



Nanofluidic Biosensor Created by Bonding Patterned Model Cell Membrane and Silicone Elastomer with Silica Nanoparticles

Tanabe, Masashi
Ando, Koji
Komatsu, Ryota
Morigaki, Kenichi

(Citation)

Small, 14(49):1802804–1802804

(Issue Date)

2018-12-06

(Resource Type)

journal article

(Version)

Accepted Manuscript

(Rights)

© 2018 WILEY - VCH Verlag GmbH & Co. KGaA, Weinheim. This is the accepted version of the following article: [Small, 14(49):1802804, 2018], which has been published in final form at <https://doi.org/10.1002/smll.201802804>. This article may be used for non-commercial purposes in accordance with the Wiley Self-Archiving Policy

(URL)

<https://hdl.handle.net/20.500.14094/90005608>



Nanofluidic Biosensor Created by Bonding Patterned Model Cell Membrane and Silicone Elastomer with Silica Nanoparticles

Masashi Tanabe¹, Koji Ando¹, Ryota Komatsu¹, Kenichi Morigaki^{1, 2 *}

1: Graduate School of Agricultural Science, Kobe University, Rokkodaicho 1-1, Nada, Kobe 657-8501, Japan

2: Biosignal Research Center, Kobe University, Rokkodaicho 1-1, Nada, Kobe 657-8501, Japan

*Corresponding author:

Kenichi Morigaki: E-mail: morigaki@port.kobe-u.ac.jp, Fax: +81-78-803-5941

Key words: biosensor; nanofluidics; lipid bilayer; silica nanoparticle; gap-junction

Abstract

Selective and sensitive detection of specific molecules in a solution containing diverse coexisting molecules is important in many biomedical and environmental applications, including diagnostics and pollutant detection. Here, a nanofluidic biosensor is developed to detect specific target molecules (e.g. toxin proteins) in the presence of non-target molecules by bonding a patterned model cell membrane and a silicone elastomer (polydimethylsiloxane: PDMS) sheet using surface-modified-silica-nanoparticles as the adhesive layer. Owing to the uniform size of nanoparticles, a nanometric gap junction is formed between the fluid bilayer and PDMS (nanogap-junction). The thickness of nanogap-junction is controlled by the size of silica nanoparticles. Target molecules that specifically bind to the receptor molecules in the fluid bilayer are selectively transported into the nanogap-junction by the lateral diffusion of lipid membrane. A thinner gap formed with smaller nanoparticles can enhance the sensitivity (signal-to-background ratio) more effectively, owing to the suppression of nonspecific penetration of coexisting molecules. Silica nanoparticles also provide excellent mechanical robustness, realizing long-term stability of the gap structure. Nanogap-junction using silica-nanoparticles provides a versatile platform for highly selective and sensitive sensing by realizing detection of specific target molecules in a solution containing more concentrated non-target molecules.

Introduction

Sensitive detection of specific target molecules in solutions containing diverse coexisting molecules is important in many biomedical and environmental applications. An effective means for enhancing the sensitivity is to suppress the background noise by reducing the detection volumes,^[1] as exemplified by the total-internal-reflection fluorescence microscopy,^[2] zero-mode-waveguide,^[3] nanofluidics,^[4] micro-chambers,^[5] and nanopores.^[6] However, these techniques are generally not capable to detect specific target molecules in the presence of diverse and often much more concentrated coexisting molecules. Here, we report a bioinspired sensing methodology that combines the reduced detection volume with unique features of the biological membrane.

In the nature, sensing specific molecules in an environment containing coexisting molecules is ubiquitous. Biological membranes composed of lipid bilayer and associated proteins work as a platform for this highly selective and sensitive detection, owing to its unique structural and physicochemical properties.^[7] Biological membranes are two-dimensional fluid, in which membrane-bound molecules can diffuse laterally. In addition, the phospholipid-based membrane surface can suppress nonspecific adsorption of proteins, thus enhancing the specific binding by molecular recognition. To reproduce these unique properties of the biological membrane in a technologically amenable format, we use a substrate-supported model membrane having lithographically patterned lipid bilayers.^[8] The model membrane comprises two distinctive lipid bilayer regions, *i.e.* polymerized phospholipid bilayer and natural phospholipid bilayer composed of 1,2-dioleoyl-*sn*-

glycero-3-phosphocholine (DOPC). The polymerized bilayer (hereafter called "polymeric bilayer") provide the mechanical stability, whereas the natural phospholipid bilayer (hereafter called "fluid bilayer") possesses the lateral mobility of molecules (fluidity) and the biomimetic functionality (*e.g.* molecular recognition by surface receptors). The reduced detection volume is realized by attaching the surface of polymeric bilayer with a silicone elastomer (polydimethylsiloxane: PDMS) sheet using an adhesion material (glue) having a finite thickness, which generates a nanometric gap structure between the fluid bilayer and PDMS (nanogap-junction) (Figure 1). The small gap thickness and the biomimetic property of fluid lipid bilayer enable to selectively transport and detect target molecules that bind to the membrane surface receptor by the molecular recognition, whereas nonspecific penetration of coexisting molecules is suppressed.

