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Short cationic peptides induce pH-sensitive non-leaky membrane fusion

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Abstract: pH-sensitive, non-leaky and complete membrane fusion mediated by two short peptides is shown. Depending on the specific fusogenic peptides, the observed fusion occurs at different pH ranges.

Key words: membrane fusion; peptide; liposome; pH-sensitive

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短链阳离子多肽引发 pH 敏感的非泄漏膜融合

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摘要:介绍了两种短链多肽,它们可以引发 pH 敏感的、不泄露的、完全的膜融合.利用这两种特定的融合多肽,观察到不同 pH 范围内的膜融合现象发生.

关键词:膜融合;多肽;囊泡;pH 敏感

0 Introduction

Membrane fusion is the fundamental process that allows specific cargo transport within and between cells[1]. In this process, two separate membrane bilayers are brought into intimate contact for lipids in their proximal leaflets to interact and eventually merge into one[2-4]. These fusion events are catalyzed by fusion protein[4]. Typically large and complex, natural fusion proteins are difficult to handle, which has inspired the development of artificial membrane fusion systems[5-6]. In principle, artificial membrane fusion systems can not only help unveil the still elusive mechanisms of membrane fusion[7-8] but also promise promoted cytoplasmic delivery of cell-impermeable therapeutics (e.g., DNA in gene therapy)[9-10].

One major strategy toward an artificial membrane fusion system is to use peptides (e.g., derivatives of the fusion peptide domains in natural fusion proteins[11], designer peptides[12-13], and

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antimicrobial peptides[14]) as fusogens for inducing liposomal membrane fusion[15], possibly because a short amphiphilic peptide domain (i. e., fusion peptides[16-17]) of a fusion protein is essential to natural membrane fusion process.[15, 18] In contrast to the natural fusion proteins which cause a non-leaky process[19], artificial peptidicfusogens in general induce leaky membrane fusion[6] — which could cause disastrous consequences to the cell/organelle[20]. Therefore, peptides capable of inducing non-leaky membrane fusion are highly desired.

In this work, we report on a non-leaky, pH-sensitive, complete fusion mediated by two short peptides, ORB-KK (LKGCWTKSIPPKPCFK, stabilized by one intra-molecular disulfide bond) and its open form (ORB-KK_{open}), which are analogues of ORB, an antimicrobial peptide secreted by skin of *Odorrana grahami* frog.[21]

1 Materials and Methods

1.1 Materials

All peptides (purity ≥98%) used in this work were purchased from Chinapeptides Co., Ltd. (Shanghai, China) and used as supplied. All lipids used in this work including DOPG, DOPE, and DOPC were purchased from Avanti Polar Lipids (Alabama, USA) and used without further purification. Dil, DPX, HPTS were purchased from Life Technologies (Shanghai, China). Lucigenin was purchased from Sigma-Aldrich (Shanghai, China). All other reagents used in this work were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). All reagents were used as supplied without further purification unless specified otherwise.

Buffers used in this work are as follows unless specified otherwise: ① pH=3.0 buffer: 10 mmol/L H_3PO_4, 150 mmol/L NaCl; ② pH=4.0 buffer: 10 mmol/L NaAc, 150 mmol/L NaCl; ③ pH=5.0 buffer: 10 mmol/L NaAc, 150 mmol/L NaCl; ④ pH=7.4 buffer: 10 mmol/L HEPES, 150 mmol/L NaCl.

1.2 Large unilamellar vesicle (LUV) preparation

DOPG stock solution was mixed with DOPE and DOPC stock solutions at desired mass ratios; all stock solutions were at 20 mg/mL in chloroform. The resulting mixtures were dried to a film under a stream of N_2, and desiccated under vacuum overnight. The resulting thin film was rehydrated with an inner solution (50 mmol/L NaCl, or solution of an expected probe) to a final lipid concentration of 10 mg/mL at 40℃ for 2 h; to preload a probe, solution of the probe at an expected concentration was used as the inner buffer instead. The resulting lipid dispersion were subjected to seven freeze-thaw cycles, and extruded through Nucleopore membrane with a pore size of 0.2 μm (Whatman) for 11 times using mini-extruder (Avanti Polar Lipids), which yielded the expected LUVs. All LUV dispersions were stored at 4℃ prior to use.

