[23] Mechanism of Leakage of Contents of Membrane Vesicles Determined by Fluorescence Requenching

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Introduction

A commonly used method for studying the permeabilization of membranes by peptides or proteins utilizes vesicle-encapsulated fluorescent dyes and quenchers that change fluorescence intensity on release. Self-quenching dyes such as carboxyfluorescein or calcein and dye/quencher pairs such as ANTS/DPX (8-aminonaphthalene-1,3,6 trisulfonic acid/p-xylene-bis-pyridinium bromide) and terbium/dipicolinic acid are the most frequently used marker systems. There are several mechanisms by which leakage can occur and distinguishing among them is of fundamental importance. For the most part, leakage can be graded, in which all of the vesicles lose some of their contents, or it can be all-or-none, in which vesicles either lose all of their contents or none (Fig. 1). These two mechanisms are commonly distinguished by measuring the degree of quenching inside of vesicles that have been physically separated from released contents.¹⁻⁶ The requenching method we describe here utilizes the dye/quencher pair ANTS/DPX to give a quantitative description of the mechanism of release without physical separation of the vesicles from entrapped material. This is accomplished through measurements of the intensity changes that occur when the quencher DPX is titrated into a suspension of vesicles that have leaked some of their contents.

We first present the theory of the requenching method and show how it distinguishes between leakage mechanisms. We then describe in detail the experimental implementation, discuss the potential sources of errors, and present several examples in which the requenching method is used to determine the exact release mechanism of several antimicrobial peptides.

Fig. 1. Mechanisms of peptide-induced release of bilayer vesicle contents. (◇) Fluorescent dye; (●) quencher. In this schematic representation, vesicles are initially loaded (A) with a self-quenching dye or a binary dye–quencher pair. Graded nonpreferential release (B) leads to partial release of dye and quencher from all of the vesicles while graded preferential release (C) occurs when dye and quencher are different molecules that leak out unequally. All-or-none leakage (D) can occur as a result of peptide-induced pores in the membranes or peptide-induced lysis. Examples of each type of leakage can be found in the literature.\textsuperscript{1-8,10,12} Graded release can be distinguished from all-or-none release by means of the fluorescence quenching method\textsuperscript{7,8} described in text.

The examples demonstrate that the leakage mechanism can be affected by the changes in the physical state of a peptide or by changes in lipid composition. Finally, we show through an example how the kinetic measurements of fluorescence intensities can be converted into true effluxes of the encapsulated dye and quencher.

Theory

Principal Definitions

The general idea of the quenching method\textsuperscript{7,8} is to determine the degree to which ANTS dye molecules remaining inside vesicles are quenched by the remaining DPX after partial leakage has occurred. This is done by titrating the vesicle suspension with DPX at some chosen time after the addition of a leakage-inducing agent.


The total fluorescence observed at any time will be \( F = F_o + F_i \), where \( F_o \) is the fluorescence originating from outside the vesicles and \( F_i \) that from inside. If there were no quenching, the observed total fluorescence from ANTS inside and outside the vesicles would have the maximal value \( F_{\text{max}} = F_{o}^{\text{max}} + F_{i}^{\text{max}} \). When quencher is present in the vesicles, the addition of Triton X-100 causes lysis of the vesicles and dilution of the DPX to a negligible concentration so that the fluorescence observed in that case will essentially be \( F_{\text{max}} \). We define quenching outside \((Q_{\text{out}})\) and inside \((Q_{\text{in}})\) the vesicles and the total quenching \((Q_{\text{total}})\) as follows:

\[
Q_{\text{out}} = \frac{F_o}{F_{o}^{\text{max}}} \\
Q_{\text{in}} = \frac{F_i}{F_{i}^{\text{max}}} \\
Q_{\text{total}} = \frac{F}{F_{\text{max}}}
\]