We developed a nanogap-junction using surface-functionalized silica nanoparticles as the adhesion layer. The surface of silica nanoparticles was modified with biotin so that the nanoparticles could bind the polymeric bilayer and PDMS. A prototype of nanometric gap structure was previously constructed using lipid vesicles as the adhesion material.^[9] However, the use of soft lipid material compromised the structure and utility of nanogap-junction due to the fact that the gap thickness was not controlled and the long-term stability of the gap structure was limited. The use of silica nanoparticles as the adhesion layer offers two critical advantages. First, the thickness of nanogap-junction can be accurately controlled due to the uniform sizes of silica nanoparticles. By using silica nanoparticles having a smaller size, we can generate a thinner nanogap-junction which realizes a

higher selectivity of detection (*vide infra*). Second, silica nanoparticles are mechanically robust, and nanogap-junctions formed with silica nanoparticles can have a long life time ($> \text{week}$). This allows to store a pre-assembled biochip for use, avoiding elaborate onsite assembly process. These features are important in realizing a highly selective and sensitive biosensing platform suitable for biomedical/ environmental applications. We first describe a mathematical model that predicts the influences of gap thickness on the signal-to-background noise ratio (S/B ratio), and verify the theoretical predictions by constructing the nanogap-junction using silica nanoparticles having different sizes.

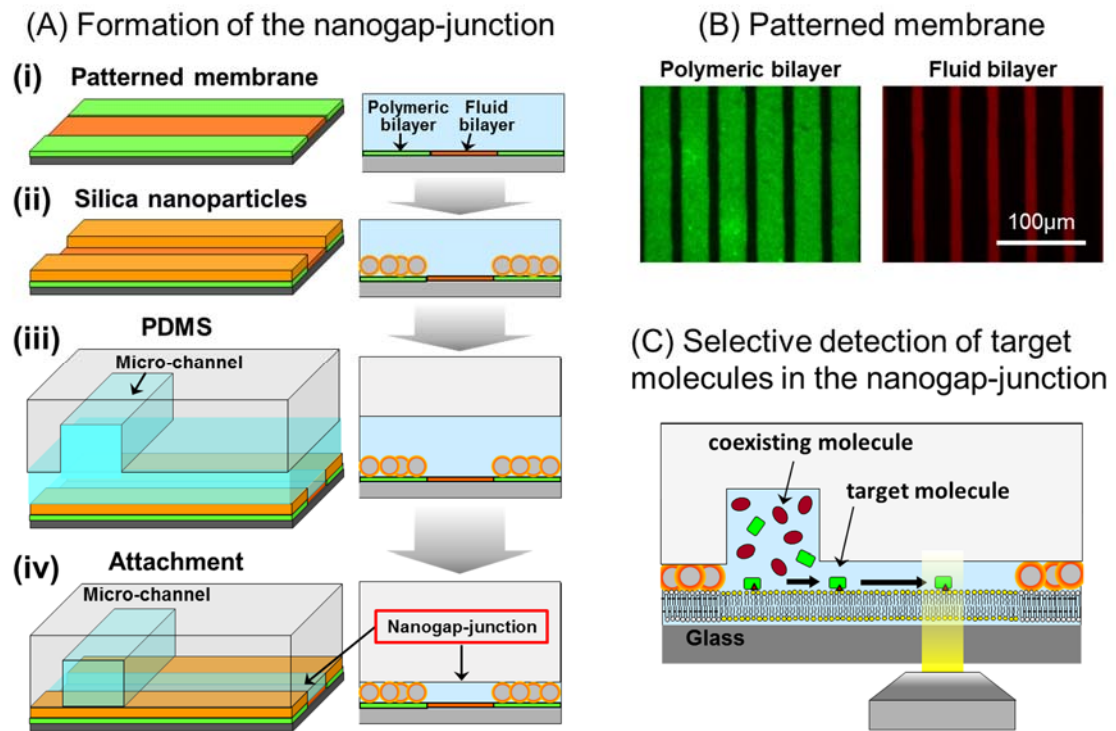


Figure 1: The nanogap-junction between a patterned membrane and PDMS formed with silica nanoparticles as the adhesion layer. (A) Schematic of the formation process: (i) A patterned membrane composed of polymeric and fluid bilayers, (ii) adsorption of the silica nanoparticles (adhesion layer),

(iii) place a PDMS sheet on the patterned bilayer with the adhesion layer (an aqueous solution is sandwiched between the membrane and PDMS), (iv) attachment of the PDMS and the adhesion layer (formation of the nanogap-junction). (B) Fluorescence micrographs of patterned polymeric (green fluorescence)/ fluid (red fluorescence) bilayers observed at the same position. (C) Selective detection of target molecules that bind to the membrane and diffuse into the nanogap-junction. The objects are not drawn in size, *i.e.* thickness of the patterned bilayer (ca. 4 nm) and the nanogap-junction (ca. 100 nm or less) is enlarged for clarity.

Results and Discussion

Selective detection in the nanogap-junction

We first formulate a theoretical model that describes the influences of gap thickness on the selective detection. We consider the concentrations of target (T) and non-target (NT) molecules in the solution (C_T^s and C_{NT}^s), and assume that molecules partition into the adhesion layer or bind onto the membrane with a defined affinity. The concentrations in the adhesion layer (C_T^a and C_{NT}^a), and on the membrane (C_T^b and C_{NT}^b) can be expressed with the following equations.

$$\text{Target molecule in the adhesion layer: } C_T^a = k_T^a \times C_T^s \quad (1a)$$

$$\text{Non-target molecule in the adhesion layer: } C_{NT}^a = k_{NT}^a \times C_{NT}^s \quad (1b)$$

$$\text{Membrane-bound target molecule: } C_T^b = k_T^b \times C_T^s \quad (1c)$$

$$\text{Membrane-bound non-target molecule: } C_{NT}^b = k_{NT}^b \times C_{NT}^s \quad (1d)$$

k^a and k^b are the partitioning constants to the adhesion layer and the bilayer membrane, respectively.