1.3 Dynamic light scattering (DLS) characterizations

To assess whether ORB-KK induces an increase in liposome size, we performed DLS characterizations. The distribution in hydrodynamic diameters of LUVs was monitored with a Zetasizer (Nano-ZS90, Malvern). In a sample cuvette, the LUV dispersion was diluted with a corresponding buffer at an expected pH to a final volume and a final lipid concentration of 1 mL and 0.1 mmol/L, respectively. The data was collected every 60 s for a total of 1500 s, and every data point was the average of 3 individual scans. At 300 s after the initiation of recording, ORB-KK solution at an expected concentration (10 μL) was added into the sample cuvette, to achieve the expected peptide to lipid molar ratio, P/L. Controls are those assayed similarly but without ORB-KK addition (i. e., adding 10 μL of the corresponding buffer instead). The reported results are averages of two independent trials.

1.4 Inter-vesicular lipid mixing assays

Dil-labelled LUVs (DOPG/DOPE = 20/80): Dil (10 mg/mL in chloroform) was added into the resulting lipid mixture in chloroform to achieve a

final Dil to lipid molar ratio of 8%[22]. The resulting mixture in chloroform was then dried under gentle N_2 flow, desiccated under vacuum, and rehydrated with 50 mmol/L NaCl solution to a final concentration of 10 mg/mL. The resulting suspension was subjected to seven freeze-thaw cycles, followed by sonication to clarity (at an output power of 9.3 W for 5 min). The resulting suspension was subsequently extruded through a Nucleopore membrane (pore size of 0.2 μm) for 11 times using a mini-extruder.

Dil (a membrane dye) is self-quenched once its content in a lipid bilayer reaches ≥8% (molar ratio) but becomes brightly fluorescent upon dilution (for example, *via* fusion with unlabelled vesicles)[22]. We hence use Dil as the fluorescent membrane dye for inter-vesicular lipid mixing assays. Inter-vesicular lipid mixing assays were carried out according to a previously reported protocol[23-26] with slight modification. Briefly, in a quartz sample cuvette, dispersion of unlabelled LUVs was mixed with that of Dil-labelled LUVs at an unlabelled to labelled liposomal lipid molar ratio of 3:1, followed by dilution with a corresponding buffer at an expected pH to a final volume and a final lipid concentration of 1 mL and about 0.2 mmol/L, respectively; both unlabelled and Dil-labelled LUVs have the same lipid composition. Kinetics of fusion-induced Dil dilution was monitored by recording Dil's fluorescence intensity ($\lambda_{ex}/\lambda_{em}$ = 552nm/571 nm) for 1,000 s using a fluorimeter (Fluorolog-3-Tou, HORIBA Jobin Yvon). At 200 s after the initiation of recording, a peptide solution (ORB-KK, or ORB-KK_{open}) (10 μL, at an expected concentration in a corresponding buffer) was added into the cuvette, to achieve an expected peptide to lipid molar ratio, P/L. Relative enhancement in Dil's fluorescence intensity is determined by F_t/F_0(%), where F_t and F_0 are Dil's fluorescence intensity readings at time t (s) and 0 (s), respectively. Reported results are averages of two independent trials.

1.5 intra-vesicular content mixing assays

HPTS-preloaded LUVs (DOPG/DOPE = 20/80): the desiccated lipid thin film was rehydrated to a final lipid concentration of 10 mg/mL with HPTS solution (2 mmol/L HPTS in 50 mmol/L NaCl). The resulting lipid dispersion was subjected to seven freeze-thaw cycles and extruded through a Nucleopore membrane (pore size of 0.4 μm) for 11 times using a mini-extruder.