Defined in this way, the \( Q \) parameters have a value of 1 when there is no quenching and 0 when there is complete quenching. The total fluorescence \( F \) is given by \( F = Q_{\text{total}} F_{\text{max}} = Q_{\text{out}} F_{o}^{\text{max}} + Q_{\text{in}} F_{i}^{\text{max}} \). In the absence of quenching, the ratio of fluorescence coming from inside the vesicles to that coming from the outside equals the molar ratio of dye molecules inside and outside. Therefore, the fractions of ANTS outside and inside the vesicles are \( f_{\text{out}} = \frac{F_o}{F_{\text{max}}} \) and \( f_{\text{in}} = \frac{F_i}{F_{\text{max}}} \) and it must be true that \( f_{\text{out}} + f_{\text{in}} = 1 \). The total quenching now can be expressed as

\[
Q_{\text{total}} = Q_{\text{out}} f_{\text{out}} + Q_{\text{in}} (1 - f_{\text{out}})
\]

The goal of the requenching experiment is to determine the behavior of \( Q_{\text{in}} \) with the change in \( f_{\text{out}} \) because it reflects the state of the vesicle contents. To accomplish this, one measures \( Q_{\text{total}} \) as a function of \( Q_{\text{out}} \) to obtain \( Q_{\text{in}} \) and \( f_{\text{out}} \) by fitting the data with Eq. (2). This is described in detail under Experimental Procedures later in this chapter.

**Leakage Mechanism**

The dependence of \( Q_{\text{in}} \) on \( f_{\text{out}} \) reveals the mechanism of leakage: For all-or-none leakage, \( Q_{\text{in}} \) is constant, and equals the initial value, while for graded leakage \( Q_{\text{in}} \) increases with \( f_{\text{out}} \) in a way that depends on the selectivity of the leakage pathway for quencher and dye. To account for this selectivity, a parameter of preferential release, \( \alpha \), can be defined that relates the fractions of quencher and dye inside or outside of the vesicle. Keeping in mind that \( f_{\text{in}} \) and \( f_{\text{out}} \) refer to the fraction of ANTS inside and outside, we define \( \alpha \) through the relations

\[
f_{\text{in}}^{\text{DPX}} = (f_{\text{in}})^\alpha \\
1 - f_{\text{out}}^{\text{DPX}} = (1 - f_{\text{out}})^\alpha
\]
One can then obtain an expression\(^8\) for the dependence of \(Q_{in}\) on \(f_{out}\) for graded release that contains only two unknown parameters, the initial concentration of encapsulated quencher, \([DPX]_0\), and the parameter of preferential release \(\alpha\):

\[
Q_{in} = \left[\frac{1 + K_d[DPX]_0(1 - f_{out})^\alpha}{1 + K_a[DPX]_0(1 - f_{out})^\alpha}\right]^{-1}
\]

(4)

where \(K_d = 50 \text{ } M^{-1}\) is the dynamic quenching constant and \(K_a = 490 \text{ } M^{-1}\) is the association constant for static quenching.\(^9\)

Equation (4) is the basic equation of the quenching technique and may be used to distinguish all-or-none from graded release and to estimate the parameter \(\alpha\).

**Simulations**

Simulations using Eq. (4) allow one to explore the effect of \([DPX]_0\) and \(\alpha\) on the \(Q_{in}\) dependence of \(f_{out}\). The results are shown in Fig. 2. The solid lines in Fig. 2A correspond to equal release of dye and quencher in a graded manner, while the dashed lines correspond to all-or-none release. Experimentally, one is limited to certain values of \(f_{out}\) (not more than 0.9) because the reduction in the fraction of molecules remaining inside increases the experimental error. The ability to distinguish the two mechanisms is affected by the choice of \([DPX]_0\) because the two curves become more similar at lower \(f_{out}\) values as \([DPX]_0\) increases. The optimal conditions are met when moderate concentrations of 4–8 mM DPX are encapsulated in vesicles.

