mg/mL), and extrusion sizes (100 nm, 350 nm, and 430 nm). The extruded samples are denoted by the asterisk (shown only for the longest d_c image). With the exception of T_2 relaxation effects, the signal intensity for the longest correlation distances shows no difference between the water and liposome samples, as well as no differences for the different concentrations or extrusion sizes. As the correlation distance decreases, the water signal also decreases due to diffusion effects. The rms distance for water at TE = 20 ms causes the water signal to drop off at correlation distances below 10 μm ; in contrast, the rms distance for water at TE = 360 ms

causes the water signal to drop off at correlation distances below 40 μm . The concentration and extrusion size determines the encapsulation volume (ranging from approximately 9.5% to 64% here, according to Table 1). The top two rows show unextruded and 100 nm extruded liposomes at a lipid concentration of 25 mg/mL for TEs of 20 and 360 ms. The 100 nm extruded liposomes have a short T_2 and therefore appear dark on the long TE images. The T_2 of the unextruded liposomes is much longer, and the signal is much higher at the longer TE because iDQCs grow in with time. Unextruded and 100 nm extruded liposomes are shown in the third row at a higher concentration with TE = 20 ms. The higher lipid concentration results in a higher encapsulation volume, which is noticeably reflected in the higher signal intensity for the extruded liposomes. The bottom two rows show extruded liposomes of different extrusion sizes, 350 nm and 430 nm. These larger extruded liposomes encapsulate more water, as evidenced by the higher extruded liposome signal.

At the short correlation distances, the only signal is due to restricted diffusion spins in extruded and unextruded liposomes. Each image is scaled to show the maximum signal, and the signal intensity does decrease as the correlation distance decreases, as expected because there are fewer spins that have sufficient restricted diffusion to be observable at 8 μm correlation distance. Although the signal is significantly reduced at these distances, it is certainly detectable at several standard deviations above the noise. At the short correlation distances, the increasing signal intensity for the extruded liposomes as the size is increased reflects differences in the encapsulation volume, while the unextruded liposomes are the same formulation and concentration for each row and have similar signal intensities.

Figure 6 shows the iDQC signal for water and 25 mg/mL extruded (350 nm) and unextruded liposomes for correlation distances ranging from 60 to 2 μm (TE = 360 ms). At correlation distances greater than 60 μm , the water and liposomes have about the same amount of signal. However, as the correlation distance decreases, the water signal quickly falls off due to diffusion. In contrast, the extruded (350 nm) and unextruded liposome signals decrease, but not as much as would be expected for free diffusion. Even at distances as short as 4 μm , the liposome signal is sufficiently restricted to detect iDQCs. The extruded data shown here is for the median extrusion size (350 nm); the 430 nm extruded signal follows a similar trend, while the 100 nm extruded signal falls off more rapidly due to the small encapsulation volume.

Similar images can be obtained for unextruded and extruded polymersomes. Figure 7 shows the iDQC images for 400 nm extruded and unextruded polymersomes at the same range of correlation distances. The polymer concentration of both polymersome samples is 30 mg/mL. As with the liposomes, the polymersomes and water have signal for the longest correlation distance, which highlights all water spins. As the correlation distance decreases, the water signal decreases due to the unrestricted diffusion, while the unextruded polymersomes have sufficient restricted diffusion to have iDQC signal at distances as short as 6 μm . On the other hand, the extruded polymersome signal decreases similar to the water signal. This most likely reflects the small aqueous encapsulation volume of the extruded polymersomes (approximately 13%), leading to a small (unobservable) restricted volume.

Based on the obvious signal dependence on the percent of entrapped water, we also considered a range of lipid concentrations for the 350 nm extruded and unextruded liposomes. The concentrations considered ranged from 100 mg/mL, where almost all of the water is entrapped, to 1.5625 mg/mL, where almost none of the water is entrapped. All of the data presented thus far has been magnitude images, but a wealth of information may lie in the phase-sensitive images. Figure 8 shows the real and imaginary images for these liposomes from 100 to 1.5625 mg/mL at a short correlation distance (8 μm). The phases are very similar for the lower concentrations, while the highest concentrations have vastly

different (negative) phase characteristics. The highest concentrations have a substantial portion of the water entrapped and were extremely viscous. Future analysis of phase-sensitive images at extremely high concentrations may yield important insights into percolation dynamics and fluidity.

