On the mobility of biomolecules
Bogaart, Geert van den

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Chapter 3

Dual-color fluorescence-burst analysis to probe protein efflux through the mechanosensitive channel MscL

Geert van den Bogaart, Victor V. Krasnikov, and Bert Poolman

3.1 Abstract

The mechanosensitive channel protein of large conductance, MscL, from *Escherichia coli* has been implicated in protein efflux, but the passage of proteins through the channel has never been demonstrated. We used dual-color fluorescence-burst analysis (DCFBA) to evaluate the efflux of fluorescent labeled compounds through MscL. The method correlates the fluctuations in intensity of fluorescent labeled membranes and encapsulated (macro-) molecules (labeled with second fluorophore) for each liposome diffusing through the observation volume. The analysis provides quantitative information on the concentration of macromolecules inside the liposomes and the fraction of functional channel proteins. For MscL, reconstituted in

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large unilamellar vesicles, we show that insulin, bovine pancreas trypsin inhibitor and other compounds smaller than 6.5 kDa can pass through MscL, whereas larger macromolecules cannot.

3.2 Introduction

Mechanosensitive channels are found in bacteria, fungi, plants, and animals and play a vital role in cell physiology [18]. Upon a dilution of the external osmolyte concentration, bacterial cells eject cytoplasmic molecules, including ions, metabolites [72], and possibly proteins [73]. For instance, Escherichia coli releases a subset of proteins, up to 10% of total protein content, when cells are dispersed in water [74]. At least part of the cytoplasmic molecules released has been attributed to membrane channel proteins gated by mechanical forces [75]. For a basic understanding of protein release by microorganisms and for biomedical applications, it is important to establish whether channel proteins of large conductance can facilitate the passage of macromolecules. For instance, liposomes containing mechanosensitive channels have been proposed as drug delivery vehicles for the controlled release of therapeutic peptides and proteins [26]. In the case of the mechanosensitive channel of large conductance (MscL) from E. coli, the protein or membrane environment has been engineered to allow the channel to open and close in response to light or pH [76, 77].

MscL is the biophysically and biochemically best characterized mechanosensitive channel protein and has a large conductance of 2.5 to 4 nS [18]. On the basis of the crystal structure of the homologous MscL protein from Mycobacterium tuberculosis [78] and a protein fusion study [79], MscL is predicted to form a pentamer. From molecular modeling [28, 80], patch-clamp studies [28], and in vivo and in vitro flux measurements [75, 81, 82, 83], the diameter of the pore of MscL has been estimated to be between 30 and 40 Å. This diameter should be sufficient for the release of small proteins, but it is not known how large a molecule can actually pass through MscL [28, 74, 75, 77, 82, 83].

Here, we used dual-color fluorescence-burst analysis (DCFBA, chapter 2), to monitor efflux of a broad range of (macro-) molecules through a membrane pore. This analysis is based upon the fluorescent DiO labeling of MscL-containing liposomes and encapsulating macromolecules labeled with a second fluorophore Alexa fluor 633 (Fig. 3.1). The fluorescent lipid analogue DiO and Alexa fluor 633 have non-overlapping excitation and emission spectra and can be detected separately. For both labels, the fluorescence intensity fluctuations, resolving from the liposome diffusing through the observation volume of a confocal microscope, are correlated with
each other. To activate MscL, a cysteine was engineered at the constriction site of the pore (G22C) for labeling with thiol-specific reagents. MscL (G22C) is pushed open when a charge is introduced at the Cys-22 site [63]. Labeling of MscL G22C with MTSET introduces five (positively-charged) choline moieties at the constriction site of the pore [77], whereas MTSES introduces (negatively-charged) sulfonaethyl moieties [63]. A set of peptides and stably folded proteins were chosen as substrates.

Figure 3.1: Principle of DCFBA. (a) The focal geometry of a two-channel confocal microscope and diffusion of DiO-labeled liposomes, containing Alexa fluor 633-labeled macromolecules (■). Note that the diffusion of the two fluorophores coincides. (b) Like (a), but after opening of MscL and release of the macromolecules. Note that the diffusion no longer coincides. (c) Fluorescence count traces resulting from the diffusion of DiO labeled liposomes (upper panel, $I_{\text{DiO}}$) with Alexa fluor 633-labeled glutathione encapsulated (lower panel, $I_{\text{AF633}}$). Between $t_1$ and $t_2$ the intensity of the DiO signal is above the offset.