It should be noted that the concentrations in the solution and adhesion layer are defined as the number of molecules in a unit volume, whereas the concentrations of membrane-bound molecules represent the number of molecules in a unit area. Therefore, the proportionality constant for membrane-bound molecules (k^b) have the dimension of length, whereas the proportionality for the adhesion layer (k^a) is dimensionless. Assuming that both target and non-target molecules have the same fluorophore, the fluorescence intensities in the nanogap and adhesion regions (I_1 and I_2) should be proportional to the

total number of molecules in each region, as described in the following equations.

(Nanogap region)

$$I_1 \propto h \times (C_T^s + C_{NT}^s) + C_T^b + C_{NT}^b$$

$$= h \times (C_T^s + C_{NT}^s) + k_T^b \times C_T^s + k_{NT}^b \times C_{NT}^s \quad (2a)$$

(Adhesion region)

$$I_2 \propto h \times (C_T^a + C_{NT}^a) = h \times (k_T^a \times C_T^s + k_{NT}^a \times C_{NT}^s) \quad (2b)$$

h represents the height of the nanogap-junction. The fluorescence arising from the adhesion region corresponds to the background noise ($B = I_2$). On the other hand, the fluorescence intensity difference between the nanogap and adhesion regions corresponds to the signal ($S = I_1 - I_2$).

Therefore, the S/B ratio can be defined as follows.

$$\frac{S}{B} = \frac{I_1 - I_2}{I_2} = \frac{h \times ((1 - k_T^a) \times C_T^s + (1 - k_{NT}^a) \times C_{NT}^s) + k_T^b \times C_T^s + k_{NT}^b \times C_{NT}^s}{h \times (k_T^a \times C_T^s + k_{NT}^a \times C_{NT}^s)} \quad (3)$$

For a very small h , we can assume the following relation:

$$h \times [(1 - k_T^a) \times C_T^s + (1 - k_{NT}^a) \times C_{NT}^s] \ll k_T^b \times C_T^s + k_{NT}^b \times C_{NT}^s,$$

which simplifies Equation (3) as the following:

$$\frac{S}{B} \approx \frac{k_T^b \times C_T^s + k_{NT}^b \times C_{NT}^s}{h \times (k_T^a \times C_T^s + k_{NT}^a \times C_{NT}^s)} \quad (4)$$

Thus obtained Equation (4) has three important implications on the factors that influence the sensitivity and selectivity of nanogap-junction. First, the sensitivity (S/B ratio) increases as the gap thickness (h) decreases. Second, the selectivity is directly affected by the difference in membrane binding constants of target and non-target molecules (k_T^b and k_{NT}^b). If k_{NT}^b is large, we would have a false signal from non-target molecules. Third, the S/B ratio can be heightened if non-specific adsorption of molecules in the adhesion layer is lowered. This analysis clearly shows that a thin gap structure, suppression of nonspecific binding on the fluid bilayer, and restricted penetration of molecules into the adhesion layer are the key factors for realizing high selectivity and sensitivity in the nanogap-junction.

Formation of nanogap-junction with silica nanoparticles

Lipid-coated silica nanoparticles were used to selectively bond the polymeric bilayer and PDMS, forming a nanometric gap space between the fluid bilayer and PDMS. Figure 2 shows the fluorescence micrographs of a patterned bilayer with attached silica nanoparticles. The green fluorescence in the upper panel shows the polymeric bilayer (DiynePC/ DiynePE). Red fluorescence in the lower panels is from TR-PE in the fluid bilayer (DOPC/ TR-PE) and on the silica nanoparticles (DOPC/ Biotin-PE/ TR-PE). The molar fraction of TR-PE was the same for both bilayers (1%). Silica nanoparticles adsorbed specifically onto the polymeric bilayer regions by the biotin-streptavidin linkage. As a result, the red fluorescence in the polymeric regions increased after the addition of silica nanoparticles, whereas the fluorescence in the fluid bilayer regions remained unchanged. We

estimated the surface density of silica nanoparticles on polymeric bilayer by comparing the fluorescence intensities in the polymeric and fluid bilayer regions. The estimated surface coverage was 0.48, 0.38, and 0.25 for 30, 100, and 200 nm silica nanoparticles, respectively. The surface coverage was higher for smaller nanoparticles, though it was considerably lower than the theoretical value of hexagonal closest packing (*i.e.* 0.90). This is presumably due to kinetic limitation of colloidal systems to attain the most densely packed (and hence energetically most stable) state.