DPX-preloaded LUVs (DOPG/DOPE = 20/80): the desiccated lipid thin film was rehydrated to a final lipid concentration of 10 mg/mL with DPX solution (90 mmol/L DPX in Millipore water). The resulting lipid dispersion was subjected to seven freeze-thaw cycles and extruded through a Nucleopore membrane (pore size of 0.4 μm) for 11 times using a mini-extruder.

HPTS is a fluorescent probe and can be quenched upon mixing with DPX[27]. We hence use HPTS and DPX as the distinct intra-vesicular contents confined by the liposome mergers. Intra-vesicular content mixing assays were performed according to a previously reported protocol[27] with some modification. Briefly, HPTS-preloaded LUVs were separated from free HPTS *via* gel filtration (Sephadex G-25, GE Healthcare), using the buffer at an expected pH as the eluent. The resultant dispersion of HPTS-preloaded LUV was subsequently added into a quartz cuvette, followed by addition of DPX-preloaded LUV dispersion to achieve a final "HPTS-preloaded LUV" to "DPX-preloaded LUV" molar ratio of 1:5 (lipid:lipid). The resulting mixture was subsequently diluted further with the corresponding eluent buffer to a final lipid concentration of 0.2 mmol/L. Kinetics of change in HPTS' fluorescence intensity was monitored by recording the fluorescence intensity of HPTS ($\lambda_{ex}/\lambda_{em}$ = 403nm/510 nm) for 900 s using a fluorimeter (Fluorolog-3-Tou, HORIBA Jobin Yvon). At 200 s after the initiation of recording, ORB-KK solution was added into the cuvette to achieve the expected P/L ratio. Relative change in fluorescence intensity of HPTS is

determined by F_t/F_0 (%), where F_t and F_0 are HPTS's fluorescence intensity readings at time t(s) and 0 (s), respectively. The reported results are averages of two independent trials.

1.6 Vesicle leakage assays using lucigenin as indicator

Lucigenin-preloaded LUV (20/80 DOPG/DOPE): the desiccated lipid thin film was rehydrated with lucigenin solution (1 mmol/L lucigenin in 50 mmol/L $NaNO_3$ aqueous solution) to a final lipid concentration of 10 mg/mL at 40℃ for 2 h. The resulting lipid dispersion were subjected to seven freeze-thaw cycles, and extruded through Nucleopore membrane (pore size of 0.4 μm) for 11 times using a mini-extruder.

We assess whether ORB-KK induces membrane permeabilization (i. e., leakage) by performing lucigenin quenching assays[28], because lucigenin is a membrane-impermeant dye and gets quenched upon mixing with halide anions,[28] which can indicate the cross-membrane transportation of lucigenin and/or chloride ions.

Prior to a vesicle leakage assay, lucigenin-preloaded LUV was separated from the free lucigenin *via* gel filtration. The resultant LUV dispersion was added into a quartz sample cuvette, followed by dilution with the same eluent buffer to a final volume and a final lipid concentration of 1 mL and about 0.2 mmol/L, respectively. Kinetics of change in lucigenin's fluorescence intensity ($\lambda_{ex}/\lambda_{em}$=368nm/505 nm) was monitored for 900 s with a fluorimeter (Fluorolog-3-Tou, HORIBA Jobin Yvon)[28]. At 100 s and 800 s after the initiation of recording, ORB-KK solution (10 μL) and Triton X-100 solution (20%, 50 μL) were added into the cuvette to achieve the expected P/L and to establish 100% leakage, respectively.

