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# Urea-Mediated Anomalous Diffusion in Supported Lipid Bilayers

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## Abstract

Diffusion in biological membranes is seldom simply Brownian motion; instead, the rate of diffusion is dependent on the timescale of observation and so is often described as anomalous. In order to help better understand this phenomenon, model systems are needed where the anomalous subdiffusion of the lipid bilayer can be tuned and quantified. We recently demonstrated one such model by controlling the excluded area fraction in supported lipid bilayers (SLBs) through the incorporation of lipids derivatised with polyethylene glycol. Here we extend this work, using urea to induce anomalous subdiffusion in SLBs. By tuning incubation time and urea concentration, we produce DCPC bilayers that exhibit anomalous behaviour on the same scale observed in biological membranes.

## Key Words

Anomalous, diffusion, lipid bilayers, membranes, urea.

## Introduction

Diffusion is a vital process that underpins many cellular functions, including protein organisation [1], signalling [2, 3], and cell survival [4]. In living systems diffusion rarely follows the Brownian motion predicted by a simple random walk model but instead exhibits ‘anomalous’ subdiffusion, whereby the rate of diffusion is dependent on the timescale of observation [5]. Anomalous subdiffusion has been observed in 3D in the cytosol [6] and in 2D in plasma membranes [7–9]. The underlying mechanism for anomalous subdiffusion in membranes is thought to involve molecular crowding [10], with contributions from slower-moving obstacles [11, 12], pinning sites, and compartmentalisation [8, 10, 13]; reviewed comprehensively elsewhere [14]. The notion that the cell membrane is a homogenous entity in which lipids and proteins are free

25 to diffuse unhindered, as per the ‘fluid mosaic model’ [15], has in recent years been  
26 re-evaluated to accommodate increased levels of complexity [10].

27 Anomalous diffusion can be modelled by a power law:

$$\langle \Delta r^2 \rangle = 4\Gamma \Delta t^\alpha, \quad (1)$$

28 where the conventional diffusion coefficient  $D$  is replaced by an anomalous trans-  
29 port coefficient  $\Gamma$ , whose dimensions change for different degrees of anomalous be-  
30 haviour. The anomalous coefficient  $\alpha$  defines whether the diffusion is normal ( $\alpha = 1$ ),  
31 sub-diffusive ( $\alpha < 1$ ) or super-diffusive ( $\alpha > 1$ ). The units of  $\Gamma$  vary with the degree  
32 of anomalous behaviour, which presents a challenge of interpretation. However, by  
33 de-dimensionalising the observation time [5] with a ‘jump time’  $\tau$ ,

$$\langle \Delta r^2 \rangle = 4D\Delta t \left( \frac{\Delta t}{\tau} \right)^{\alpha-1}, \quad (2)$$

34 the length-scale  $\lambda$  associated with the 2D anomalous behaviour can be defined ( $\lambda =$   
35  $\sqrt{4D\tau}$ ).

36 Artificial bilayers have been critical in furthering our understanding of anomalous  
37 diffusion [16–21]. In supported lipid bilayers (SLBs), phase separation [17], protein  
38 binding [18], and defect formation [22] have been used to generate anomalous diffu-  
39 sion. Simulations have also played a vital role [5, 23–30], in particular those linking  
40 the role of mobile and immobile obstacles within the bilayer to the phenomenon [11,  
41 12]. Simulations have also provided the means to better interpret single particle track-  
42 ing (SPT) data [31], as well as methods for discriminating between distinct classes of  
43 anomalous diffusion [32].

44 In order to elucidate the specific molecular mechanisms giving rise to anomalous  
45 subdiffusion *in vivo*, there is a need for experimental models which are able to exhibit  
46 readily tuneable anomalous subdiffusion of a biologically relevant magnitude [14]. Re-

47 cently we used SPT to sample anomalous behaviour over four orders of magnitude  
48 of time by forming SLBs containing varying mole fractions of lipids functionalised  
49 with polyethylene glycol (PEG), thereby controlling nanoscale obstacle formation [22].  
50 Here, we make use of urea as a chaotropic agent, with reported ability to alter the phys-  
51 ical properties of lipid bilayers [33–36]. Urea is present at high concentrations in the  
52 tissues of deep-sea elasmobranchs (sharks, skates, rays) [37] and is also part of the  
53 Natural Moisturising Factor in skin [38], where it is thought to offer cell membranes  
54 protection from osmotic shock due to highly saline or dehydrating conditions by sta-  
55 bilising the lamellar liquid phase. Here we use single-molecule total internal reflection  
56 fluorescence (smTIRF) and perform SPT to evaluate urea as a means to induce anoma-  
57 lous diffusion in pre-formed SLBs.

