Quantifying membrane permeability of amphotericin B ion channels in single living cells

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Recently, the structure–function relationships between amphotericin B (AmB) and ergosterol have been solved using synthetic techniques that require a mycosamine-mediated direct binding interaction between AmB and ergosterol to form AmB ion channels. However, studies to directly probe the AmB-induced membrane permeability changes have not been conducted. In the present work, we investigate the following fundamental question: does AmB induce concentration- and time-dependent permeability changes across ergosterol-containing membranes? Herein, we employ fluorescent dyes of known average diameter to quantify the diameters of AmB ion channels. In addition, we take a single-particle tracking approach to define the intracellular microrheology in the absence and presence of AmB ion channels. Present results show that increasing AmB concentration tends to increase the preferential accumulation of AmB ion channels in the presence of the excess membrane-embedded ergosterol. We found that AmB induces time-dependent membrane permeability; increases approaching 50% in both the velocity fluctuations and diffusion coefficients of vesicles occur on the same time scale as the efflux of potassium ions (≅30 min). Furthermore, we propose a two-dimensional, semi-regular tessellation model to geometrically assess the pore size of the AmB ion channels in response to the AmB dose. This approach offers one possibility for the design of AmB ion channels with tunable aqueous pore size, which could provide an opportunity to replace damaged membrane water channels of the aquaporin family in future applications.

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1. Introduction

The polyene antibiotic amphotericin B (AmB) has been considered the standard in antifungal therapy for deep-seated systemic fungal infections since its release in the 1950s [1–3]. The antifungal activity of AmB is most closely related to its ability to form hydrophilic aqueous pores via binding to ergosterol in the fungal cell membrane. Pore formation not only promotes plasma membrane destabilization but also allows for the leakage of intracellular components such as potassium ions, which are responsible for cell lysis. This process ultimately results in rapid cell death [4,5]. AmB has also been reported to have a substantially higher affinity for ergosterol than cholesterol [6,7]. Specifically, AmB interacts more rapidly with fungal cells than human cells [8]. The substitution of cholesterol with ergosterol in tumor cells has thus been proposed as a possible opportunity for anticancer therapy [9,10].

The binding paradigm for the interaction between AmB and ergosterol has been determined by nuclear magnetic resonance spectroscopy [6]; the aqueous pores consist of an annulus of eight AmB molecules that are hydrophobically linked to eight ergosterol molecules [11–15]. In addition, the eight subunits that make up the aqueous pore are arranged as the staves of a barrel, with their hydrophilic sides forming a central channel approximately 8 Å in diameter [11,14]. Recently, the structure–function relationships between AmB and ergosterol have been solved using a synthetic technique based on the synthesised enabled deletion of functional groups from the polyene macrolide skeleton. This technique reveals key underpinnings of amphotericin B ion channel and antifungal activities. Specifically, a mycosamine-mediated direct binding interaction between AmB and ergosterol is required to both form ion channels and kill yeast cells [16,17]. A voltage clamp planar lipid bilayer system has also been used to probe the formation of discrete ion channels and investigate the membrane-permeabilizing activity of AmB [16]. However, studies to directly probe the real-time changes in the intracellular responses of living cells caused by the presence of AmB ion channels have not been conducted.
Recently, it has been recognized that the extract of *Taiwanofungus camphoratus* possesses extensive biological activity, such as hepatoprotective, antihypertensive, anti-hyperlipidemic, immunomodulatory, antioxidant, anti-inflammatory and anticancer properties [18–20]. In our preliminary experiments, we found that treating cancer cells with the ethanolic extract of *T. camphoratus* might increase the susceptibility of the plasma membrane of cancer cells to AmB [21]. In addition, pretreatment with the ethanolic extract of *T. camphoratus* followed by AmB treatment dramatically inhibited cell proliferation in HT29 cells. This result implies that cholesterol, the dominant sterol in mammalian cell membranes, was substituted with ergosterol. This effect can be attributed to the presence of ergosterol in the ethanolic extract of *T. camphoratus* [18].