The iDQC signal characteristics at a range of concentrations can lead to information about the local spin environments. The iDQC signal between coupled water spins that are entrapped in two different liposomes should have a concentration dependence that scales quadratically. The iDQC signal between coupled water spins that are entrapped in the same liposome should have a concentration dependence that scales linearly. These relations should hold for the medium to low concentrations, although the dynamics are significantly complicated at the highest concentrations. Figure 9 shows graphs of the iDQC signals at 6, 8, 10, and 12 μm (normalized to the 100 μm signal and reported as a percent). For the highest concentration 50 mg/mL (as well as 100 mg/mL, not shown), the signal falls off rapidly due to local resonance frequency heterogeneity over short distances. More specifically, the signal is sensitive to dynamical dephasing that occurs over milliseconds, which arises from a combination of effects such as susceptibility variations due to vesicle reorientation. For concentrations lower than 25 mg/mL, the signal for the extruded liposomes depends quadratically on the concentration. This indicates that the majority of signal for extruded liposomes arises from coupled water spins in separate liposomes. The signal characteristics for the unextruded liposomes are complicated due to the highly variable size and lamellar structure. Overall, the signal for the unextruded liposomes appears to have a combination of linear and quadratic dependences on concentration, indicating signals that arise from coupled spins in both the same liposome and in different liposomes.

Diffusion MRI can probe the microscopic movement of water molecules. Figure 10 shows conventional diffusion maps (monoexponential fit) and iDQC images at long (103 μm) and short (8 μm) correlation distances for liposomes (top) and polymersomes (bottom) over a range of lipid or polymer concentrations. From the diffusion maps, the average water diffusion coefficient is $2.18 \times 10^{-3} \text{ mm}^2/\text{s}$. At the highest lipid concentration of 50 mg/mL, the diffusion coefficients are 0.93×10^{-3} and $0.53 \times 10^{-3} \text{ mm}^2/\text{s}$ for extruded and unextruded liposomes, respectively. At the highest polymer concentration of 30 mg/mL, the diffusion coefficients are 1.84×10^{-3} and $1.27 \times 10^{-3} \text{ mm}^2/\text{s}$ for extruded and unextruded polymersomes, respectively. As the concentration decreases, the diffusion coefficient increases and approaches that of free water. For the lowest lipid concentration of 1.5625 mg/mL, the diffusion coefficients are 1.91×10^{-3} and $1.51 \times 10^{-3} \text{ mm}^2/\text{s}$ for extruded and unextruded liposomes, respectively. For the lowest polymer concentration of 1 mg/mL, the diffusion coefficients are 2.14×10^{-3} and $2.08 \times 10^{-3} \text{ mm}^2/\text{s}$ for extruded and unextruded polymersomes, respectively. Comparing liposomes and polymersomes of similar concentrations, the polymersomes have faster diffusion (i.e., higher diffusion coefficients).

For the iDQC images, the long correlation distances highlight the bulk unrestricted water signal and show little signal intensity difference at the different concentrations. At the short correlation distances, there is no signal for water, as expected, and the signal decreases with decreasing concentration. For the extruded liposomes, the minimum detectable concentration was approximately 12.5 mg/mL of lipid, which corresponds to an encapsulation percent of 16.3%. For the unextruded liposomes, the minimum detectable concentration was slightly lower at 6.25 mg/mL, indicating that the unextruded liposomes have an increased fraction of spins with restricted diffusion for the same lipid concentration compared to extruded liposomes. As the concentration is further decreased, there is no detectable signal, indicating decreased restricted diffusion. This was also confirmed by the diffusion-weighted images. For the polymersomes, the short iDQC signal is much smaller than the liposome signal, reflecting the smaller encapsulation percent. Moreover, the

extruded polymersomes signal is unobservable at all tested concentrations, while the unextruded polymersomes have signal at concentrations greater than 10 mg/mL of polymer.

Although the conventional diffusion maps were produced using monoexponential fits, the physical model for vesicles clearly supports multicompartmental (and thus multiexponential) diffusion. To confirm multiexponential behavior, Figure 11a shows semi-log diffusion plots for water and extruded and unextruded vesicle ROIs. Water exhibits obvious monoexponential behavior, as evidenced by the linear fit on the semi-log plot. In contrast, all four vesicle ROIs deviate from linear (i.e., monoexponential) behavior. The lines through the low b -values reflect the results from the exponential weighted linear least squares analysis, which fails to account for both components in the vesicles. The diffusion data was subsequently fit to a biexponential model using the simulated annealing fitting algorithm with a fast and slow diffusing component (as shown in Figure 11b for the highest concentrations). Figure 11c graphs the amplitudes A_1 (free) and A_2 (entrapped) and diffusion coefficients D_1 (free) and D_2 (restricted) for liposomes and polymersomes versus concentration. These results show that the vesicles have an entrapped component whose amplitude (A_2) increases linearly with concentration. As expected, the unextruded vesicles have larger entrapped amplitudes compared to the extruded vesicles, and the polymersomes have smaller entrapped amplitudes compared to liposomes. This corresponds well with the calculations and experimental iDQC results. The restricted diffusion coefficients (D_2) were extremely long (i.e., very slow diffusion associated with entrapped water) and could not be quantitatively extracted. Conversely, the free diffusion coefficient (D_1) changes with lipid (or polymer) concentration: it approaches the diffusion coefficient of water for 0 mg/mL lipid (or polymer). Moreover, D_1 decreases (corresponding to slower diffusion) with increasing concentration, which is reasonable because the diffusion would be slightly more constricted due to presence of the vesicles. Overall, these biexponential fitting results validate the iDQC characterizations for liposomes and polymersomes. Moreover, using the iDQC method, we can directly observe the restricted diffusion component.