## 58 **Materials and Methods**

### 59 **Materials**

60 1,2-dicapryl-*sn*-glycero-3-phosphocholine (DCPC) was purchased from Avanti Polar  
61 Lipids (Alabaster, AL). Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine  
62 triethylammonium salt (TR-DHPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-  
63 N-[methoxy(polyethylene glycol) - 5000] ammonium salt (PEG(5K)-DPPE) was pur-  
64 chased from Lipoid (Ludwigshafen, Germany). Unless stated, all other chemicals were  
65 purchased from Sigma-Aldrich. All aqueous solutions were prepared using doubly  
66 deionized 18.2 M $\Omega$  cm MilliQ water.

### 67 **Supported Lipid Bilayers**

68 SLBs were prepared on glass coverslips by fusion of small unilamellar vesicles (SUVs)  
69 [39] made from 1.77 mM DCPC doped with 1.0 mol% PEG(5K)-DPPE and  $3 \times 10^{-6}$   
70 mol% TR-DHPE. The addition of PEG-functionalised DPPE (below the mol% required

71 to induce anomalous diffusion [22]) helps improve bilayer fluidity by raising the bi-  
72 layer, thereby reducing interactions between the lipids in the lower leaflet and under-  
73 lying glass [40]. Texas Red-labelled DHPE was also included in order to assess the  
74 diffusive properties of the bilayer using smTIRF.

75 Lipid mixtures were first dried with nitrogen and placed under vacuum overnight.  
76 The dried lipids were hydrated with water and vortexed before tip sonication (Vibracell  
77 VCX130PB with CV188 tip, Sonics & Materials, Newtown, CA) for 15 minutes at 25%  
78 amplitude. The resulting clear vesicle suspension was centrifuged (3 minutes; 14000  
79  $\times g$ ) before the supernatant was retained and any titanium residue (from the sonicator  
80 probe) was discarded. SUV preparations were stored at 4°C for up to 48 hours.

81 Glass coverslips were rigorously cleaned using stepwise bath sonication with DECON-  
82 90, MilliQ water, and propan-2-ol for 20 minutes each. Immediately before use, the  
83 glass was dried under nitrogen and cleaned with oxygen-plasma treatment for 3 min-  
84 utes (Diener Electronic, Femto). A well was created on each coverslip using vacuum  
85 grease (Dow Corning). The coverslip was heated to 37°C before 50  $\mu\text{L}$  of SUV stock  
86 were diluted 1:1 in buffer (250 mM NaCl, 10 mM EDTA, 20 mM Tris pH 7.0) and  
87 added to the chamber immediately. DCPC SLBs were produced by fusion of the SUVs  
88 onto the glass coverslip. The vesicles were incubated for 30 minutes before the mem-  
89 branes were washed thoroughly with degassed MilliQ water followed by buffer.

90 Urea was added (or removed) by buffer exchange via pipetting; all but 50  $\mu\text{L}$  of  
91 fluid above the SLB was replaced with 200  $\mu\text{l}$  of the new buffer (containing 0.2, 0.5,  
92 or 1M urea), a minimum of 5 times. Bilayers were imaged 15 seconds after buffer  
93 exchange.

## 94 **Total Internal Reflection Fluorescence Microscopy**

95 532 nm continuous-wave laser light was focussed at the back aperture of an objective  
96 lens (60 $\times$  TIRF oil-immersion NA 1.49, Nikon,  $\sim 1.4 \text{ kW cm}^{-2}$ ) such that total inter-

97 nal reflection occurred at the coverslip/sample interface. The excited TR-DHPE fluo-  
98 rescence was transmitted through 545 nm dichroic and 550 nm longpass filters before  
99 being imaged with an electron-multiplying CCD camera (Andor iXon). The inverted  
100 microscope objective was heated to maintain 37°C at the sample throughout imaging;  
101 above the transition temperature for this lipid to ensure the bilayer was in the liquid  
102 phase. Bilayers were imaged at an exposure time of 20 ms for 5000 frames.

### 103 **Single Particle Tracking**

104 SPT was performed using TrackMate [41], a plugin for ImageJ [42]. The space-time  
105 co-ordinates of the output tracks were used to calculate mean-squared displacements  
106 calculated for different observation times using custom-written procedures in MAT-  
107 LAB (MathWorks) as described previously [22].