The number of DiO molecules per liposome is independent of the state (open or closed) of MscL, whereas the number of Alexa fluor 633-labeled macromolecules does depend on this state. With MscL in the open state, the labeled molecules, depending on their size, might be released. When DiO labeled liposomes, with encapsulated Alexa fluor 633-labeled molecules, diffuse through the observation volume of the confocal microscope, the DiO and the Alexa fluor 633 signals correlate (Fig. 3.1a). If the macromolecules are small enough to efflux through MscL, opening of MscL G22C by chemical modification with MTSET or MTSES [63] will reduce this correlation (Fig. 3.1b). Molecules smaller than bovine pancreas...
Methods

3.3 Methods

3.3.1 MscL purification and reconstitution in liposomes

MscL G22C [63] was expressed in the *E. coli* mscL-knockout strain PB104 [84] using vector pB10b [85]. Cells were grown to mid-exponential growth phase (OD at 660 nm of 0.5) and expression of *mscL* was induced by adding 1 mM IPTG (isopropyl-D-thiogalactopyranoside). Membrane vesicles were then prepared by rupturing the cells with a high pressure homogenizer (Kindler Maschinen AG, Zürich, Switzerland)
and solubilized by 3% octyl-β-glucoside. MscL was purified using nickel affinity chromatography as described previously [9].

Purified MscL G22C was inserted into Triton X-100 destabilized liposomes as described [76, 86]. Briefly, 4 mg ml\(^{-1}\) liposomes were extruded 13× through 200 nm pore-size polycarbonate filters (Avestin, Ottawa, Canada) and titrated with Triton X-100 until saturation. The lipid mixtures were composed of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS, Avanti Polar-Lipids, Alabaster, AL) and the fluorescent lipid analog 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO; Invitrogen, Carlsbad, CA; excitation and emission wavelengths of 484 and 499 nm respectively) in a 30,000 : 10,000 : 1 mol ratio. MscL was added to the mixture in a protein to lipid ratio of about 1 : 50,000 (mol pentameric MscL / mol lipid) as estimated by a Folin reagent based DC-protein assay (Bio-Rad, Hercules, CA). Subsequently, the detergent was removed by incubating the mixture overnight at 4°C with stepwise addition of 100 mg ml\(^{-1}\) Bio-Bead SM-2 polystyrene beads (Bio-Rad). Membrane reconstitution was performed in 50 mM potassium phosphate buffer, pH 7.0.

### 3.3.2 Substrate labeling and encapsulation

To measure the upper size of molecules that can diffuse through MscL, several proteins were labeled with the fluorophore Alexa fluor 633 C\(_5\)-maleimide (Invitrogen; excitation and emission of 621 and 639 nm, respectively). The hormone insulin from bovine pancreas (Sigma), trypsin inhibitor from bovine pancreas (BPTI, Sigma-Aldrich, St. Louis, MO), HPr of the phosphotransferase system (PTS) from E. coli (purified as described in [87]), thioredoxin from Spirulina sp. (Sigma-Aldrich) and the calcium metalloprotein α-lactalbumin from bovine milk (Sigma-Aldrich) were labeled by adding 1 ml 100 µM protein solution in 30 mM K-Hepes, pH 8.5, to 40 nmol Alexa fluor 633, and the mixture was incubated for 30 min at room temperature. At this pH, maleimides not only react with sulfhydryl groups but also with primary amines such as the side-chain of lysines and the N-terminus of proteins. Reaction conditions (2.5-fold excess of protein over Alexa fluor 633; pH 8.5) were chosen to preferentially label the N-terminus. The buffer was exchanged to 50 mM potassium phosphate, pH 7.0, using gel-filtration with a Sephadex G-25 column (Nap10 column, Amersham Biosciences, Piscataway, NJ). Glutathione and the peptide RPPGFSPFC (bradykinin R9C, Genscript, Piscataway, NJ) were labeled by adding 1 ml 500 µM solution in 50 mM potassium phosphate, pH 7.0, to 40 nmol Alexa fluor 633 C\(_5\)-maleimide and incubating for 30 min at room temperature.

The mass of Alexa fluor 633 C\(_5\)-maleimide was determined using matrix-assisted
laser desorption / ionisation time of flight (MALDI-TOF) mass spectrometry. 0.5 µl of a 1 µM solution of Alexa fluor 633 in water with 0.1% TFA was spotted on the MALDI target and immediately mixed 1 : 1 with the matrix solution consisting of 10 mg ml\(^{-1}\) \(\alpha\)-cyano-4-hydroxycinnamic acid (dissolved in 70% acetonitril plus 0.065% trifluoroacetic acid). The spots were allowed to dry completely before the MALDI-TOF experiment was carried out on an Applied Biosystems 4700 Proteomics Analyzer (Foster City, CA).

The Alexa fluor 633 labeled macromolecules were encapsulated into MscL-containing liposomes by at least three fast freezing (liquid nitrogen), and slow thawing (at 4°C) steps, in concentrations ranging from 1 to 10 µM and sample volumes of 200 µl. After this, 1 M of the membrane permeable osmolyte glycerol was added to slow down diffusion. The liposomes were extruded through 200 nm pore filters at a total lipid concentration of 2.5 mg ml\(^{-1}\). Dynamic light scattering (DLS) experiments were performed to determine the size of the liposomes. DLS was performed at a fixed angle of 90° with a Malvern Instruments Sizer 5,000 (Worcestershire, United Kingdom), using the Contin analysis mode.