As mentioned above, the ability to monitor dynamic changes in vesicle structures is crucial to understanding the entrapment, transport, and controlled release of small molecules and drugs. These liposomes are temperature sensitive in that they are specially formulated to rapidly release their contents at mild hyperthermic conditions, around 41°C. Figure 12 shows the long and short distance iDQC signals as the lipid permeability changes when the liposomes go through the phase transition temperature. The samples here are in a slightly different setup; there are three NMR tubes filled with water (right in each image), unextruded liposomes (top in each image), and extruded liposomes (bottom in each image). The left column shows the long correlation distance images that highlight all signal, while the right column shows the short correlation distance images that highlight restricted diffusion. The top row of images is at room temperature (20°C), the middle row is at an elevated temperature around 50°C, and the bottom row images were acquired after the sample was cooled back to room temperature.

As the lipid bilayer goes through the transition temperature induced by the heating, permeability across the membrane increases, and the entrapped volume is released. At the elevated temperatures, the restricted diffusion signal decreases significantly for both the extruded and unextruded liposomes. Additionally, the short distance iDQC signal for the extruded liposomes (showing restricted diffusion) decreases more than the unextruded liposome signal. The unextruded liposomes have a multilamellar structure, which would still cause restricted diffusion even under increased membrane permeability. In this case, the signal decreased by approximately 23% at the elevated temperature. On the other hand, the water in extruded liposomes is entrapped by a single bilayer (unilamellar) membrane, and thus the effects of increased membrane permeability are much more apparent. The signal

decreased by approximately 55% for the extruded liposomes at the elevated temperature. As the liposomes are cooled back down to room temperature, the liposomes reform, leading to the reappearance of the restricted diffusion iDQC signals from the liposomes.

VI. Conclusions

We have demonstrated that iDQC signals can be obtained at extremely short correlation distances, significantly below the normal lower detection limit. Because the dipolar field that refocuses the iDQC signal depends on angle between the two spins, diffusion typically eliminates signals at short correlation distances. On the other hand, samples with restricted diffusion would permit the angular dependence of the dipolar field to be maintained, and iDQC signals at short correlation distances can be observed. The ability to observe spins with restricted diffusion has important implications in materials science for the study of highly structured materials.

In this case, water in uni- and multilamellar vesicles provided the necessary restricted diffusion for short distance iDQC signals. Liposomes and polymersomes have medical applications for entrapment, transport, and release of small molecules or drugs. The iDQC signal depends on the correlation distance, with the longer correlation distances highlighting all signals and the shorter correlation distances highlighting only the restricted diffusion signals. The lower detection limit for free water is easily determined from the rms diffusion distance and corresponds well with the iDQC imaging results. The short distance iDQC signal can probe the entrapment of water inside vesicles as a function of the composition, lamellar structure, size, and concentration. These parameters directly relate to the percent of water encapsulated. Moreover, the restricted diffusion results correspond well with the diffusion coefficients obtained with diffusion weighted MR imaging. The short distance iDQCs were used to monitor dynamic changes in the lamellar structure as specially formulated temperature-sensitive liposomes released their contents.

Short distance iDQCs may have several advantages over diffusion studies to probe restricted diffusion. The iDQC signal arises from interactions between coupled spin separated by the tunable correlation distance, which permits selective imaging of only restricted diffusion spins. In contrast, conventional diffusion imaging provides a bulk measure of the diffusion characteristics. The iDQC signal is also more sensitive to its local environment, which was exploited to differentiate between inter-liposomal, cross-liposomal, and intra-liposomal interactions (information not obtainable using conventional diffusion imaging). The iDQC signal directly relates to number of restricted diffusion spins; although these encapsulation percents can be determined using bi-exponential fitting of conventional diffusion data, many b-values were required (significantly increasing the scan time), and the bi-exponential fitting tends to be computationally expensive. Finally, we note that the high concentration limit, where the restricted diffusion behavior becomes very complex, is quite interesting in its own right (although probably not for biological applications). iDQC measurements could nicely complement diffusion studies in this regime because they are inherently sensitive to an adjustable distance scale (the distance scale set by the correlation distance).