## 108 **Results**

109 Diffusion of TR-DHPE in the DCPC SLB was fast ( $6 \mu\text{m}^2 \text{s}^{-1}$ ) and normal ( $\alpha = 1.01$   
110  $\pm 0.01$ ) in the absence of urea (Fig. 1A&B). In the presence of 1M urea, the diffusion  
111 became slower and more anomalous over time (Fig. 1C).  $\alpha$  decreased roughly linearly  
112 to 0.38 and the transport coefficient ( $\Gamma$ ) showed an approximately exponential decrease  
113 to  $0.02 \mu\text{m}^2 \text{s}^{-\alpha}$  (Fig1D) over a 10 minute period. Although  $\Gamma$  values cannot be directly  
114 compared (because they depend on  $\alpha$ , which is also changing), a linear change in  $\alpha$   
115 would be expected to cause an overall exponential change in  $\Gamma$ , as we report.

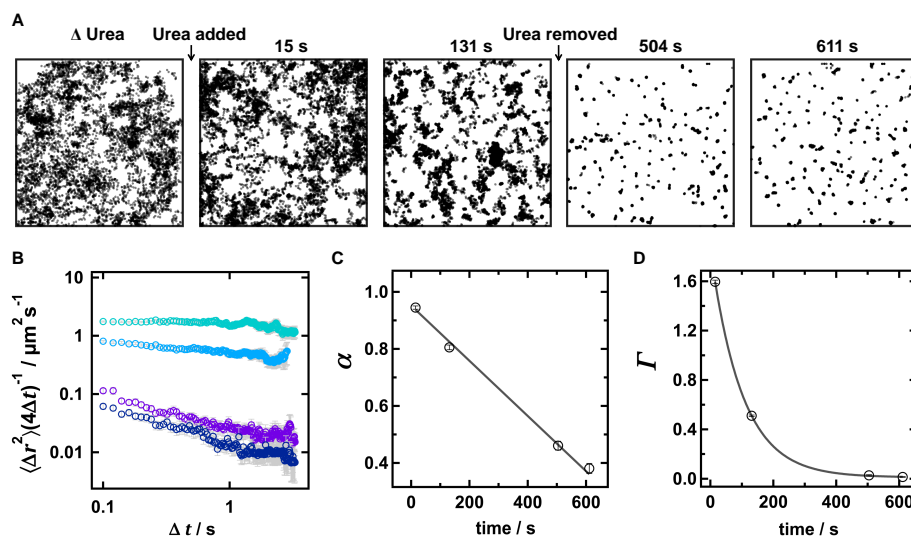


Figure 1: **Time dependence of anomalous behaviour induced by 1 M urea** (A) Spot locations of tracked TR-DHPE in the absence of urea (left) and after the addition of 1M urea at four time points. Urea was removed by buffer exchange at 200-300 s. Image size:  $3 \times 3 \mu\text{m}$  (B) Anomalous sub-diffusion increases over time from 15 seconds (turquoise) to 10 minutes (dark blue). (C) Linear decrease of  $\alpha$  over time, at a rate of  $9.7 \times 10^{-4} \text{ s}^{-1}$ . (D) Exponential decrease of  $\Gamma$  over time,  $t_{1/2} = 69 \text{ s}$ . Error bars throughout represent standard errors from a minimum of 250 tracks.

116        Increasing the urea concentration of the buffer surrounding the SLB incrementally  
 117 from 0 to 1 M, with a fixed short incubation time (15 s), resulted in increasingly slower  
 118 diffusion (Fig. 2A). The behaviour is largely normal at this short interval, with only a  
 119 modest decrease of  $\alpha$  (to 0.94) at the highest concentration tested (Fig2B). An expo-  
 120 nential decrease in  $\Gamma$  with increasing urea concentration was observed (Fig. 2C). From  
 121 the linear relationship between  $\log_{10}(\Gamma/D)$  and  $\alpha$  (Fig. 2D) the characteristic length-  
 122 scale ( $\lambda$ ) associated with the system was calculated to be 45.1 nm, with a jump time  
 123 ( $\tau$ ) of 86.1  $\mu\text{s}$ .