The liposomes were diluted to a final concentration of 1 mg lipid ml\(^{-1}\) into 50 mM potassium phosphate, pH 7.0, plus 1 M glycerol in the presence or absence of 1 mM of either MTSET ([2-(trimethylammonium)ethyl] methanethiosulfonate bromide) or MTSES ([2-sulfonatoethyl] methanethiosulfonate sodium salt, Anatrace, OH) for opening of the MscL channels, and incubated for 30 min at 18°C. FCS or DCFBA was then measured for 5 to 10 min at 18°C in a 2 µl volume.

### 3.3.3 Optical setup

DCFBA and FCS measurements were carried out on a laser scanning confocal microscope [9], based on an inverted microscope Axiovert S 100 TV (Zeiss, Jena, Germany), in combination with a galvanometer optical scanner (model 6860, Cambridge Technology, Watertown, MA) and a microscope objective nano-focusing device (P-721, PI Electronics AG, Baden-Dättwil, Switzerland). For excitation of the fluorescent lipid analog DiO, an argon ion laser (488 nm, Newport Corporation, Irvine, CA), and for excitation of Alexa fluor 633 a He-Ne laser (633 nm, JDS Uniphase, Milpitas, CA) were focused by a Zeiss C-Apochromat infinity-corrected 1.2 NA 63× water immersion objective. The laser power for both channels did not exceed 10 µW at the back aperture. Emission was collected through the same objective, separated from the excitation beams by a beam pick-off plate (BSP20-A1, ThorLabs, Newton, NJ), and split into two channels by a dichroic beam splitter (585dcxr, Chroma Technology, Rockingham, VT) and finally directed through emission filters.
(HQ 535/50 and HQ675/50, Chroma Technology) and pinholes (diameters of 30 µm) onto two avalanche photodiodes (SPCM-AQR-14, PerkinElmer Optoelectronics, Fremont, CA). The fluorescence signals were digitized and auto- and cross-correlation curves were calculated using a multiple \( \tau \)-algorithm. The autocorrelation curves were fitted with a two step analytical model (section 3.3.5). For the DCFBA, the fluorescence count traces for both colors were recorded for more then 300 s with a binwidth of 1 µs.

The setup was calibrated by measuring the known diffusion coefficients of Alexa fluor 488 and 633 in water (Invitrogen; \( D = 300 \text{ m}^2 \text{ s}^{-1} \) [88]). The lateral radii \( \omega_{xy} \), defined as the point where the fluorescence count rate per molecule decreased \( e^2 \) times, for 488 and 633 nm excitation, were 180 and 240 nm, respectively, corresponding to detection volumes of \( \sim 0.20 \text{ fl} \) and 0.45 fl. For the DCFBA, the overlap between the two focal volumes was optimized by maximizing the cross-correlation signal of a double labeled, doublestranded oligonucleotide. For this, the oligonucleotides 5'-ATTAT TGAGT GGTCA CTTTA AA and 5'-TTTAA AGTGA CCACT CAATA AT, labeled with Alexa fluor 488 and 633, respectively (Invitrogen), were boiled together for 5 min at a final concentration of 0.5 µM and subsequently slowly cooled to 4°C to allow annealing. Afterwards, 1 mM NaN3 was added as a preservative.

### 3.3.4 DCFBA analysis

DCFBA (see also chapter 2) was used to probe efflux through MscL. First, Alexa fluor 633 labeled macromolecules were encapsulated in MscL containing liposomes labeled with a second fluorophore DiO. Then, the fluorescence-bursts emerging from diffusion of the liposomes through the focal volume of the dual-color confocal laser-scanning microscope was measured (Fig. 3.1c). For all the bursts in the DiO fluorescence above a certain offset, the area of the \( i \)th peak \( (L_i) \) was then calculated:

\[
L_i = \frac{1}{t_2 - t_1} \int_{t_1}^{t_2} I_{\text{DiO}} dt, \tag{3.1}
\]

where the fluorescence in the DiO channel \( I_{\text{DiO}} \) is above the offset between times \( t_1 \) and \( t_2 \) (Fig. 3.1c). For the fluorescence in the Alexa fluor 633 channel \( I_{\text{AF633}} \), the corresponding signal \( (S_i) \) was also calculated:

\[
S_i = \frac{1}{t_2 - t_1} \int_{t_1}^{t_2} I_{\text{AF633}} dt. \tag{3.2}
\]

Because the fluorescence intensities are linearly proportional to the number of molecules, the concentration of macromolecules in arbitrary units, \( C_{\text{arb}} \), could now be
calculated:

$$[C_{arb}]_i = \frac{S_i}{L_i^{3/2}}.$$  (3.3)