1.7 Vesicle leakage assays using HPTS as the indicator

HPTS-preloaded LUVs for vesicle leakage assays: the desiccated lipid thin film was rehydrated to a final lipid concentration of 10 mg/mL with HPTS solution (0.1 mmol/L HPTS in 50 mmol/L NaCl aqueous solution). The resulting lipid dispersion was subjected to seven freeze-thaw cycles and extruded through a Nucleopore membrane (pore size of 0.4 μm) for 11 times using a mini-extruder.

Prior to a vesicle leakage assay, HPTS-preloaded LUV was separated from free HPTS *via* gel filtration using a buffer at expected pH as the eluent. The resulting vesicle dispersion was added into a quartz sample cuvette, followed by dilution with the same eluent buffer to achieve a final volume and a final lipid concentration of 1 mL and about 0.2 mmol/L, respectively. Prior to the vesicle leakage assay, free DPX (12.06 μL, 90 mmol/L) was added into the resulting liposome dispersion and mixed well gently. Kinetics of change in HPTS' fluorescence intensity ($\lambda_{ex}/\lambda_{em}$= 403nm/510 nm) was monitored for 1000 s with a fluorimeter (Fluorolog-3-Tou, HORIBA Jobin Yvon). At 200 s after the initiation of recording, a peptide solution (ORB-KK, or ORB-KK_{open}) was added into the cuvette to achieve an expected P/L ratio.

2 Results and discussion

2.1 Research on ORB-KK

Membrane fusion must result in an increase in liposome size. Dynamic light scattering characterizations reveal that, upon ORB-KK addition, LUVs at pH = 3.0 (Fig. 1) exhibit consistent increase in average hydrodynamic diameter whereas those at pH = 4.0 exhibit negligible change in size (Fig. 1). Controls are those assayed similarly but without ORB-KK addition.

In the inter-vesicular lipid mixing assays, mixing between a Dil-labelled liposome and an unlabelled liposome makes the originally self-quenched probe become brightly fluorescent, due to the fusion-induced dilution (Fig.2(a)). Kinetics of change in Dil's fluorescence intensity show that, upon addition of ORB-KK (at peptide to lipid molar ratio, P/L, of 1/20), mixture of Dil-labelled and unlabelled LUVs at pH=3.0 exhibits significant increase in fluorescence intensity

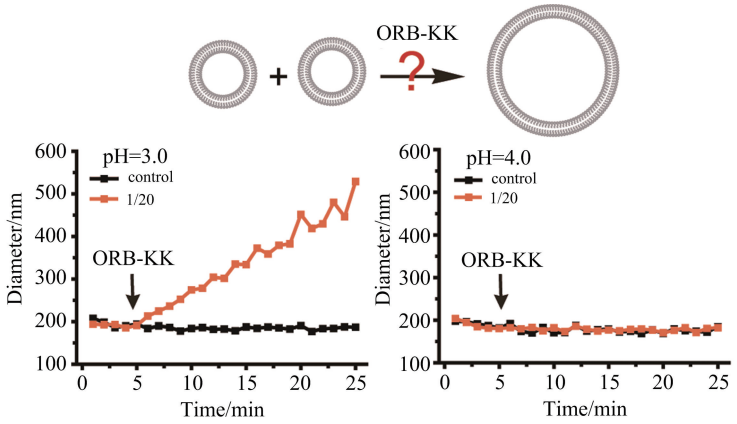

Fig.1　ORB-KK induced a gradual increase in vesicle size for liposomes at pH=3.0 but did not affect the size for those at pH=4.0

whereas that at pH=4.0 fails to do so, indicative of pH-sensitive inter-vesicular lipid mixing induced by ORB-KK. Controls are those assayed similarly but without ORB-KK addition (i.e., adding buffer instead).

In the intra-vesicular content mixing assays, complete fusion between an HPTS-preloaded LUV and a DPX-preloaded LUV makes the originally fluorescent HPTS get quenched by DPX (Fig. 2(b)). Kinetics of change in HPTS' fluorescence intensity show that, upon ORB-KK addition (P/L=1/20), mixture of HPTS-preloaded and DPX-preloaded LUVs at pH=3.0 exhibits a significant decrease in HPTS' fluorescence intensity whereas that at pH=4.0 fails to do so, indicative of pH-sensitive intra-vesicular content mixing. Controls are those assayed similarly but without ORB-KK addition.