The variation in \(\alpha\) has a dramatic effect on the shape of the \(Q_{in}(f_{out})\) curves and consequently on the determination of the leakage mechanism, as shown in Fig. 2B. While preferential release of DPX (\(\alpha > 1\)) increases the difference between all-or-none and graded leakage, the preferential release of ANTS diminishes it. In the limiting case of complete absence of DPX release (\(\alpha = 0\)), the graded release of ANTS becomes indistinguishable from the all-or-none release of ANTS and DPX.

\(^{9}\) The interpretation of the quenching experiment depends little on the exact form of the dependence of fluorescence intensity, \(F\), on DPX concentration [in our case \(F_0/F = (1 + K_d[DPX])/(1 + K_a[DPX])\)]. Therefore one can use any empirical formula as long as it describes the value of \(F\) when [DPX] is changed between zero and [DPX]\(_0\). The reported values of \(K_d\) and \(K_a\) were measured in 50 mM KCl with 10 mM HEPES buffer (pH 7.0) at 25° as described by Ladokhin et al.\(^8\) These values, however, might depend on pH and buffer ionic strength and, therefore, they need to be determined separately for each set of experimental conditions. Note that the domination of static quenching prevents the direct resolution of leakage mechanism with lifetime measurements.
Fig. 2. Simulations of internal quenching ($Q_{in}$) of ANTS inside vesicles as a function of the ANTS released ($f_{out}$). Dashed lines correspond to all-or-none release and solid lines to graded release simulated using Eq. (4). (A) The effect of initial DPX concentration for a nonpreferential release ($\alpha = 1$). (B) The effect of preferential release for a fixed initial DPX concentration. Because one is experimentally limited to $f_{out} < 0.9$, both [DPX]$_0$ and $\alpha$ affect the ability to distinguish all-or-none from graded release in a quenching experiment. (Reprinted from Ladokhin et al., with permission.)

Experimental Procedures

Overview

To determine how $Q_{in}$ varies with $f_{out}$, one first establishes a calibration curve by disrupting untreated vesicle-encapsulated ANTS/DPX preparations with Triton X-100, making incremental additions of DPX, and measuring the normalized fluorescence $F_{DPX}/F_{max} = Q_{out}$ following each addition so that $Q_{out}$ for a particular DPX addition can be determined. One then carries out the leakage experiment. The peptide (or other agent that induces leakage) is added to an ANTS/DPX-containing vesicle solution and the system incubated long enough to reach a plateau level of fluorescence. $F_{total}$ as a function of DPX concentration for the preparation is then measured after each incremental addition of DPX. Following the last addition of DPX, Triton X-100 is added to determine $F_{max}$ from Eq. (1c). Using those measurements and the $Q_{out}$ calibration curve, one plots $Q_{total}$ versus $Q_{out}$ and thereby obtains a linear curve with slope $f_{out}$ and intercept $Q_{in}(1 - f_{out})$ by means of standard linear least-squares fitting procedures.
### TABLE I

**Typical Experimental Results Obtained Using Requenching Protocol**

<table>
<thead>
<tr>
<th>Sample</th>
<th>((F - F_{\text{background}}) \times \text{volume})</th>
<th>(F_{\text{norm}})</th>
<th>(Q_{\text{total}})</th>
<th>(Q_{\text{out}})</th>
</tr>
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<td>POPC + Triton</td>
<td>1602</td>
<td>3552</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>+DPX</td>
<td>1019</td>
<td>2259</td>
<td>0.636</td>
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<td>737</td>
<td>1634</td>
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<td>1271</td>
<td>0.358</td>
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<td>984</td>
<td>0.277</td>
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<tr>
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<td>1000</td>
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<td></td>
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<td>2812</td>
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<td>1000</td>
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<tr>
<td>+Triton</td>
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<td>1000</td>
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</tr>
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</table>

\(^a\) See explanations in text.