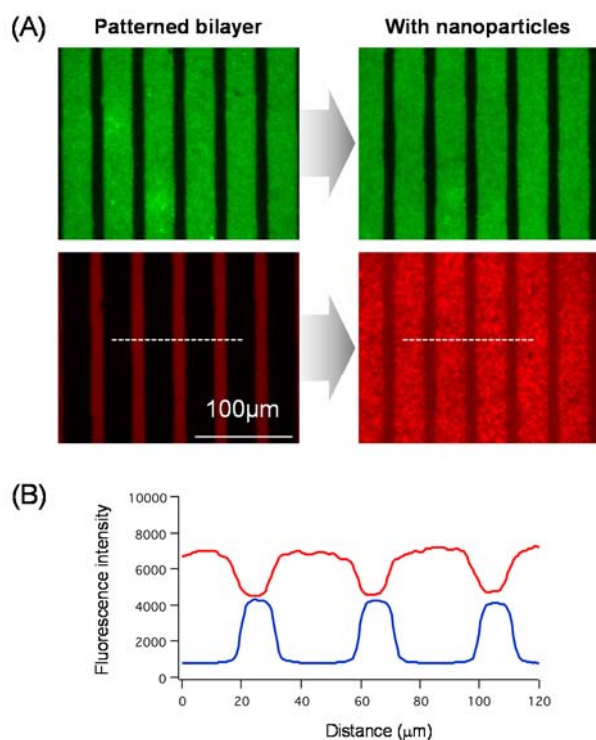


Figure 2: (A) Fluorescence micrographs of a patterned bilayer before and after the adsorption of silica nanoparticles. The upper and lower images show the fluorescence from polymeric bilayer (green) and fluid bilayer/ silica nanoparticles (red), respectively. The fluid bilayer consisted of DOPC/ TR-PE (1mol%). The silica nanoparticles were coated with a lipid bilayer (DOPC/ Biotin-PE (5mol%)/ TR-PE (1mol%)). (B) Fluorescence intensity profiles before (blue) and after (red) the adsorption of

nanoparticles (measured along the dashed line in (A)). The fluorescence in the polymer region increased by the adsorption of silica nanoparticles.

A nanogap-junction was formed by attaching a streptavidin-coated PDMS sheet onto the patterned bilayer with adsorbed silica nanoparticles (Figure 1(A)). For evaluating the gap thickness, we used a PDMS sheet having a step structure (height: 150 nm) as the height reference (Figure 3). A calcein solution (100 μ M) was sandwiched between the PDMS sheet and patterned membrane, and its fluorescence was microscopically monitored. Contrast in fluorescence intensity was observed at the PDMS step and nanogap (Figure 3B). The polymeric bilayer regions had a slightly weaker fluorescence compared with the gap regions, because silica nanoparticles partially excluded the calcein solution. The relative thickness of nanogap ($t_{nanogap}$) was evaluated by comparing the fluorescence intensity increment at the nanogap ($\Delta I_{nanogap}$) and PDMS step (ΔI_{step}).

$$t_{nanogap} = \frac{\Delta I_{nanogap}}{\Delta I_{step}} \quad (5)$$

For the nanogap junctions formed using 30 nm, 100 nm, and 200 nm silica nanoparticles, we estimated the relative thickness to be 0.17, 0.64, and 0.90, respectively (Figure 3C left). The gap thickness correlated with the size of silica nanoparticles, supporting the premise that the gap thickness could be controlled by the silica nanoparticle sizes. We further corrected the relative nanogap thickness by taking the particle densities into account. From the experimentally determined surface

coverage of 30, 100, and 200 nm nanoparticles (0.48, 0.38, and 0.25), we estimated the volume occupation of nanoparticles in the adhesion layer (V) to be 0.32, 0.26, 0.16, respectively, using the ratio between volume and area a spherical particle occupies. Since the fluorescence intensity difference between the nanogap and polymeric regions ($\Delta I_{nanogap}$) should be proportional to the volume occupied by nanoparticles, we can obtain the corrected relative nanogap thickness ($T_{nanogap}$) by the following equation.

$$T_{nanogap} = \frac{t_{nanogap}}{V} \quad (6)$$

Figure 3(C) right shows the plot of corrected relative thickness versus sizes of nanoparticles determined by the dynamic light scattering (25.9, 101.8, and 200.4 nm for 30, 100, and 200 nm silica nanoparticles, respectively). The corrected relative thickness was normalized with the value of 200 nm silica nanoparticles. The corrected relative thickness and the measured average nanoparticle sizes have a very good linear correlation (see the linear fit in Figure 3(C) right), further supporting the controlled gap thickness using nanoparticles.

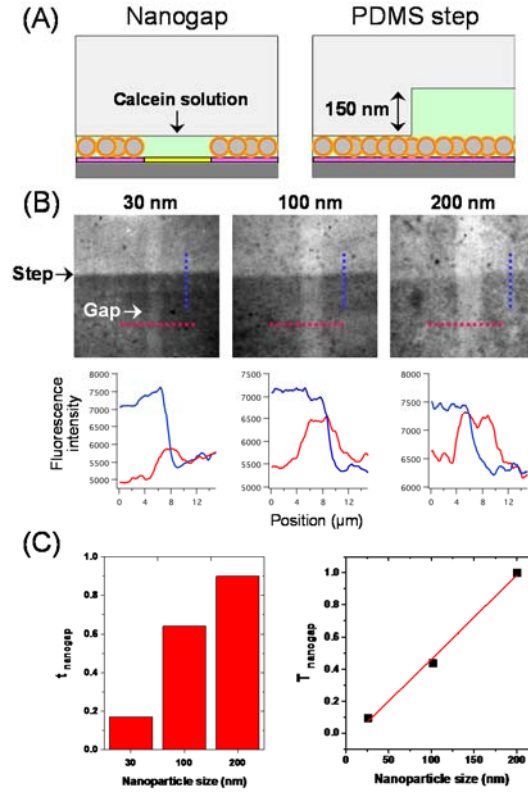


Figure 3: Evaluating the gap thickness: (A) Schematic illustration: A calcein solution was trapped between the bilayer and PDMS, and its fluorescence was observed. The PDMS sheet had a step structure with a known height (150 nm) to calibrate the gap thickness. (B) Fluorescence micrographs of the nanogap-junctions prepared with silica nanoparticles having the sizes of 30 nm, 100 nm, and 200 nm. Fluorescence intensity profiles were measured at the nanogap-junction (red lines) and PDMS step (blue lines). (C) The relative thickness of nanogap-junction was estimated from the fluorescence intensity profiles at the nanogap and PDMS step ($t_{nanogap}$: left), and further corrected taking into account the coverage of nanoparticles ($T_{nanogap}$: right).