In this study, we also apply the organic solvent n-hexane to extract solid state cultivated *T. camphoratus*. The main purpose of this study is to provide direct evidence to prove whether pretreatment with *T. camphoratus* n-hexane extract (TCHE) followed by AmB treatment leads to a change in the cell-membrane permeability. In addition, we propose a new cell membrane permeabilization method for the detection of intracellular microrheology in the absence and presence of AmB ion channels. Furthermore, we hypothesize that increasing AmB concentrations tend to increase the preferential accumulation of AmB ion channels; this consequence can be explained by our semi-regular tessellation model. Finally, conclusions are offered, and areas for further study are identified.

2. Materials and methods

2.1. AmB and TCHE preparation

The AmB was purchased from Sigma Aldrich Co. (St. Louis, MO). A pulverized crude extract of solid state cultivated *T. camphoratus* was provided by Well Shine Biotechnology Development (Taipei, Taiwan) and prepared as previously described; the resulting compound contained 15–20% triterpenoids and 1–2% polysaccharides [21]. In this study, we used the organic solvent n-hexane to extract solid-state cultivated *T. camphoratus*. The resultant extract proved to be more effective than ethanolic extract in preventing the proliferation of HT29 cells. In addition, the n-hexane extract of *T. camphoratus* was separated by preparative thin layer chromatography (TLC) with Kiesel gel 60 F254 (Merck), as shown in Fig. S1. One fraction (# 8) in panel (A) was found to be active in synergistic to AmB, where the stationary phase was silica gel and the mobile phase was CHCl₃. This fraction could be further separated using the TLC silica gel plate developed in a mixture of CHCl₃–MeOH (98:2 v/v), as shown in Fig. S1(B). Here the separated extract (# 8) in panel (B), denoted as TCHE in this study, was scratched and subjected to further purification. We found that pretreatment with TCHE followed by AmB treatment remarkably and dramatically inhibited the proliferation of HT29 cells.

2.2. Cell culture and drug treatments

The human colon cancer cell line HT29 was purchased from the Food Industry Research and Development Institute (FIRDI) (Hsinchu, Taiwan, Republic of China) and cultured up to 70% confluence in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Gibco, Grand Island, NY) under standard culture conditions (37 °C, 95% humidified air and 5% CO₂). The HT29 cells wereTryspinized, counted and resuspended at the appropriate density (2 × 10⁵ cells/ml) in fresh culture medium. These HT29 cells were then seeded onto collagen-1-coated glass coverslips for 3 h at 37 °C. Next the cells were pretreated with 10 μg/ml of TCHE for 21 h at 37 °C. During cell seeding and TCHE pre-treatment processes, the flow cell was stored in a covered Petri dish and kept in the incubator at 37 °C and 5% CO₂. Finally, the flow cell was assembled into the flow delivery system; this allowed us conduct real-time probing the time- and dose-dependence of AmB induced permeability changes across ergosterol-containing membranes. Note that, during experiments the flow cell was carefully sealed and protected to prevent changes in drug concentration by medium loss.

2.3. The specially designed flow cell

We have designed a flow cell system assembled by placing one layer of parafilm (100 μm thick) cut in chamber shape between a microscope glass slide and a collagen-1-coated coverglass (170 μm thick). Two holes drilled through glass slide form the entrance and exit ports for the flow cell [26]. In addition, an automated syringe attached to the flow cell is used for buffer exchange and delivering various drugs, i.e., AmB, Alexa Fluor® 635 phalloidin, QD-EGF, in an optimally controlled manner. Finally, this temperature-controlled microfluidic flow cell is incorporated into microscopy systems. In this study, HT29 cells were delivered to the flow cell and seeded onto collagen-1-coated glass coverslips for 3 h at 37 °C. Next the cells were pretreated with 10 μg/ml of TCHE for 21 h at 37 °C. During cell seeding and TCHE pre-treatment processes, the flow cell was stored in a covered Petri dish and kept in the incubator at 37 °C and 5% CO₂. Finally, the flow cell was assembled into the flow delivery system; this allowed us conduct real-time probing the time- and dose-dependence of AmB induced permeability changes across ergosterol-containing membranes. Note that, during experiments the flow cell was carefully sealed and protected to prevent changes in drug concentration by medium loss.