In equation 3.3, the signals are normalized because DiO is associated to the surface of the liposomes and Alexa fluor 633 is present inside the vesicles; as a function of liposome radius, $L_i$ and $S_i$ thus scale to the power 2 and 3, respectively. From $C_{arb}$, the average concentration over all the liposomes ($C_{av}$) could be calculated:

$$C_{av} = \frac{\sum_{i=1}^{N_L} [C_{arb}]_i}{N_L},$$  (3.4)

with $N_L$ corresponding to the number of bursts. In addition to $C_{av}$, the number of empty liposomes was also determined. A burst in the DiO count rate corresponded to an empty liposome, when a corresponding burst in the Alexa fluor 633 count rate was absent. The above analysis assumes that the liposome diameter is smaller than that of the observation volume and that the observation volumes of the two channels spatially coincide. In general, both assumptions can be satisfied by a proper adjustment of the objective fill-factors of the excitation laser beams and the diameters of the confocal pinholes.

### 3.3.5 Fluorescence correlation spectroscopy

To verify the results obtained by DCFBA, the diffusion of macromolecules was assessed using FCS. With FCS (chapter 1), one can determine diffusion constants and concentrations for ensemble averages [8, 86]. The fluorescence autocorrelation signals of the DiO and the Alexa fluor 633 channels were measured and the data were fitted with a two-step analytical model to quantify the release of macromolecules. The fitting model for a normalized autocorrelation function of $P$ different compounds, assuming 3-dimensional Brownian motion, is given by [86]:

$$G(\tau) = \frac{\sum_{i \in P} C_i \alpha_i^2}{V} \left( \frac{1}{1 + \frac{4\tau D_i}{\omega_{xy}^2}} \right)^2 \sqrt{1 + \frac{4\tau D_i}{z_0^2}},$$  (3.5)

where $\alpha_i$ is the product of the fluorescence quantum yield and the absorption cross-section, $C_i$ is the concentration of the molecules, $\omega_{xy}$ and $z_0$ are the effective radii perpendicular and parallel to the focal axis, respectively, and $V$ is the effective confocal volume, defined as $V = \pi^{2/3} \omega_{xy}^2 z_0$.

Because not all liposomes were of the same size and the diffusion constant is inversely related to their radius (Einstein-Stokes relationship, see Eqn 1.2), the fluorescence autocorrelation curves could not be fitted with equation 3.5 and assuming
two components (Eqn. 3.5; \( P = 2 \)). Therefore, the model was modified so that it included the distribution of the liposomes. The radii of the liposomes follow a Maxwell distribution, which can be approximated with a Gaussian distribution, with average radius \( R \) and spread \( a \). The fraction of liposomes with radius \( r \) is then given by:

\[
k(r) = \frac{2 \exp \left( -\frac{(r - R)^2}{a^2} \right)}{a \sqrt{\pi} \left( 1 + \text{erf} \left( \frac{R}{a} \right) \right)},
\]

(3.6)

The number \( W(r) \) of DiO molecules per liposome is related to the radius \( r \) by:

\[
W(r) = \frac{8\pi c}{A} r^2,
\]

(3.7)

where \( c \) is the ratio of DiO to lipid molecules and \( A \) is the surface area of a lipid (0.6 – 0.7 nm\(^2\) [44, 45]). The number of macromolecules \( S \) inside a liposome is related to the radius of the liposome by:

\[
S(r) = \frac{4}{3} \pi C_S r^3,
\]

(3.8)

where \( C_S \) is the concentration of substrate inside the liposome. The diffusion coefficient \( D_l \) of a liposome also depends on its radius according to the Einstein-Stokes relationship:

\[
D_l(r) = \frac{\gamma}{r},
\]

(3.9)

where \( \gamma \) is a constant determined by the viscosity and temperature of the medium.

Assuming that \( \alpha_i \) is linearly dependent on the number of DiO or substrate molecules in the liposome, the fluorescence autocorrelation of DiO (Eqn. 3.10) and substrate (Eqn. 3.11) are given by:

\[
G(\tau) = \frac{1}{VC_1} \int_0^\infty W(r)^2 k(r) \frac{1}{1 + \frac{4\pi D_l(r)}{\omega_{xy}}} \frac{1}{\sqrt{1 + \frac{4\pi D_l(r)}{\omega_{xy}}}} dr \left( \int_0^\infty W(r) k(r) dr \right)^2,
\]

(3.10)

\[
G(\tau) = \frac{f C_1}{V_r} \int_0^\infty S(r)^2 k(r) \frac{1}{1 + \frac{4\pi D_l(r)}{\omega_{xy}}} \frac{1}{\sqrt{1 + \frac{4\pi D_l(r)}{\omega_{xy}}}} dr + C_f \frac{1}{1 + \frac{4D_f}{\omega_{xy}}} \frac{1}{\sqrt{1 + \frac{4D_f}{\omega_{xy}}}} \left( f C_1 \int_0^\infty S(r) k(r) dr + C_f \right)^2,
\]