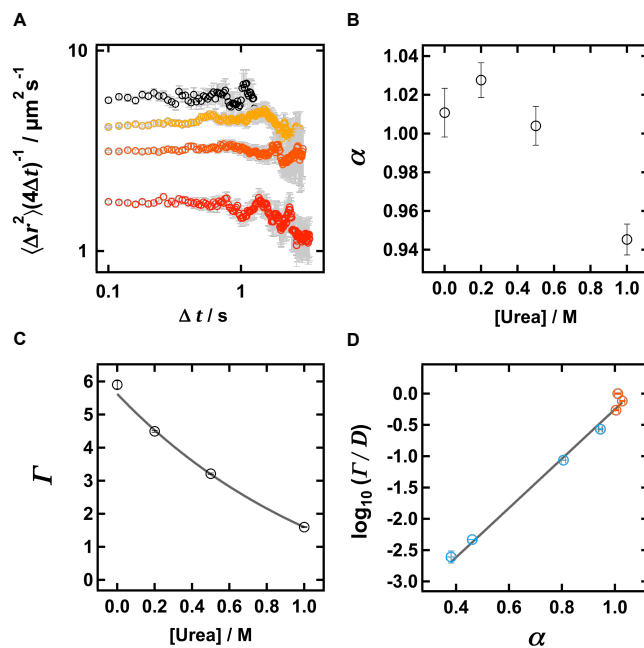


Figure 2: **Effect of urea concentration on lipid diffusion in an SLB** (A) Diffusion of lipids becomes slower as urea concentration of the surrounding buffer is increased from 0 (black) to 1M (red). (B) Decrease of  $\alpha$  with increasing urea concentration. (C) Exponential decrease of  $\Gamma$  with increasing urea concentration. (D) Plot of  $\log_{10}(\Gamma/D)$  vs.  $\alpha$  with linear fit. Blue: Data from 1M urea timecourse (see Fig. 1); Orange: Data from urea titration (This figure). Error bars represent standard errors.

## 124 Discussion

125 We observe that urea causes diffusion in DCPC SLBs to become irreversibly slower  
 126 and more anomalous in a time and concentration-dependent manner. Given our previ-  
 127 ous experiments reporting defect-mediated anomalous diffusion using PEG-doping of  
 128 SLBs [22], it is appealing to suggest that a similar mechanism must operate for urea.  
 129 For this case, urea would associate with the bilayer, where its chaotropic nature would  
 130 act to induce the removal of bilayer patches from the glass coverslip surface, producing  
 131 defects visible as excluded areas of the surface corresponding to those observed in Fig  
 132 1A. However, there is little evidence that urea acts directly to solubilise or otherwise

133 permeabilise lipid bilayers [34], and this hypothesis would rely on urea acting at the  
134 glass-lipid interface.

135 An alternative explanation for our results would be the action of urea to alter lipid  
136 phase behaviour, inducing phase co-existence phases[33]. Unfortunately, the evidence  
137 supports a mode of action whereby urea stabilizes the liquid disordered phase [33, 34],  
138 suppressing phase separation, rather than encourage it. In our experiments, we observe  
139 a decrease in the area fraction of mobile lipids, which is the opposite trend.

140 A final hypothesis would be the action of urea not on the bilayer, but on the PEG-  
141 DHPE. A chaotropic effect on the PEG might act to increase the area fraction occupied  
142 by the PEG, which would then again drive the formation of defects in the membrane  
143 [22].

144 The effect that urea has on diffusion appears not only irreversible, but appears to  
145 progress even once urea is removed from the bulk solution. The half-life for this pro-  
146 cess at 1M urea was short (69 s) and was finished after approximately 500 s. We  
147 speculate that either our (1000-fold dilution) washing procedure must be ineffective, or  
148 there is a more long-lived, direct, interaction between urea and the bilayer. Given the  
149 low partition coefficient for urea in lipid bilayers [43] and the evidence from studies of  
150 multilamellar phases that it remains primarily in the aqueous layers between bilayers  
151 [34], it is difficult to rationalize this as a possible mechanism.

152 Further work is needed to distinguish between these different possible mechanisms  
153 either by viewing the defects directly (e.g. by atomic force microscopy) or by restoring  
154 the defects by addition of fresh SUVs.

## 155 **Conclusion**

156 We have presented preliminary findings demonstrating a novel approach to controlling  
157 anomalous subdiffusion in SLBs on a scale relevant to biological systems [13, 16]  
158 by incorporating urea into the aqueous medium surrounding a supported lipid bilayer.

159 Although this work involved the use of DCPC, it would be interesting to extend the  
160 method to other, more biologically-relevant lipid compositions. As a complementary  
161 method to the inclusion of PEG-lipids, we see potential for this approach for producing  
162 a simple membrane model with defined anomaleity,

## 163 **Authors' Contributions**

164 EEW performed the experiments, HLEC and MRC performed the analysis, MIW se-  
165 cured the funding; all authors wrote and reviewed the manuscript.

## 166 **Competing Interests**

167 The authors declare no competing interests.

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