2.4. Epi-fluorescence imaging

Fluorescence images of cells were obtained with an inverted microscope (TE2000U, Nikon) equipped with an objective lens (Plan Apo, 60×/1.40 oil, Nikon), band-pass filters for QD 565 and Alexa 635, and an EMCCD camera ( Luca EM, DL6581, Andor). These images were acquired with a spatial resolution of 112 nm per pixel and with a temporal resolution of 50 ms.

2.5. Single particle tracking and image analysis

Intracellular microrheology is related to the characterization of the viscoelastic properties of the cytoplasm of cells [27]. Particle-tracking microrheology is a powerful technique that allows us to probe the mechanical properties of the cytoskeleton in its natural environment. We applied our Matlab-based FFT software for cross-correlation processing [26] to track the lateral displacement of the vesicles within the living cells. This displacement was then used to determine the two-dimensional mean square displacement (2-D MSD). For the 2-D random walk, the 2-D MSD increases linearly with time according to the relationship $MSD = 4DΔt$, where $D$ and $Δt$ are the diffusion coefficient and time lag, respectively. Hence, the diffusion coefficient of the vesicles could be obtained from the linear slope of the MSD plot; values of $D$ could then be used to probe the effect of AmB ion channels on membrane permeability and intracellular microrheology. By tracking multiple vesicles within the living cells, particle-tracking microrheology can measure
simultaneously the micromechanical responses to stimuli in various parts of the cell.

3. Results

3.1. Effect of AmB on cell-membrane permeability

We examined the cytotoxicity of combined AmB and TCHE treatment in human colon HT29 cells using an MTT assay (Fig. 1). The data show that pretreatment with 10 μg/ml TCHE followed by 6 μg/ml AmB dramatically inhibited the proliferation of HT29 cells. The inhibitory concentration that induced 50% cell viability was further adapted to conduct the present experiments at the single cell level.

A straightforward approach to characterize the concentration-dependence of AmB-induced permeability changes across ergosterol-containing membranes is to use fluorescent dyes of known average diameter to distinguish the cell-membrane permeability and quantify the pore size of the AmB ion channels. First, HT29 cells were seeded onto collagen-I–coated glass coverslips for 3 h at 37 °C. Next, the cells were pretreated with 10 μg/ml of TCHE for 21 h at 37 °C, followed by a further incubation with 6 μg/ml AmB for 1 h at 37 °C. To assess membrane permeability, HT29 cells were treated with either 10 nM QD-EGF at 4 °C for 30 min (Fig. 2) or Alexa Fluor® 635 phalloidin at 37 °C for 30 min (Fig. S2). A large accumulation of QD-EGF (red pseudo-color) was observed along the periphery of the cell membrane but not in the cytoplasm for untreated (Fig. 2D) and TCHE-treated HT29 cells in the absence of AmB (Fig. 2E). However, the cellular distribution of the QD-EGF fluorescence signal was strongly localized inside the cell in the presence of AmB (Fig. 2F). Specifically, pretreatment with TCHE followed by AmB treatment led to a change in the cell-membrane permeability such that the QD-EGF could penetrate the cell membranes at 4 °C.