Selective transport and detection of target molecules

Influence of the gap thickness on the selective detection was assessed by comparing the nanogap-junctions made with silica nanoparticles having different sizes (30 nm and 100 nm). Fluorescently labeled cholera toxin subunit B (CTB-488) and bovine serum albumin (BSA-488) were used as the model target and coexisting molecules, respectively. Both proteins were labelled with the same fluorophore emitting green fluorescence. CTB-488 can specifically bind to G_{M1} ganglioside (G_{M1}) in the fluid bilayer, and is selectively transported into the nanogap-junction by the lateral diffusion of lipid molecules. On the other hand, BSA-488 cannot bind onto the fluid bilayer, and its transport into the nanogap-junction is limited to the nonspecific penetration via three-dimensional diffusion in the nanometer-sized gap structure. A mixed solution of CTB-488 ($5\text{ }\mu\text{g/ mL} \approx 3.0 \times 10^{-7}\text{ M}$) and BSA-488 ($200\text{ }\mu\text{g/ mL} \approx 3.0 \times 10^{-6}\text{ M}$) (phosphate buffer, pH 6.6) was introduced into the microchannel, and migration of molecules into the nanogap-junction was observed (Figure 4). Since the concentration of BSA-488 was ten-times higher compared with CTB-488, fluorescence in the microchannel was dominated by BSA-488 (Figure 4(A) left side). In the nanogap-junctions aligned perpendicular to the micro-channel, fluorescence of BSA-488 was suppressed and green fluorescence of CTB-488 was observed to gradually migrate (Figure 4(A) right side). As we used BSA-594 instead of BSA-488, we observed that CTB-488 penetrated into the nanogap-junction, whereas BSA-594 remained in the micro-channel, clearly demonstrating the selective transport of CTB. Furthermore, no fluorescence was observed in the nanogap-junction, if the fluid bilayer did not contain G_{M1} (Figure 4(C)), strongly corroborating the premise that the green fluorescence in the nanogap-junction was emitted from CTB-

488 that specifically bound to the membrane. Absence of fluorescence in the nanogap-junction also indicates that the substrate surface is mostly covered with a lipid bilayer, since defects would cause nonspecific adsorption of proteins molecules. A weaker green fluorescence was observed in the adhesion region composed of polymeric bilayer and adsorbed silica nanoparticles (Figure 4)). Since lipid bilayers on silica nanoparticles did not contain G_{M1} , this fluorescence should stem from nonspecific penetration of CTB-488 and BSA-488 into the adhesion layer. The fluorescence in the adhesion layer was observed also in the absence of G_{M1} in the fluid bilayer (Figure 4(C)).

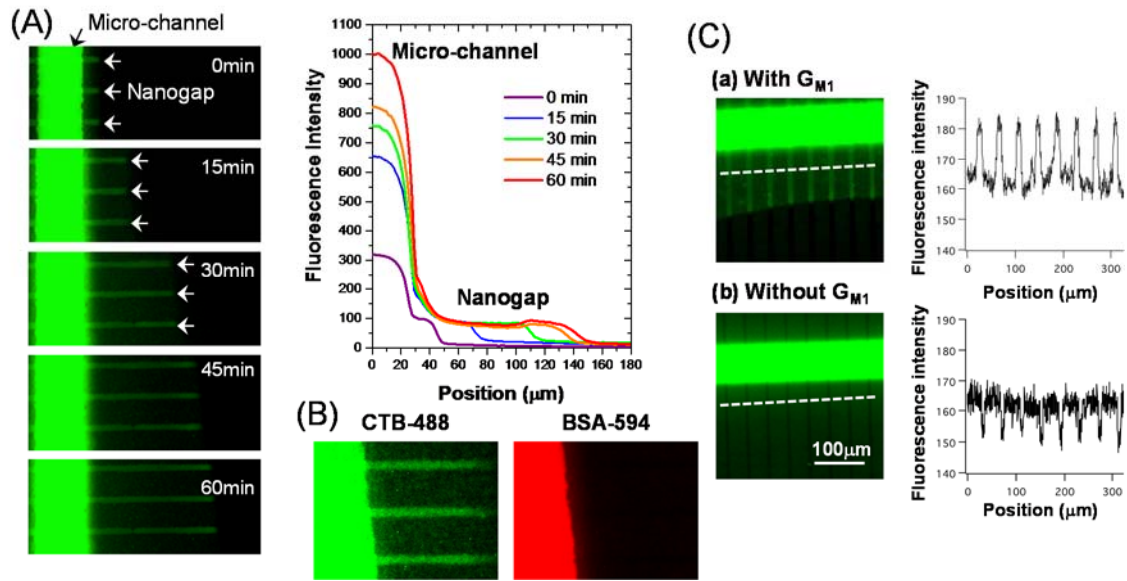


Figure 4: Selective transport of target molecules into the nanogap-junction: (A) A mixed solution of CTB-488 ($5 \mu\text{g/mL}$: $\text{ca. } 3.0 \times 10^{-7} \text{ M}$) and BSA-488 ($200 \mu\text{g/mL}$: $\text{ca. } 3.0 \times 10^{-6} \text{ M}$) was introduced into the micro-channel (width $50 \mu\text{m}$, height $40 \mu\text{m}$), and fluoresce in the nanogap-junction (three horizontal stripes indicated with white arrows) was observed to elongate with time. Fluorescence intensity profiles in the nanogap-junction were plotted (right). (B) A mixed solution of CTB-488 ($5 \mu\text{g/mL}$: $\text{ca. } 3.0 \times 10^{-7} \text{ M}$) and BSA-594 ($200 \mu\text{g/mL}$: $\text{ca. } 3.0 \times 10^{-6} \text{ M}$) was used to separately observe

CTB and BSA. (C) Effect of G_{M1} on the selective penetration: If G_{M1} was present in the fluid bilayers, we observed the fluorescence of CTB-488 in the nanogap-junction (a). No fluorescence was observed in the nanogap-junction, if G_{M1} was lacking in the fluid bilayer (b).