(3.11)

where \( C_1 \) is the concentration of liposomes, \( D_f \) is the diffusion constant of the free substrate, \( C_f \) is the concentration of free label, and \( f \) is the fraction of liposomes.
containing substrate. If substrate effluxes through MscL, the percentage of fast component $F$ increases after opening of the channel, where $F$ is defined as:

$$F = \frac{C_f}{fC_1 \int_0^{\infty} S(r)^2 k(r)dr + C_f} \times 100.$$  \hspace{1cm} (3.12)

The numerators of equations 3.10 to 3.12 cannot be analytically obtained and were therefore approximated numerically, using Simpson’s method in 8 steps from $r = R - 2a$ to $r = R + 2a$.

### 3.4 Results

To assess the diffusion of macromolecules through MscL, MscL G22C was reconstituted in liposomes that were labeled with the fluorescent lipid analogue DiO. Figure 3.2a shows the result of DLS measurements on liposomes that were extruded through 200 nm diameter filters. An average radius of the liposomes of $107\pm5$ nm was found, with a spread of $16.5\pm1.5$ nm. The solid line is a fit, assuming a Gaussian distribution. A series of Alexa fluor 633-labeled macromolecules was encapsulated in the liposomes. The mass of Alexa fluor 633 C$_5$-maleimide was 1,089 Da in both the unconjugated (Fig. 3.2b) and the conjugated state (not shown). SDS-PAGE gels of Alexa fluor 633-labeled insulin, BPTI, HPr, thioredoxin, and α-lactalbumin, are shown in figure 3.3, where the proteins are visualized by UV-illumination or Coomassie brilliant blue (CBB) staining. All the tested macromolecules were present in a monomeric form, according to the literature [89, 90, 91, 92] and confirmed by FCS and size-exclusion chromatography (not shown). The oligomeric state of insulin depends on its concentration and is monomeric at the concentration used here [93].

For the DCFBA, the fluorescence intensity fluctuations resulting from the liposomes diffusing through the confocal volume was measured for 5 to 10 min. During this time, 100 to 1,000 liposomes passed through the focal volume, estimated from the fluorescence trace, where each burst corresponds to a liposome (Fig. 3.1). To verify whether the DCFBA measurements were quantitative, Alexa fluor 633-labeled glutathione at 1, 5, and 10 µM was encapsulated inside liposomes containing DiO. The fluorescence intensities were accumulated for 10 minutes and the $C_{arb}$-values were calculated according to Eqn. 3.3 (Fig. 3.4a). The $C_{av}$ values (Eqn. 3.4) were $1.2\pm0.3$ for 1 µM, $3.8\pm0.6$ for 5 µM and $7.9\pm0.7$ for 10 µM (Fig. 3.4b), which is in fair agreement with the expected ratio of 1 : 5 : 10. The percentages of empty liposomes were $53\pm12\%$ for 1 µM, $24\pm3\%$ for 5 µM and $5.5\pm1\%$ for 10 µM (Fig. 3.4c).
These values are close to the values of 76%, 26% and 6.6%, respectively, calculated from the average size of the liposomes (Fig. 3.2a), assuming a Poisson distribution of the number of molecules contained in the liposomes. Deviations between the measured and expected values are likely due to pipetting errors and uncertainties in the concentration of Alexa fluor 633 labeled glutathione, which was estimated from the absorption at 630 nm. Spatial differences in the observation volumes of the channels could also have contributed to the observed deviations.

The efflux from MscL-containing liposomes was studied by encapsulating Alexa fluor 633 labeled macromolecules. 4 µM glutathione, 8 µM insulin, 2 µM BPTI, 2 µM HPr, 2 µM thioredoxin or 8 µM α-lactalbumin were encapsulated into the DiO-labeled liposomes. The macromolecules did not associate measurably with the membrane, as was verified by the addition of the Alexa fluor 633 labeled macromolecules to DiO containing liposomes and performing DCFBFA experiments (data not shown). The complete histograms of BPTI and thioredoxin are shown in figure 3.5a and 3.5b. Figure 3.5c shows the average concentration $C_{av}$ of the tested compounds in the liposomes, and figure 3.5d shows the percentages of empty liposomes. Upon opening of the MscL G22C channel by modification with MTSET or MTSES, the concentration of glutathione, insulin and BPTI decreased significantly, whereas in the case of HPr, thioredoxin and α-lactalbumin there was no significant change (Fig. 3.5c). Furthermore, the fraction of empty liposomes increased for glutathione from 37.5 ± 0.5% to 62 ± 5%, for insulin from 11 ± 2% to 36 ± 2%, and for BPTI from