To test whether AmB-induced permeability changes were due to the substitution of cholesterol, the dominant sterol in mammalian cell membranes, with ergosterol, we further examined membrane permeability of HT29 cells pretreated with either 10 μg/ml pure ergosterol (Exp. I) or 10 μg/ml TCHE (Exp. II), respectively, to address this question. Furthermore, HT29 cells pretreated with 10 μg/ml of TCHE for 1 h at 37 °C followed by 6.5 nM AmB for 1 h at 37 °C (Exp. III), together with HT29 cells treated with 6.5 μM AmB alone for 1 h at 37 °C (Exp. IV), were performed as control experiments. Finally, membrane permeability of these four experiments was assessed using Alexa Fluor® 635 phalloidin, as shown in Fig. S3. Here, the bright-field cell images of HT29 cells and the corresponding epi-fluorescence images of the cellular uptake of Alexa Fluor® 635 phalloidin were shown in the left (A, C, E and G) and right (B, D, F and H) panels, respectively. As shown in the epi-fluorescence images, Alexa Fluor® 635 phalloidin can freely penetrate the cell membrane in the cases of Exp. I and II (B and D). However, in the case of Exp. III, cell membrane permeability change was not seen with shorter TCHE incubation time tested. In addition, the penetration of Alexa Fluor® 635 phalloidin was not observed in HT29 cells treated with 6 μg/ml AmB alone (in the case of Exp. IV). This phenomenon demonstrated that the substitution of cholesterol with ergosterol has occurred when HT29 cells pretreated with either pure ergosterol or TCHE for 21 h at 37 °C.

3.2. Quantifying the pore size of the AmB ion channels

In an attempt to quantify whether the pore size of the AmB ion channels depends on the AmB dose, we applied fluorescent probes based on Alexa Fluor 635 phalloidin and QD-EGF. The procedures for cell treatments were similar to those employed to generate Fig. 2, where HT29 cells were incubated with AmB at four different concentrations ranging from 0 h at 37 °C: 40 pg/ml, 50 pg/ml, 1 ng/ml, and 2 ng/ml. The corresponding bright-field images of HT29 cells are shown in Fig. 3A–D. Here, HT29 cells were treated with Alexa Fluor® 635 phalloidin at 37 °C for 30 min; this treatment allowed us to determine the pore size of the AmB ion channels at low AmB concentrations. To determine the pore size of the AmB ion channels at higher AmB concentrations, HT29 cells were treated with 10 nM QD-EGF at 4 °C and fixed in 4% PFA. As shown in the epi-fluorescence images, Alexa Fluor® 635 phalloidin cannot freely penetrate the cell membrane at 40 pg/ml AmB (Fig. 3E), but it can do so at 50 pg/ml (Fig. 3F). This phenomenon implied that the pore diameter was nearly 16 Å when the concentrations of AmB fell within the range of 40–50 pg/ml. Similarly, when the AmB concentration was higher than 2 ng/ml, QD-EGF (red pseudo-color) could freely penetrate the cell membrane, which demonstrated that the pore diameter was nearly 16 nm when the concentrations of AmB fell within the range of 1–2 ng/ml (Fig. 3G and H). This observation revealed that permeability changes across ergosterol-containing membranes are dependent on the AmB dose. Note that these two simultaneous fluorescence images of QD-EGF (Fig. 3G and H) were taken using an inverted epi-fluorescent microscope equipped with an EMCCD camera at the same image focal plane; however, all two-dimensional optical slices have an in-focus plane and a contribution from the out-of-focus fluorescence that obscures the image and reduces contrast. This may explain why some QDs-EGF were concentrated close to membrane especially when the pore size of the AmB ion channels was almost the same as the size of the QD-EGF, as shown in Fig. 3H.

Besides, it is not known whether channel or pore formation influences EGFR-mediated internalization when the pore diameter is smaller than the size of QD-EGF (16 nm). The cellular uptake of QD-EGF in HT29 cells at 37 °C (Fig. S4 and Mov. S1) demonstrated that EGFR-mediated internalization still occurred in the presence of 1 ng/ml AmB, indicating that EGFR dimerization was not inhibited.