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Highlights

- We present our ability to selectively image restricted water diffusion in vesicles.
- This technique is based on previously unobservable short-distance iMQC signals.
- The signal can provide information on the vesicle spin environment.
- These results correlated well with conventional diffusion weighted images.
- We can also monitor dynamic changes in the vesicle structure.

Figure 1.
Schematic of unextruded and extruded vesicles.

Figure 2.
The standard CRAZED iDQC sequence.

Figure 3. Microscopy of unextruded liposomes (left) and polymersomes (right). Scale bar is 10 μm .

Figure 4.

Spin echo ($TR = 1$ s; $TE = 14$ ms) and iDQC images ($TR = 6$ s; $TE = 20$ ms) at long and short correlation distances (100 and $8\ \mu\text{m}$). The sample is two 5 mm NMR tubes filled with extruded (denoted by asterisk) and unextruded liposomes that are submerged in a tube of water (30 mm in diameter).

Figure 5. iDQC images for a range of correlation distances (60 to 8 μm), echo times (20 or 360 ms), concentrations (25 or 40 mg/mL), and extrusion sizes (100, 350, and 430 nm, denoted by asterisk on the longest d_c image). The concentration and extrusion size determines the encapsulation volume. There is no difference between the unextruded liposomes for each concentration.

Figure 6. iDQC signal (log scale) vs. correlation distance for water and 25 mg/mL extruded (350 nm) and unextruded liposomes (TE = 360 ms). The error bars indicate the ROI standard deviation, and the error bars for the liposomes and long- d_c water fall within the symbols. The dotted black line indicates the noise level. The water signal falls off quickly at the shortest correlation distances, while the liposome signals do not.

Figure 7. iDQC images of extruded (400 nm, denoted by asterisk on the longest d_c image) and unextruded polymersomes for a range of correlation distances (60 to 8 μm). The concentration and extrusion size determines the encapsulation volume ($\approx 13\%$ for these extruded polymersomes).

Figure 8.
Real and imaginary iDQC images at 8 μ m correlation distance for liposomes at various concentrations.

Figure 9.

Graphs of iDQC signals at short correlation distances (6, 8, 10 and 12 μm), normalized to the 100 μm signal, at different lipid concentrations. The extruded liposome signal has a quadratic dependence on concentration, while the unextruded liposome signal has a combined linear and quadratic dependence on concentration.

Figure 10.

Conventional diffusion weighted images and iDQC images at long ($103 \mu\text{m}$) and short ($8 \mu\text{m}$) correlation distances for liposomes (top) and polymersomes (bottom) at a range of lipid and polymer concentrations (in mg/mL). The asterisk denotes the extruded samples. The average water diffusion coefficient is $2.18 \times 10^{-3} \text{ mm}^2/\text{s}$. The diffusion coefficients increase with decreasing concentration, while the short-dc iDQC signal decreases with decreasing concentration. As the concentration decreases, the percent of water entrapped decreases.

Figure 11.

a: Natural log of signal vs. concentration for a water ROI, 2 liposome ROIs, and 2 polymersome ROIs (25 mg/mL; extruded and unextruded), showing obvious multi-exponential behavior for the liposomes and simple monoexponential behavior for water. b: Bi-exponential fits of diffusion data for vesicles in (a). Inset shows earlier diffusion points. c: Amplitudes (A_1 and A_2) and diffusion coefficients (D_1 and D_2) for extruded and unextruded liposomes and polymersomes vs. concentration (in mg/mL of lipid or polymer, respectively). Inset on diffusion graphs shows narrow range around D_2 .

Figure 12.

Long (left column) and short (right column) correlation distance iDQC images of temperature-sensitive liposomes (extruded and unextruded) and water (right tube in each image). As the liposomes are heated (middle row), permeability increases across the membrane, which leads to a loss of the restricted diffusion iDQC signal. The extruded liposome signal is reduced more than the unextruded liposome signal (55% and 23%, respectively) because water molecules are entrapped by only a single bilayer membrane. As liposomes are cooled down to room temperature, the liposomes reform, and the restricted diffusion iDQC signal returns.

Table 1

Entrapped volume and percent entrapped calculations for liposome and polymersome populations of varied sizes and concentrations.

Diameter (nm)	Entrapped Volume (mL) ^a	Lipid Concentration (mg/mL)	Percent Entrapped ^b (%)
100	380	100.0	38.0
		50.0	19.0
		25.0	9.5
		12.5	4.8
350	1300	100.0	100.0
		50.0	65.0
		25.0	32.5
		12.5	16.3
430	1600	100.0	100.0
		50.0	80.0
		25.0	40.0
		12.5	20.0
400 ^c	330	30.0 ^d	13.1

^aCalculated from Equation 1 [34; 35].

^bCalculated from Equation 2.

^cPolymersomes.

^dPolymer concentration.