### Example Protocol

The following is a description of a requenching experiment\(^{10}\) intended to establish the mode of leakage caused by the antimicrobial peptide indolicidin.\(^{11}\) The detailed protocol, which is repeated over a range of concentrations of leakage-inducing peptide, is presented in Table I. A 25-μl aliquot of a stock solution of 25 mM POPC vesicles preloaded with ANTS/DPX was added to a cuvette containing a solution (1.025 ml) of 0.24% Triton X-100 (sample A, in Table I), leakage-inducing peptide (in this case, 30 μM indolicidin, sample B), or just buffer (sample C). After the fluorescence had stabilized, each sample was titrated with 45 mM DPX solution in four 25-μl additions. Fluorescence intensities were subsequently corrected for background and dilution (Table I). Then, 25 μl of 10% Triton is added to samples B and C and 25 μl of buffer to sample A. The intensities resulting

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from these final samples were used for normalization. By dividing the normalized intensities by the initial intensity of sample A (3552), $Q_{\text{total}}$ is obtained from samples B and C, and $Q_{\text{out}}$ from sample A. A fit of $Q_{\text{total}}$ against $Q_{\text{out}}$ with Eq. (2) is used to determine $Q_{\text{in}}$ and $f_{\text{out}}$. The following values were obtained: For sample B, $Q_{\text{in}} = 0.079$, $f_{\text{out}} = 0.778$ and for sample C, $Q_{\text{in}} = 0.019$, $f_{\text{out}} = 0.097$. The observed values of $f_{\text{out}}$ are corrected for the amount of unencapsulated ANTS (sample C) as described under Incomplete Entrapment. $f_{\text{out}}$ is found to be $(0.778 - 0.097)/(1 - 0.097) = 0.754$ for sample B and, similarly, 0 for sample C. These points are among the data plotted in Fig. 4B, which were used in further analysis with Eq. (4).

Common Sources of Errors

There are a number of factors that can seriously complicate the implementation and analysis of requenching experiments. In our work on leakage-inducing peptides, we have observed each of these complexities. Here we briefly describe some of them.

Back-Leakage of DPX

The requenching technique is based on the assumption that additions of DPX affect only $Q_{\text{out}}$ but not $Q_{\text{in}}$ or $f_{\text{out}}$. For graded release, however, the quenching of ANTS molecules remaining inside might be affected by externally added DPX, due to the leakage of DPX back into the vesicles. This possibility is supported by experimental evidence. Under these circumstances, the acquisition time for the requenching experiment should be as short as possible to reduce DPX backflow effects. When the DPX backflow is particularly fast, the titration of the same sample with consecutive additions of DPX is not recommended. Instead, experiments on several identical samples, one for each concentration of added DPX, should be performed, and the size of the initial drop in fluorescence should be used as $F_{\text{total}}$. However, the very fact of a strong back-leakage of DPX in the requenching experiment indicates that the leakage-inducing agent causes graded leakage with the preference for DPX.

Incomplete Entrapment

The removal of ANTS from outside the vesicles during sample preparation is sometimes incomplete. For this reason, it is essential that the fraction of ANTS outside the vesicles be assessed before the requenching experiment is performed. This is accomplished through a requenching analysis.

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of the vesicles that have not been treated with peptide (see previous text). In such cases, \( f_{\text{out}} = f_{\text{o}} \) is the fraction of ANTS that is outside and \( Q_{\text{in}} \) is the quenching inside the vesicles at the beginning of the experiment. If \( f_{\text{o}} \) is significant, then the experimentally observed values of \( f_{\text{out}} \) should be corrected: \( f_{\text{corr}} = (f_{\text{obs}} - f_{\text{o}})/(1 - f_{\text{o}}) \). Some vesicle preparations will spontaneously leak a fraction of their contents during prolonged storage of stock solution and we therefore recommend repeating the determination of \( f_{\text{o}} \) each time the leakage experiments are conducted.