3.3. Effect of AmB on intracellular micro rheology

Because we have shown that AmB induces permeability changes across ergosterol-containing membrane in a concentration-dependent manner, the present study also aims to present a real-time cell membrane permeabilization method for the detection of intracellular micro rheology in the absence and presence of AmB ion channels. In this study, particle-tracking micro rheology, as determined by measurement of the lateral displacement of the vesicles within the living cells, was applied to follow the kinetics of the permeability changes that were induced by the presence of the AmB ion channels. Simultaneous bright-field images of the same living, single HT29 cell pretreated with 10 μg/ml TCHE for 21 h at 37 °C were captured before (A), (see also Mov. S2), during (B–D) and after (E) (see also Mov. S3) treatment.
with 6 μg/ml AmB at 37 °C (Fig. 4). This process allowed us to determine whether the AmB induced time-dependent permeability changes across ergosterol-containing membranes.

We then applied particle-tracking microrheology to determine the lateral displacement of vesicles within living cells. Thus, the transport velocity, MSD, and diffusion coefficient of a single vesicle could be obtained at each time point (A through E). The image-processing procedures for this analysis are shown in Fig. 4. For example, six individual vesicles were arbitrarily selected from the bright-field image at time point E (left), as shown on the upper right of Fig. 5, revealing the lateral displacements of the vesicles in microdomains. The corresponding two-dimensional trajectories of each vesicle were also plotted. Below are two MSD plots obtained from trajectories of vesicles 1 and 2, respectively, from which the diffusion coefficient of the vesicles could be obtained by determining the linear slope of the MSD plot.

The average values of both the velocity fluctuations and diffusion coefficients of vesicles within the living cell at each time point (A through E) are demonstrated in Fig. 6. The experimental conditions used to obtain Fig. 6 were the same as those for Fig. 4; during the measurements, more than 140 vesicles at each time point were tracked offline for 100 frames (5 s) from the same single, living cell. The resultant data show that increased AmB exposure time resulted in a rapid increase in both the velocity fluctuations (root mean square velocity) and diffusion coefficients of vesicles within the living cells until a plateau was reached. This time-dependent increment was caused by the addition of 6 μg/ml AmB in conjunction with the TCHE pretreatment. In contrast, no variations occurred with the addition of 6 μg/ml AmB alone (Fig. 5).

As a straightforward measure of observing osmosis at the single cell level, the real-time changes in the dimensionless cell thickness were used to characterize whether the AmB-induced osmotic lysis can be completely prevented by the incubation of HT29 cells into a sucrose-containing medium. The time-dependence of the dimensionless cell thickness changes across ergosterol-containing membrane was shown in Fig. S6, where the dimensionless cell thickness was defined as the ratio of the cell thickness during experiment to cell thickness at the beginning of the AmB flow (time point a). The data showed that pretreatment with 10 μg/ml TCHE followed by 6 μg/ml AmB caused a dramatic increase in the cell thickness of HT29 cell during time points a through c. The cell thickness reached its maxima after treatment with 50 mM sucrose for 10 min at 37 °C, and then decreased slightly to finally remained constant after time point d. In contrast, cell thicknesses was remained constant (≈10 μm) when HT29 cells pretreated with 10 μg/ml of TCHE for 21 h at 37 °C followed by 6.5 nM AmB for 1 h at 37 °C. This phenomenon also suggested that AmB-induced osmotic lysis was occurred due to the presence of larger pore size of the AmB ion channels.

3.4. Geometry model for predicting the pore size of the AmB ion channels

The other main focus of the present study is to present a theoretical model proposing a mechanism by which the AmB concentration controls the diameter of AmB ion channels on the ergosterol-containing membrane. A recent study has found that ergosterol may increase membrane fluidity more than cholesterol [28]. Thus, we hypothesize that the local interfacial area expansion induced by increasing AmB concentrations may be attributed to the preferential accumulation of AmB ion channels. To test this idea, we applied a two-dimensional semi-regular tessellation model to analyze and categorize the effects of the AmB molecule concentration on the membrane permeability in the presence of excess membrane-embedded ergosterol. Specifically, we sought to determine whether increasing AmB concentration tends to increase the preferential accumulation of AmB ion channels.