Figure 5 compares the fluorescence signals in the micro-channel and nanogap-junction. In the micro-channel, the background fluorescence of BSA-488 was much more intense than the fluorescence from membrane-bound CTB-488 in the fluid bilayer (stripe regions) (Figure 5(A) and (B)). The signal from CTB-488 was more clearly visible in the nanogap-junction due to suppressed background fluorescence from BSA-488. The intensity profiles in Figure 5(B) also show an enhanced S/B ratio in the nanogap-junction. In the micro-channel, the background fluorescence level was very high and the binding of CTB-488 only slightly elevated the fluorescence intensity (Figure 5(B) left, line (a)). In the nanogap-junction, the background fluorescence level was vastly suppressed and the locally enhanced fluorescence due to the binding of CTB-488 was more clearly detected (Figure 5(B) left line (b) and the enlarged plot in Figure 5(B) right). The fluorescence in the adhesion region decreased with the distance from the micro-channel, whereas the fluorescence intensity remained constant in the nanogap-junction (Figure 5(C)), suggesting that the fluorescence signal in the nanogap-junction arose primarily from membrane-bound CTB-488. The rather sharp boundary of fluorescence at the edge was presumably due to the absence of CTB-488 in further regions. Since CTB forms pentameric molecular clusters, its diffusion on the membrane is expected to be rather slow.^[10] For molecules that diffuse freely, one would expect a gradual decrease of the fluorescence intensity along the nanogap-junction.

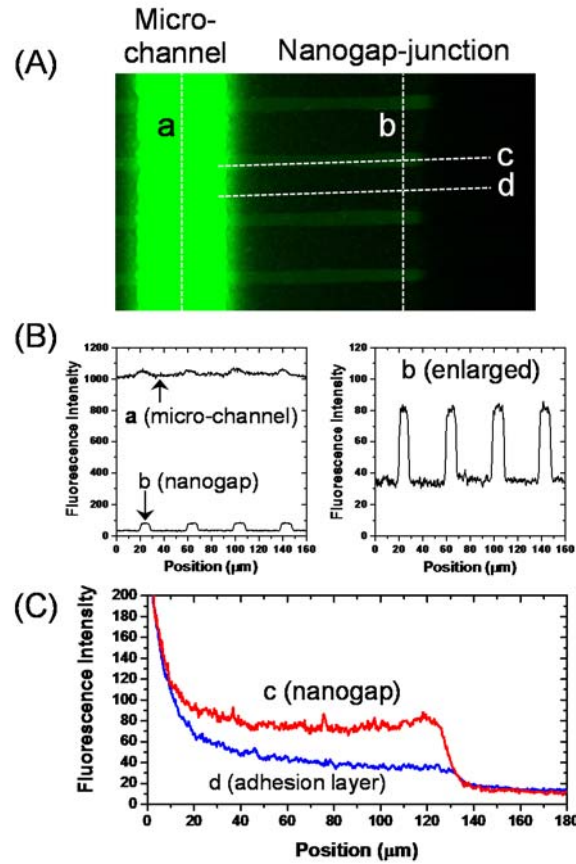


Figure 5: Selective transport of target molecules into the nanogap-junction: (A) A mixed solution of CTB-488 (5 $\mu\text{g/mL}$: ca. 3.0×10^{-7} M) and BSA-488 (200 $\mu\text{g/mL}$: ca. 3.0×10^{-6} M) was introduced into the micro-channel and incubated for 60 min. CTB-488 bound onto the stripe-shaped fluid bilayers having GM_1 . In the micro-channel, fluorescence from CTB-488 was much weaker than the background fluorescence from BSA-488. In the nanogap-junction, stripes of fluid bilayers could be more clearly observed due to selective lateral diffusion of CTB-488 and suppressed penetration of BSA-488. (B) Left: The fluorescence intensity profiles in the micro-channel (a) and in the nanogap-junction (b). Right: Enlarged plot of the fluorescence intensity in the nanogap-junction (b). (C) The fluorescence intensity profiles in the nanogap-junction (c) and adhesion layer (d).

Figure 6 compares the selectivity in nanogap-junctions made with silica nanoparticles having different average sizes. Fluorescence in the polymeric region (background noise) was lower for 30 nm nanoparticles compared with 100 nm nanoparticles. On the other hand, the fluorescence intensity in the fluid bilayer region (signal) was not changed. These results clearly demonstrate that nonspecific penetration of molecules was more effectively suppressed in the nanogap-junction made with smaller nanoparticles, whereas penetration of membrane-bound molecules was not affected by the gap thickness. As a result, a higher S/B ratio was attained for the nanogap-junction formed with smaller silica particles. The S/B ratio was calculated from the average fluorescence intensities in the nanogap and adhesion regions in the distance between 50 μm and 150 μm from the micro-channel. The obtained values were 0.95 and 0.28 for 30 nm and 100 nm silica nanoparticles, respectively. These results could be fitted to Equation (4), which shows the inverse proportionality of the S/B ratio to the gap thickness. The plot of S/B ratio versus the silica nanoparticle size is shown in Figure S2 to compare the theoretical prediction and experimental results. The heightened S/B ratio for the nanogap-junction with 30 nm nanoparticles is consistent with the theoretical prediction that the S/B ratio is inversely proportional to the gap thickness (*vide supra*).