**Nonreleasable ANTS/DPX**

The presence of multilamellar or oligolamellar vesicles can lead to the entrapment of ANTS/DPX that cannot be released by peptides acting on the vesicle surfaces. However, this nonreleasable fraction will be released by Triton. A nonreleasable fraction can be included in the analysis of quenching experiments by adding an extra term to Eq. (2):

\[
Q_{\text{total}} = Q_{\text{out}} f_{\text{out}} + Q_{\text{in}} (1 - f_{\text{out}} - f_{\text{NR}}) + Q_{\text{NR}} f_{\text{NR}}
\]  

(5)

where \( f_{\text{NR}} \) and \( Q_{\text{NR}} \) are the fraction of ANTS and the quenching of the nonreleasing fraction, respectively. These parameters can either be estimated from independent experiments or used as parameters to fit \( Q_{\text{in}} \) to \( f_{\text{out}} \). If ignored, a significant nonreleasable fraction leads to a bimodal dependence of \( Q_{\text{in}} \) on \( f_{\text{out}} \) in the case of graded release where \( Q_{\text{in}} \) initially increases with \( f_{\text{out}} \), and then decreases as the nonreleasable fraction begins to dominate the apparent \( Q_{\text{in}} \). Obviously, the best way of dealing with this problem is to make sure that the stock vesicle preparation is homogeneous.

**Complex Leakage Mechanism**

The previous analysis and comments assume that a single mechanism is responsible for leakage of a vesicles contents. However, both graded and all-or-none release can be present simultaneously in a system; this seriously complicates the analysis. Consider a peptide that is capable of releasing \( f_{\text{graded}} \) and \( f_{\text{a-o-n}} \) fractions of the dye in graded and all-or-none manners, respectively. However, the effect of graded leakage is limited to only the portion of vesicles that have not already been completely emptied via an all-or-none mechanism. Therefore, the total fraction of dye released is

\[
f_{\text{out}}^{\text{total}} = f_{\text{a-o-n}} + (1 - f_{\text{a-o-n}}) f_{\text{graded}}
\]

(6)
Fig. 3. Simulations of internal quenching ($Q_{in}$) of ANTS inside vesicles as a function of the ANTS released ($f_{out}$) for a combination of graded and all-or-none release ([DPX]$_0$ = 6 mM, $\alpha = 1$) using Eqs. (4), (6), and (7). (A) The effect of various relative contributions of all-or-none and graded mechanisms: $\beta = 0.5$ (domination by graded release), $\beta = 1$ (equal contributions), $\beta = 2$ (domination by all-or-none release). (B) The effect of complex leakage mechanism on the determination of mode of leakage with the quenching analysis (see text for details). The curves that divide the figure into zones I–IV correspond, from top to bottom, to $\beta = 0.2, 5,$ and $\infty$. See text for description and use of zones.

For the special case that all-or-none and graded leakage are proportional to each other, preference for all-or-none release, $\beta$, is given by

$$\beta = \frac{f^{a-o-n}}{f^{graded}}$$

(7)

After solving Eqs. (6) and (7) for $f^{graded}$ and substituting it into Eq. (4) for $f_{out}$, one can easily obtain the dependence of $Q_{in}$ on $f_{out}^{total}$ for this combination of two mechanisms.\textsuperscript{13}

We have examined the predictions of this simplified model for combinations of graded nonpreferential and all-or-none mechanisms (Fig. 3A). Depending on $\beta$, the curves could appear close to pure graded (low $\beta$) or all-or-none (high $\beta$) mechanisms. Note that for intermediate values of $\beta$, the curves appear to be similar to those for graded release with the preference for ANTS, and, for example, the curve for $\beta = 1$ coincides with the one for $\alpha = 0.5$ from Fig. 2B.

\textsuperscript{13} A somewhat different approach to complex leakage has been described by Schwarz and Arbuzova.\textsuperscript{1}
This inability to differentiate certain mechanisms is a limitation of the
reducing technique and cannot be overcome by further data analysis.
Nevertheless, there can be value in understanding what models may be
consistent with the data. This can be done by dividing the $Q_{in}$ vs $f_{out}$ plane
into four different zones, as shown in Fig. 3B.

**Zone I:** Here the mechanism is quite well defined: Leakage is graded, with
the preference for DPX ranging from high preference to no preference.