In the content leakage assays, if membrane permeabilization occurred during fusion mediated by ORB-KK, it would result in an efflux of the fluorescent indicator pre-loaded within liposome and/or influx of its quencher abundant in the extra-vesicular environment, leading to quenching of the indicator by the quencher (Fig.2(c)). Here, two indicator/quencher pairs, lucigenin/Cl^- and HPTS/DPX, were used. Kinetics of change in indicators' fluorescence intensity show that, at pH=3.0, ORB-KK fails to make either (Fig.2(c)-left) lucigenin or (Fig. 2(c)-right) HPTS preloaded within LUVs exhibit an appreciable decrease in fluorescence intensity, indicative of a lack of membrane permeabilization. Controls are those assayed similarly but without ORB-KK addition.

When exposed to DOPG/DOPE = 20/80 unilamellar vesicles, a model of biological membranes, ORB-KK significantly induces both inter-vesicular lipid mixing and intra-vesicular content mixing but only when pH drops to 3.0 (Fig.2(a)～(b)), indicative of complete fusion in pH-sensitive manner. Consistently, the observed fusion is accompanied by a pH-dependent increase in liposomal size (Fig.1), an essential consequence of membrane fusion. Moreover, under comparable conditions, ORB-KK fails in causing efflux and/or influx of membrane-impermeable compounds (Fig. 2(c)), suggesting that the observed fusion is non-leaky. In addition, decreasing membrane DOPE content to 60% aborts ORB-KK's ability to cause the observed liposomal membrane fusion (Fig.3). Collectively, the results above demonstrate that ORB-KK induces a pH-sensitive, non-leaky, complete fusion between 20/80 DOPG/DOPE liposomes.

The fusogenic activity of ORB-KK requires environmental pH≤3.0, which is very acidic; in the human body, such acidic microenvironment is only found in the stomach. Instead, weakly acidic microenvironments are associated with many diseased conditions including dental caries (pH < 5.5)[29], airway surface of cystic fibrosis lung (pH about 6.8)[30], and tumors (pH 6.0～6.8)[31-32]. It