![Figure 3.2: Characterization of the liposomes and of Alexa fluor 633. (a) The size of the liposomes by DLS. The average radius was 107±5 nm, with a spread of 16.5±1.5 nm. (b) The mass of Alexa fluor 633 C₅-maleimide by MALDI-TOF was 1,089 Da in both the unconjugated (shown) and the conjugated state (not shown).](image-url)
Figure 3.3: The size-marker molecules. SDS-PAGE gels after UV-illumination (UV) or Coomassie brilliant blue staining (CBB) of insulin, BPTI, HPr, thioredoxin, and α-lactalbumin, all labeled with Alexa fluor 633. The 3-dimensional structures of the macromolecules encapsulated in the liposomes were taken from the RCSB protein data bank. Shown are the NMR structure of human insulin [94], and the X-ray structures of BPTI [95], HPr from *E. coli* [96], thioredoxin from *E. coli* [97] and human α-lactalbumin [98]. The scale bar represents 2 nm.
54.5 ± 2% to 71.5 ± 2%. In the case of insulin and BPTI, modification of MscL with the negatively charged MTSES resulted in an increase of empty liposomes comparable to MTSET. When HPr, thioredoxin, or α-lactalbumin were incorporated into liposomes, there was no increase in the fraction of empty liposomes upon MscL-modification with either MTSET or MTSES (Fig. 3.5d). Addition of MTSET and MTSES to the labeled macromolecules in solution did not result in a change in fluorescence. Furthermore, MTSET and MTSES did not effect efflux of Alexa fluor 633 labeled glutathione or BPTI, encapsulated in liposomes without MscL, indicating that efflux occurred through MscL. Finally, steady-state levels of efflux were reached and the liposomes were not leaky due to for instance residual detergent, since the concentration distributions were stable for > 24 hrs.

To verify the results obtained by DCFBA, the diffusion of the liposomes and the macromolecules was assessed using FCS. With FCS, one can determine diffusion constants and concentrations for ensemble averages [8, 86] and not of individual particles as with DCFBA. The fluorescence autocorrelation signals of the DiO and the Alexa fluor 633 channels were measured and the data were fitted with Eqn. 3.10 and 3.11 to quantify the release of macromolecules. The spread in the sizes of the liposomes was determined by performing fluorescence autocorrelation spectroscopy of liposomes containing the fluorescent lipid analogue DiO. Fitting the autocorrelation function with equation 3.10 showed reasonable agreement (Fig. 3.6). For coefficient γ (Eqn. 3.9), a value of 0.23 ± 0.05 µm³ s⁻¹ was found, corresponding to a diffusion

Figure 3.4: Proof of principle of DCFBA. (a) Histogram shows the distribution of liposomes loaded with 0, 1, 5, or 10 µM of glutathione as a function of $C_{arb}$ (Eqn. 3.3). (b) The average concentration $C_{av}$ (Eqn. 3.4) of the Alexa fluor 633-labeled molecules in the liposomes. (c) The percentages of empty liposomes. Error bars were calculated from three measurements.
Figure 3.5: Protein passage through MscL probed with DCFBA. Histogram shows the distribution of MscL G22C containing liposomes loaded with (a) 2 µM BPTI and (b) 2 µM thioredoxin, unmodified (- control) or modified with MTSET or MTSES, as a function of $C_{arb}$ (Eqn. 3.3). (c) The average concentration $C_{av}$ of the Alexa fluor 633-labeled molecules in the liposomes (Eqn. 3.4). (d) The percentages of empty liposomes. Error bars were calculated from three measurements.
constant of $D \approx 2.3 \mu m^2 s^{-1}$ for a liposome with a radius of 100 nm in 50 mM potassium phosphate, pH 7.0, plus 1 M glycerol. This $D$ is about 60 times smaller than that of free Alexa fluor 633 which has a diffusion constant $D = 135 \pm 6 m^2 s^{-1}$ in 50 mM potassium phosphate, pH 7.0, plus 1 M glycerol. These diffusion constants are in accordance with the Stokes-Einstein relationship. Furthermore, fitting the autocorrelation curves with equation 3.10 (Fig. 3.6), yielded an average radius $R$ of $112 \pm 7$ nm and spread $a$ of $17.5 \pm 2.5$ nm. Both numbers are in excellent agreement with DLS measurements, where $R = 107 \pm 5$ nm and $a = 16.5 \pm 1.5$ nm were determined (Fig. 3.2a). For the measurements on Alexa fluor 633, The autocorrelation curves were fitted with Eqn. 3.11 and the fits were acceptable when $\gamma$ was given the same value as estimated from the measurements with DiO (Fig. 3.6).

Figure 3.6: Autocorrelation curve of liposomes containing fluorescent lipid analogue DiO. The solid line represents a fit with equation 3.10. An average radius $R = 110$ nm and spread $a = 19$ nm were obtained. The lower panel shows the residuals (Res.) of the fit.