**Zone II:** The mechanism is poorly defined. Multiple possibilities exist,
including graded preferential leakage of ANTS or a combination of
graded and all-or-none release. These possibilities may be distinguished
in some cases through additional independent experiments. For exam-
ple, a size-dependent leakage of large dextrans can indicate a contribu-
tion of an all-or-none mechanism.7

**Zone III:** The mechanism is reasonably well defined as mostly all-or-
none. Be sure, however, to go to the highest achievable values of $f_{out}$
to differentiate from graded leakage with strong preference for ANTS.

**Zone IV:** Data in this region are not consistent with any of the described
models. Check calculations, measurements, materials, etc., for errors.

**Applications**

Using the reducing method, we have determined the mechanism of
leakage from vesicles of different compositions induced by a variety of
natural and synthetic peptides.7,8,10,12 We present here two specific examples
that demonstrate that leakage can be sensitive to the physical state of the
leakage-inducing peptide and to lipid composition of the vesicles. Finally,
we show how the information obtained in the reducing experiments
was used to recover the efflux kinetics of dye and quencher by means
of the observed fluorescence intensities.

**Leakage Induction by a Human Neutrophil Peptide (Defensin)**

The human neutrophil peptide (defensin) HNP-2 is one of a potent
class of antimicrobial peptides found in the dense granules of neutrophils
isolated from several species.14 Neutrophil defensins are cationic arginine-
rich peptides of 30 or so residues that form a triple-stranded $\beta$-sheet struc-
ture stabilized by three disulfide bonds.15–17 Unlike known neutrophil defen-

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sins from other species, human defensins form extremely stable dimers that may be related to their ability to form large-diameter multimeric pores.\textsuperscript{7} Fluorescence quenching studies of leakage induction in anionic palmitoyl oleoylphosphatidylglycerol (POPG) vesicles by the native and reduced (de-natured) forms of HNP-2 provide excellent examples of, respectively, all-or-none and graded leakage.\textsuperscript{7} The results are summarized in Fig. 4A. As can be seen, the leakage induced by reduced HNP-2 is in zone 1 and therefore unequivocally graded. Furthermore, the leakage is preferential for leakage of DPX with $\alpha = 1.7$. The data for native HNP-2 lie in zone

![Graph (A)](image1)

![Graph (B)](image2)

**Fig. 4.** Examples of results of fluorescence quenching experiments that demonstrate both graded and all-or-none leakage of the fluorophore–quencher pair ANTS/DPX. (A) Quenching of ANTS/DPX released from POPG vesicles by native (■) and reduced (□) forms of HNP-2. (Data replotted from Wimley et al.\textsuperscript{7}) Reduced and unfolded HNP-2 induces graded leakage of ANTS/DPX from POPG vesicles (solid line, $\alpha = 1.7$). Native HNP-2 induces either all-or-none leakage (dashed line) or highly preferential leakage of ANTS (dotted line, $\alpha = 0.12$). Wimley et al.\textsuperscript{7} distinguished between these two possibilities by examining the leakage of fluorescent dextrans of different sizes. They found that native HNP-2 appears to cause all-or-none leakage through pores approximately 25 Å in diameter. These data demonstrate that the quenching method is sensitive to changes in the mechanism of release resulting from changes in the state of the peptide. (B) The release of ANTS/DPX from POPG (□) and POPC (■) vesicles induced by indolicidin. (Data replotted from Ladokhin et al.\textsuperscript{10}) Indolicidin induces graded leakage of ANTS/DPX from POPG in a manner that is highly preferential for DPX ($\alpha = 3.1$, [DPX]$_{o} = 6.1$ mM, solid line). The mechanism of leakage from POPC vesicles is different, but not well defined by quenching experiments alone. Determination of the mechanism requires additional experiments with large dextrans (see text). The dotted line describes graded nonpreferential release ($\alpha = 1$, [DPX]$_{o} = 30$ mM) and the dashed line describes all-or-none release. These results demonstrate that the quenching method is sensitive to changes in mechanism that accompany changes in lipid.
III and are mostly consistent with all-or-none leakage, although graded release with a low value of $\alpha$ is feasible (dotted line, Fig. 4A). The later possibility has been ruled out because native HNP-2 induces leakage of large dextrans.$^7$