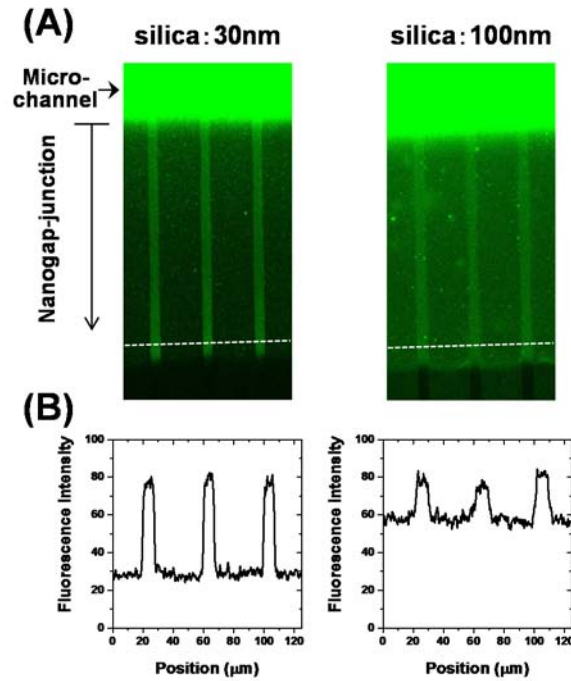


Figure 6: Effect of silica nanoparticle sizes on the suppression of background noise: (A) Fluorescence arising from a mixture of BSA-488 and CTB-488 was compared in the nanogap-junction formed with 30 nm and 100 nm silica nanoparticles. A mixed solution of CTB-488 (5 $\mu\text{g/mL}$: ca. 3.0×10^{-7} M) and BSA-488 (200 $\mu\text{g/mL}$: ca. 3.0×10^{-6} M) was introduced into the micro-channel and incubated for 60 min. (B) Fluorescence intensity profiles in the nanogap-junction. The fluorescence intensity in the adhesion region was more effectively suppressed for 30 nm nanoparticles compared with 100 nm nanoparticles, whereas the fluorescence intensity in the nanogap-junction was unchanged for both nanoparticles sizes.

Stability of the nanogap-junction

Stability of the nanometric gap structure is important for the biomedical and environmental applications. Nanogap-junction constructed with silica nanoparticles had vastly improved stability

compared with those made using lipid vesicles as the adhesion material. Nanogap-junctions made with lipid vesicles typically lasted only for several hours, and were destabilized due to dehydration. Fluid bilayers in the nanogap-junction were destroyed by direct contact with the PDMS surface. Nanogap-junctions made with silica nanoparticles were mechanically more robust, and we could maintain the structure significantly longer (more than one week). Figure 7 shows the fluorescence recovery after photobleaching (FRAP) of a fluid bilayer (DOPC/ TR-PE (1mol%)) confined in a nanogap-junction and stored at 4 °C for one week. As we partially photobleached the fluorophore in the membrane, fluorescence of the photobleached spot recovered with time, clearly showing that the integrity and fluidity of the lipid bilayer membrane were preserved. The samples stored for one week could be used for the selective detection of target molecules. Binding of CTB-488 to G_{M1} did not noticeably change, if the fluid bilayer with embedded G_{M1} was stored at 4 °C.

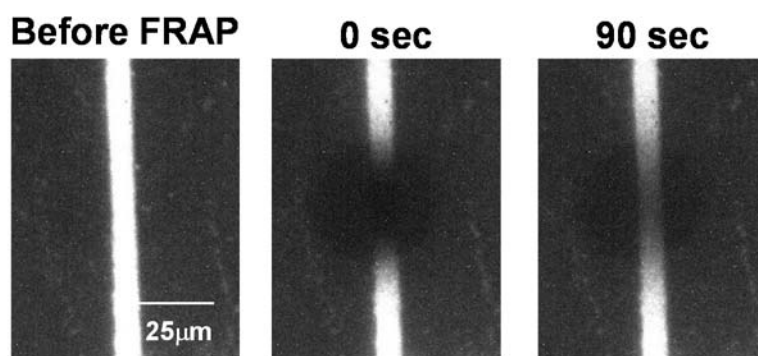


Figure 7: Continuity and fluidity of the bilayer (DOPC/ TR-PE) in the nanogap-junction stored at 4 °C for one week. A part of the bilayer was photobleached and recovery of fluorescence was observed.