In the case of glutathione, the nonameric peptide bradykinin R9C, insulin and BPTI, opening of the channel resulted in an increase of the fraction of fast component $F$ which corresponds to the released fluorophore (Fig. 3.7). No increase was observed
for HPr, thioredoxin, and α-lactalbumin. These results are in perfect agreement with the results obtained by DCFBA. The fluorescence-burst analysis, however, has the advantage over FCS of yielding a distribution of macromolecules over the liposomes, whereas FCS only provides qualitative information.

Figure 3.7: Protein passage through MscL probed with FCS. Fluorescence autocorrelation curves of MscL containing liposomes, before (black line, ■) and after (gray line, ▼) opening of the channel by modification with MTSET. Shown are typical autocorrelation measurements for liposomes containing (a) insulin and (b) thioredoxin, both labeled with Alexa fluor 633. Curves were fitted with a two step analytical model (Eqn. 3.11) and residuals are shown (Res.). (c) The percentage of particles with a high mobility (F), corresponding to released substrates, of all tested macromolecules. Error bars were calculated from 3 measurements.
3.5 Discussion

Both DCFBA and FCS revealed that the tripeptide glutathione, the nonameric peptide bradykinin R9C, insulin, and BPTI, all labeled with Alexa fluor 633, are able to efflux through MscL, whereas HPr, thioredoxin and α-lactalbumin are not. The molecular weights and dimensions of these macromolecules, derived from the three dimensional structures and presented by the Research Collaboration for Structural Bioinformatics protein data bank (RCSB pdb, http://www.rcsb.org, Fig. 3.3), are given in Table 3.1. These dimensions were obtained by fitting the structures in a box with minimal volume and do not include the bound Alexa fluor 633 C₅-maleimide label of 1,089 Da. As a result of this labeling, the dimensions of glutathione and bradykinin are dominated by the Alexa fluor 633 label. Because of the dimensions of insulin and BPTI (Table 3.1), the diameter of MscL must be larger than about 2 – 3 nm. This diameter might be overestimated, because conformational changes of the size-marker proteins are not taken into account. In addition, this diameter holds for MscL opened by MTSES or MTSET, which bind a moiety of about 5 – 6 Å to each of the subunits of the channel [63] in an unknown conformation. Patch-clamp experiments showed that the conductances of MscL channels opened by modification with MTSET and opened by stretching of the membrane by a negative pipette pressure are comparable [63], suggesting a similar pore diameter. The pore diameter estimated from the passage of insulin and BPTI is consistent with calculated estimates of 29 to 46 Å based on the conductance of the channel [28, 80].

It has been suggested that some small organic osmolytes like ATP, glycine betaine and trehalose are hindered in their diffusion through MscL [99]. In view of the here presented data on protein efflux, and reports on fluorophore [26, 77] and glycine betaine efflux via MscL [100], the earlier claims that cytoplasmic molecules are rejected from entering the pore [75, 99] seem questionable. It is possible, however, that the rates at which different types of molecules diffuse through MscL vary greatly. Owing to the limited time resolution of DCFBA, we could not obtain kinetic information on (macro-) molecule diffusion through the pore. The reason is that liposomes diffuse very slow due to their relatively large size. The measured average diffusion speed of liposomes (proportional to $\frac{1}{R}$) is about 60 times smaller than that of free Alexa fluor 633 dye. In order to measure enough liposomes to obtain an acceptable autocorrelation curve, the measurement time should typically be > 1 min and information about rates of diffusion are not readily obtained. Thus, the diffusion through MscL of glutathione, bradykinin, insulin and BPTI (and possibly that of ATP [99]) may be slow compared to that of ions.

The S3 domain of the associated C-terminal helices of the MscL pentamer are
proposed to form a pre-filter for metabolites at the cytoplasmic entrance, excluding high-molecular weight substances from entering the pore [99]. We clearly show that macromolecules up to at least 6.5 kDa can pass through MscL, but we cannot distinguish between the pre-filter or the actual pore in determining the size exclusion limits of the channel. Efflux experiments with the delta 110–136 mutant, lacking this S3 domain [101], might elucidate the role of the S3 domain in determining the pore-size. It has been claimed [75] that wild-type *E. coli* cells release thioredoxin upon an osmotic downshift, whereas thioredoxin is retained by *mscL*− cells, suggesting that thioredoxin is able to diffuse through MscL. This observation contradicts our work. Our estimates should be regarded as lower limits, because proteins may be hindered in their passage due to binding to the surface of the pore or interaction with the methanethiosulfonate label. We consider the latter less probable because MscL modification with positively- (MTSET) and negatively-charged (MTSES) labels yielded identical results. Lastly, our pore diameter matches the value obtained by patch-clamp studies, where it was found that poly-L-lysines with a diameter larger than 37 Å blocked the conductance of the channel, whereas smaller poly-L-lysines had no effect [28].