**Leakage Induction by Antimicrobial Peptide Indolicidin**

Indolicidin is a cationic 13-residue peptide amide with potent antimicrobial activity isolated from the dense granules of bovine neutrophils. Its five tryptophans and its four positive charges render it both hydrophobic and cationic. As a result, unlike neutrophil defensins, it will bind strongly to neutral (zwitterionic) palmitoyloleoylphosphatidylcholine (POPC) vesicles as well as POPG vesicles. The results,$^10$ summarized in Fig. 4B, show that the leakage-induction activity is different for the two lipids. Leakage data for POPG vesicles fall in zone I with $\alpha = 3.1$. Thus, the leakage is graded with a remarkably strong preference for DPX. For POPC vesicles, the leakage data fall in zone II so that the mechanism of leakage is not well defined. The POPC data are consistent with either graded leakage with some preference for ANTS or a combination of graded and all-or-none. Independent results on size-dependent leakage of large dextrans indicate

![Graph](image)

**Fig. 5.** Comparison of fractional changes in fluorescence ($f_{fluor}$) with the fractions of ANTS ($f_{out}$) and DPX ($f_{DPX}^{out}$) released as a function of time. The fractions are calculated as described in text.
a strong contribution of all-or-none leakage (A. S. Ladokhin and S. H. White, unpublished data).

Conversion of Fluorescence Intensity Changes to Fractional Release of Loaded Material

The fluorescence changes observed during the graded release of quenched fluorophores, unlike those observed for simple all-or-none release, do not coincide directly with the amount of material released. Unless appropriate corrections are made, the observed fluorescence over-estimates both the amount of dye released and the rate of release. These erroneous estimates can be corrected using the same basic equations developed for the requenching procedure. In the absence of the externally added DPX and infinite dilution of the DPX that has leaked out, \( Q_{\text{out}} = 1 \). Equation (2) may therefore be rewritten in combination with Eq. (1c) to give

\[
F = F^{\text{max}}[f_{\text{out}}(1 - Q_{\text{in}}) + Q_{\text{in}}]
\]

(8)

For all-or-none release, \( Q_{\text{in}} \) is constant so that the observed changes in \( F \) are proportional to \( f_{\text{out}} \). For graded release, however, \( Q_{\text{in}} \) depends strongly on \( f_{\text{out}} \) so that \( F \) is not a direct and simple measure of \( f_{\text{out}} \). This is true for self-quenching dyes as well. We have developed an analytical procedure for the analysis of efflux of dye or quencher using the parameterized dependence of \( f_{\text{out}} \) on the time elapsed after the vesicles were mixed with leakage-inducing agent. The same result can be achieved numerically as follows. (1) Using the values of [DPX]₀ and \( \alpha \) that were determined in the requenching experiment, generate a table of \( F/F^{\text{max}} \) values as a function of \( f_{\text{out}} \) values, using Eq. (8); (2) obtain from the table by interpolation the value of \( f_{\text{out}} \) that corresponds to a given experimental value of \( F/F^{\text{max}} \). The corresponding \( f_{\text{out}}^{\text{DPX}} \) is calculated with Eq. (3b). In Fig. 5 we show the time courses of the fractional releases of ANTS (\( f_{\text{out}} \)) and DPX (\( f_{\text{out}}^{\text{DPX}} \)) for [DPX]₀ = 6.1 mM and \( \alpha = 3.1 \). The fractional change in fluorescence intensity \( f_{\text{fluor}} = [F(f_{\text{out}}) - F(0)]/[F^{\text{max}} - F(0)] \) has also been plotted in Fig. 5 for comparison. The preferential nature of the release is revealed by the relative values of \( f_{\text{out}} \) and \( f_{\text{out}}^{\text{DPX}} \).

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