This model predicted that the diameter ($L_c$) of the AmB ion channels covered by this semi-regular tessellation, $n$ by $n$ AmB ion channels and the corresponding number of AmB molecules ($N_{AmB}$) could be written...
as \( L_c = n \times R + (n - 1) \times L \) and \( n \text{AmB} = 4 \times n \times (n + 1) \), respectively, where \( R \) and \( L \) were 8 Å and 3.97 Å, respectively. For example, the eight subunits (i.e., an annulus of eight AmB molecules hydrophobically linked to the eight ergosterol molecules) make up a single AmB ion channel, whereas values of 24 and 960 subunits make up the 2 by 2 and 15 by 15 preferentially accumulated AmB ion channels, respectively. It follows that the corresponding values of the AmB ion channel diameters are 8 Å, 19.97 Å, and 17.56 nm for a single AmB ion channel, 2 by 2 accumulated AmB ion channels, and 15 by 15 accumulated AmB ion channels, respectively (Fig. 7).

### 4. Discussion

In the present work, the concentration-dependence of AmB-induced permeability changes across ergosterol-containing membranes is characterized using fluorescent dyes of known average diameter at the single-cell level. We find that Alexa Fluor® 635 phalloidin, with a diameter of nearly 16 Å, and QD-EGF, with a diameter of nearly 16 nm, can freely penetrate the cell membrane at 50 pg/ml AmB and 2 ng/ml AmB, respectively. Conversely, Alexa Fluor® 635 phalloidin and QD-EGF are physically blocked from entering the smaller pores at 40 pg/ml AmB and 1 ng/ml AmB, respectively. This scenario demonstrates that the pore size of AmB ion channels depends on the concentration of AmB.

The present work also shows that the pore size of the AmB ion channels is tunable and has been verified in the range of 16 Å to 16 nm using Alexa Fluor® 635 phalloidin and QD-EGF. Note that the sizes of both Alexa Fluor® 635 phalloidin and QD-EGF are larger than a single AmB ion channel (8 Å). To quantify the relationship between the pore size of the AmB ion channels and the corresponding AmB concentration, we applied a two-dimensional semi-regular tessellation model. This model predicts the preferential accumulation of the AmB ion channels from a geometric perspective. We hypothesize that the fluorescent molecules

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**Fig. 3.** Quantitative estimation of the AmB ion channel pore size. (A–D) The bright-field images of HT29 cells in the presence of AmB at concentrations of 40 pg/ml, 50 pg/ml, 1 ng/ml, and 2 ng/ml, respectively. (E, F) and (G, H) The corresponding epifluorescence images of the cellular uptake of Alexa Fluor® 635 phalloidin and QD-EGF, respectively.
Fig. 4. Probing the time-dependence of AmB induced permeability changes across ergosterol-containing membranes in real-time. (A–E) The simultaneous bright-field live cell imaging captured before (A), during (B–D) and after (E) treatment with 6 μg/ml AmB for 2.5 h at 37 °C subsequent to pretreatment with 10 μg/ml TCHE for 21 h at 37 °C.