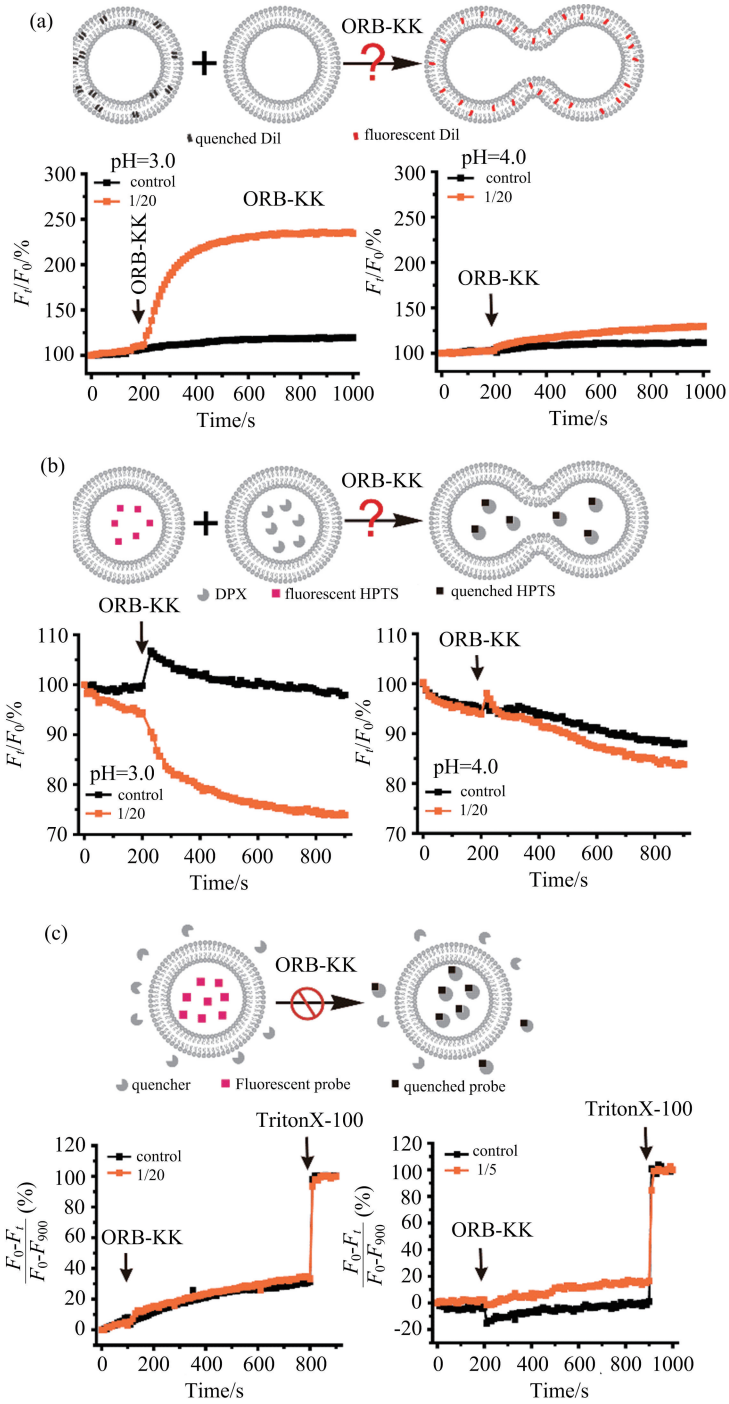

Fig.2 ORB-KK induced fluorescence changes in the inter-vesicular lipid mixing assays (a); intra-vesicular content mixing assays (b); content leakage assays (pH=3.0; two indicator/quencher pairs: lucigenin/Cl^- (c-left) and HPTS/DPX (c-right) were used)

is thus imperative to examine whether derivatives of ORB-KK cause similar fusion but at higher pH. Fortunately, one such derivative is the open form of ORB-KK (ORB-KK_{open}).

2.2 Research on ORB-KK_{open}

Assays using ORB-KK_{open} as fusogen and 20/80 DOPG/DOPE LUVs as model membranes. In the inter-vesicular lipid mixing assays (Fig.4(a)), kinetics of change in Dil's fluorescence intensity show that, upon ORB-KK_{open} addition (P/L = 1/20), mixture of Dil-labelled and unlabelled LUVs at pH = 5.0 exhibits a significant increase in fluorescence intensity while that at pH = 7.4 exhibits a weaker yet detectable increase in

fluorescence intensity, indicative of acid-facilitated inter-vesicular lipid mixing. Controls are those assayed similarly but without peptide addition. In the content leakage assays, as shown in Fig.4(b), kinetics of change in HPTS fluorescence intensity show that, at both pH 5.0 and 7.4, ORB-KK$_{open}$ fails to make HPTS-preloaded LUVs in DPX-supplemented buffer exhibit an appreciable decrease in fluorescence intensity, indicative of lack of membrane permeabilization. Controls are those assayed similarly but without peptide addition.