The 52 kDa elongation factor Tu, the 41 kDa heatshock protein DnaK and the 142 kDa enterobactin synthase polypeptide EntF have been reported to be released upon osmotic downshift [81, 82, 83], and these macromolecules have been hypothesized to pass through MscL. Due to the high molecular weight and large dimensions of these molecules, this seems rather unlikely. Moreover, a more recent study with a MscL knockout strain suggests that these molecules might be released via a mechanism independent of MscL [74]. The genome of *E. coli* K12 contains 262 open reading frames coding for proteins smaller then 9.2 kDa, of which 46 are predicted transmembrane proteins and 26 are secreted proteins. Of the remaining 190 open reading frames, more than 100 are proteins of unknown functions and for a large number it is not known whether they self-associate or complex with other proteins. Therefore, the number of proteins potentially able to diffuse through MscL upon an osmotic downshift, will most likely be smaller than 100. Nevertheless, the release of this relatively small number of proteins could be physiologically significant.

Efflux through MscL was previously assessed by two techniques: patch-clamp and calcein dequenching [28, 63, 77]. Both methods have a number of limitations compared to the fluorescence based approach used in this work. In the case of the patch-clamp technique, direct translocation of proteins through membrane pores is not readily observed, because of limited charges on the proteins relative to the free ions in the solution. Protein translocation, however, can be observed indirectly
through blocking of the channel conductance [28, 29, 30]. The calcein efflux assay is based upon the self-quenching of fluorophore [77] at very high concentrations (typically 25 – 100 mM), unlike the low concentrations used here (1 – 10 µM). Because fluorophore-labeled proteins cannot be incorporated at concentrations needed for self-quenching, calcein dequenching is not feasible for monitoring efflux of macromolecules. The fluorescence based approach used in this work has further advantages of relatively short measurement times (typically 5 min) and small volumes (about 1 µl). In this study, the diffusion through MscL was studied both by DCFBA and FCS. The FCS results, however, provide no information on the distribution of the macromolecules over the liposomes.

Similar to the fluorescence based techniques used in this study, the macromolecule efflux from liposomes could also be studied with size exclusion chromatography. However, such measurements do not yield information on the distribution of macromolecules over the liposomes. Furthermore, much more material (∼100×) is needed for size exclusion chromatography. In addition to fluorescence autocorrelation spectroscopy, efflux could be measured using dual-color fluorescence cross-correlation spectroscopy [12], since DiO and the Alexa fluor 633-labeled macromolecules have distinctive emission and excitation wavelengths. However, according to our experience, the cross-correlation curve is difficult to quantify and prone to substantial errors, since a large fraction of the liposomes does not contain an active channel or labeled macromolecule. Thus, the change of the cross-correlation curve upon opening of the channels is small. Therefore, in this particular case, dual-color fluorescence cross-correlation spectroscopy is expected to be less accurate than fluorescence autocorrelation spectroscopy.

Using DCFBA, it was shown that after opening of the channels, a large fraction of the liposomes still contained the same concentration of fluorescent labeled macromolecule as before opening, even in the case of glutathione, insulin and BPTI, which were able to diffuse through MscL. This indicates that a large fraction (between 40 and 70%) of the liposomes did not contain a functional MscL channel. Based on the reconstitution ratio of MscL pentamer to lipid of 1:50,000 (mol / mol), it is expected that each liposome should contain approximately 10 MscL channels. Because about half of the liposomes did not contain an active channel, about 90% of the channel must have been lost during the reconstitution and / or not functionally incorporated into the liposomes. This agrees with the reconstitution efficiencies reported in the literature. For instance, for MscL pentamer to lipid ratio’s of 1:50,000 to 1:10,000 (mol / mol) typically only 1 to 7 active channels are found in a patch with a diameter of ∼1 µm, instead of the expected 30 to 150 [77, 102, 103].
On the basis of thiol-labeling with membrane-impermeant reagents, the majority of ‘active’ MscL seems to be oriented right-side-in, but always a fraction of inside-out protein is present ([104]; A. Koçer, pers. comm.). When these preparations were frozen-thawed up to 8 times, we did not see a change in the efflux activity (or specificity, not shown). Although these data are consistent with the notion that MscL is active in both orientations, it was technically not possible to rule out the possibility that protein efflux occurred via the fraction of inside-out oriented molecules.

In conclusion, this is the first time that efflux of macromolecules through MscL has been unequivocally demonstrated. DCFBA provides an approach of analyzing the permeability properties of membrane proteins, with a number of merits compared to existing methods. It can be used as an universal method to study translocation of fluorophore-labeled macromolecules by membrane pores, expressed in cells or reconstituted in liposomes. The method is of particular importance for studying the effective pores of antimicrobial peptides, for which channel-formation and membrane permeabilization is still elusive [23, 105], and has been applied by us to determine the pore properties of magainin 2 and melittin (chapter 4, [19]).

3.6 Acknowledgements

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