Fig. 5. The effect of AmB-induced membrane permeability on the dynamics of intracellular vesicles within the living cell. On the right, there are six arbitrarily selected individual vesicles obtained from the bright-field image at time point e (left), revealing the lateral displacements of the vesicles in the microdomains. The corresponding two-dimensional trajectories of each vesicle are also plotted. Below are two MSD plots obtained from trajectories of vesicles 1 and 2, respectively, which can be used to further probe the intracellular micro rheology.
can penetrate the cell membrane when they are smaller than the diameter of AmB ion channels. We have determined the lower limit of AmB concentration (50 pg/ml AmB) that allows Alexa Fluor® 635 phalloidin to freely penetrate the cell membrane. However, the diameter of Alexa Fluor® 635 phalloidin is larger than the pore size of a single AmB ion channel; therefore, AmB ion channels should preferentially accumulate such that their corresponding diameter is larger than 16 Å. The present semi-regular tessellation model suggests that the 2 by 2 preferentially accumulated AmB ion channels fulfill this requirement, where \( L_c \) and \( n_{\text{AmB}} \) are 19.97 Å and 24, respectively. Furthermore, the present model suggests that the 15 by 15 preferentially accumulated AmB ion channels allow a tunable AmB ion channel pore size slightly larger than the diameter of QD-EGF, where \( L_c \) and \( n_{\text{AmB}} \) are 17.56 nm and 960, respectively. Therefore, this model requires an AmB concentration of at least 2 ng/ml—specifically, 50 pg/ml \times \frac{960}{24}—such that QD-EGF can freely penetrate the cell membrane. When the concentration of AmB is 1 ng/ml, the present model predicts that the values of \( L_c \) and \( n_{\text{AmB}} \) are \( \approx 12 \) nm and 480, respectively. Consequently, QD-EGF cannot freely penetrate the cell membrane when the concentration of AmB is less than 1 ng/ml. These scenarios and the experimental situations have been validated (Fig. 3G and H). We anticipate that, using this semi-regular tessellation model, we can properly predict the diameters of AmB ion channels as a function of the AmB concentration.

Using an MTT cytotoxicity assay, we also determined that the control group and the HT29 cells pretreated with 10 \( \mu \)g/ml TCHE followed by 1.5 \( \mu \)g/ml AmB do not differ (Fig. 1). However, cytotoxic effects were observed in HT29 cells pretreated with 10 \( \mu \)g/ml TCHE followed by treatment with concentrations of AmB greater than or equal to 3 \( \mu \)g/ml (Fig. 1). Taken together, these findings suggest that AmB ion channel formation is not causally linked to cytotoxicity in HT29 cells when the pore size of the AmB ion channels falls between 8 Å (the pore size of single AmB ion channel) and 17.56 nm; however, the treatment would result in the larger pore size of the AmB ion channels that leads to cytotoxic effects.

The present work provides additional methods to monitor the formation of AmB ion channels and to probe their influence on the temporal response of intracellular microenvironments. It has been noted that AmB ion channel formation may also mediate water influx; this osmotic stimulus triggers the mitogen-activated protein kinase (MAPK) pathways [29]. MAPK also plays a role in the regulation of cytoskeletal dynamics [30,31]. We used particle-tracking microrheology, together with our single-cell analysis platform [32], to probe the local mechanical properties within living HT29 cells. This platform allowed us to visualize the time-dependence of AmB induced permeability changes across ergosterol-containing membranes. We found that both the velocity fluctuations and diffusion coefficients of vesicles increased significantly within the living HT29 cells (Fig. 6). In particular, both the velocity fluctuations and diffusion coefficients of vesicles increases approached 50% during the earliest stages of treatment (0–30 min) with 6 \( \mu \)g/ml AmB. This increase was of the same order as the potassium ion efflux time scale caused by AmB [16]. The phenomenon of AmB-induced osmotic lysis, a dramatic increase in the cell thickness (Fig. S6), was also revealed.
in this study; therefore, MAPK pathways triggered by the formation of such large AmB ion channels may explain why this osmotic stimulus enhances the values of overall velocity fluctuations and vesicle diffusion coefficients within the living HT29 cells.

5. Conclusions

This study characterizes the concentration- and time-dependence of AmB-induced permeability changes across ergosterol-containing membranes. Our observation reveals that the pore size of the AmB ion channels is tunable and has been verified in the range of 16 Å to 16 nm (in the low AmB dose regime), where the diameters of AmB ion channels as a function of AmB dose can be properly predicted using a semi-regular tessellation model. However, the formation of AmB ion channels with a large pore size promotes water influx into cells and enhances the values of overall velocity fluctuations and vesicle diffusion coefficients within the living HT29 cells (in the high AmB dose regime). In addition, the larger the pore size of the AmB ion channels, the more rapid the water influx into cells leads to cytotoxic effects. We are currently investigating the relationship between MAPK pathways and the kinetics of the permeability changes induced by the presence of the AmB ion channels; these results will be reported in the near future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.03.021.

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