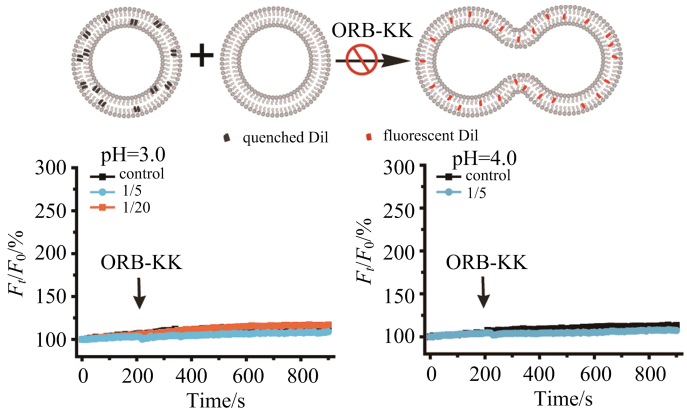

Fig.3　Decreasing DOPE content to 60% completely aborts ORB-KK's fusogenic activity

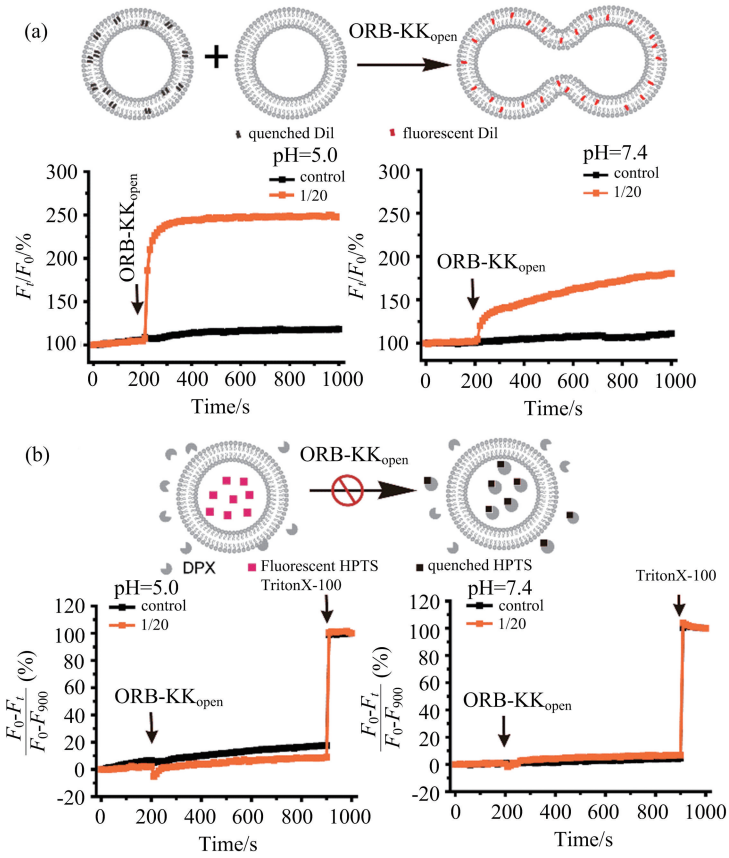

Fig.4　ORB-KK$_{open}$ induced fluorescence changes in the inter-vesicular lipid mixing assays (a); content leakage assays (indicator/quencher pairs: HPTS/DPX were used) (b)

When exposed to 20/80 DOPG/DOPE liposomes, ORB-KK$_{open}$ triggers a significant fusion at pH 5.0 (Fig.4(a)) and a weak (yet detectable) fusion at pH = 7.4 (Fig.4(a)). It is noteworthy

that the fusion mediated by ORB-KK_{open} is non-leaky through the whole examined pH range (5.0 ~7.4) (Fig.4(b)).

3 Conclusion

In summary, we report on non-leaky, pH-sensitive, complete fusion mediated by two short peptides, ORB-KK (LKGCWTKSIPPKPCFK, stabilized by one intra-molecular disulfide bond, hairpin-like peptide) and its open form (ORB-KKopen), which are analogues of ORB, an antimicrobial peptide secreted by skin of *Odorrana grahami* frog. Depending on the specific fusogenic peptides, the observed fusion occurs at different pH ranges.

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