4. Conclusions

In summary, we generated a nanogap-junction between a fluid lipid membrane and PDMS using silica nanoparticles as the adhesion material. Owing to the inherently uniform size, silica nanoparticles could precisely control the gap thickness. As theoretically predicted, selective transport and detection of specific target molecules was enhanced in a thinner nanogap-junction due to suppressed penetration of non-target molecules. Though the present study assessed only the relative thickness of gap structures, evaluating the absolute thickness is critical in the future study for extending the utility of the nanogap junction. As the gap thickness becomes smaller to quasi molecular sizes, the charge/molecular distribution effects would influence the molecular transport and properties. The stability of the gap structure was vastly improved by silica nanoparticles, due to their mechanical robustness. The nanogap structures made with silica nanoparticles were stable for more than one week. Nanogap-junction with a controlled thickness and prolonged lifetime provides a nanofluidic biosensing platform for biomedical and environmental applications, for which highly sensitive detection of specific target molecules in the presence of diverse coexisting molecules is a prerequisite. The results using a model mixture of CTB and BSA clearly demonstrate that one can selectively detect specific target molecules in the presence of much more concentrated coexisting molecules. This could possibly extended to non-labelled biomarker molecules by using membrane-bound antibodies. We envision to apply the nanogap-junction for diagnostic applications by measuring specific proteins or peptides in the blood serum. Although the fabrication process of a nanogap-junction is rather

elaborate, using it for detecting specific target molecules is facile. A pre-formed chip can be used to apply the samples into the inlet and observe the selective migration of target molecules into the nanogap-junction. The role of silica nanoparticles as a stable adhesion agent is very important in this respect, because it enables to sustain the nanometric gap structure for a long period.

Experimental Section

Materials

1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (DiynePC), 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine (DiynePE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), G_{M1} ganglioside (brain, ovine) (G_{M1}), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotiny) (Biotin-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Marina Blue-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (MB-PE), cholera toxin subunit B-Alexa Fluor 488 conjugate (CTB-488), and albumin (bovine serum)-Alexa Fluor 488 conjugate (BSA-488)/ -Alexa Fluor 594 conjugate (BSA-594) were purchased from Molecular probes (Eugene, OR). Albumin from bovine serum (BSA) was purchased from Sigma-Aldrich. *N*-hydroxysuccinimide-polyethyleneglycol-biotin (NHS-PEG₄-biotin) was purchased from Thermo Scientific. Calcein was purchased from Nacalai Tesque (Kyoto, Japan). Silica nanoparticles (30, 100, 200 nm) were purchased from Micromod Partikeltechnologie GmbH (Friedrich, Germany). The average sizes of silica nanoparticles were evaluated by the dynamic light scattering to be 25.9, 101.8, and 200.4 nm for 30, 100, and 200 nm silica nanoparticles, respectively. In the following, the three types of nanoparticles are designated as 30, 100, and 200 nm for simplicity.

Substrate cleaning

Microscopy coverslips (38 mm x 26mm) (Matsunami, Osaka, Japan) were used as the substrates for lipid film deposition. The substrates were cleaned with a commercial detergent solution, 0.5%

Hellmanex/ water (Hellma, Mühlheim, Germany), for 20 min under sonication, rinsed with deionized water, treated in a solution of NH_4OH (28%)/ H_2O_2 (30%)/ H_2O (0.05:1:5) for 10 min at 65 °C, rinsed extensively with deionized water, and then dried in a vacuum oven for 30 min at 80 °C. Before use, these substrates were further cleaned by the UV/ozone treatment for 20 min (PL16–110, Sen Lights Corporation, Toyonaka, Japan).

Preparation of polymeric bilayers

The fabrication method of patterned polymeric bilayers has been described in previous papers.^[8b, 11] Briefly, bilayers composed of monomeric diacetylene lipids (DiynePC/ DiynePE (95:5)) were deposited onto cover glass from the air/water interface by the Langmuir–Blodgett (LB) and Langmuir–Schaefer (LS) methods using a Langmuir trough (HBM-AP, Kyowa Interface Science, Asaka, Japan). The temperature of the subphase (deionized water) was controlled at 16 °C. The surface pressure was controlled at 30 mN/m. DiynePC/ DiynePE bilayers were polymerized by UV irradiation using a mercury lamp (UVE-502SD, Ushio, Tokyo, Japan) as the light source. The applied UV intensity was typically 4 mW/cm² at 254 nm and the total energy was modulated by the illumination time. The patterned area was 10 mm x 10mm. After UV irradiation, nonpolymerized DiynePC and DiynePE molecules were removed from the substrate surface by immersing in 0.1 M sodium dodecylsulfate (SDS) solution at 30 °C for 30 min and extensively rinsing with deionized water.

Subsequently, the surface of the DiynePC/DiynePE bilayer was modified with biotin by incubating in an NHS-PEG₄-biotin solution (1 mg/ mL in NaHCO₃ buffer (0.1 M, pH 8.4)) for 3 hours on ice. After rinsing with deionized water, the sample was immersed in 0.1 M SDS solution at 30 °C for 30 min and extensively rinsed with deionized water. The biotinylated polymeric bilayers were stored in deionized water in the dark at 4 °C for further experiments.

Preparation of vesicle suspensions and formation of a patterned bilayer

Two types of vesicles were prepared for the fluid bilayer and for the adhesion layer. The lipid compositions were DOPC/ G_{M1} (2mol%) for the fluid bilayers, and DOPC/ Biotin-PE (5mol%) for the adhesion layer (*vide infra*), respectively. Fluorescent lipids (either 1mol% TR-PE or 2mol% MB-PE) were added for microscopically observing the membranes. Lipids were dissolved in chloroform and mixed in a round-bottom flask. Chloroform was removed by drying under a stream of nitrogen and subsequently evaporating for at least 4 h in vacuum. The dried lipid film was hydrated in 0.01 M sodium phosphate buffer with 0.15 M NaCl (pH 6.6) (the total lipid concentration was 1 mM). Lipid membranes were dispersed by five freeze/thaw cycles. For incorporating fluid bilayers into the patterned matrix of polymerized bilayer, vesicle suspensions were sonicated by an ultrasonic homogenizer (Branson Sonifier150) on ice (30 s x 2). For forming a patterned bilayer, sonicated vesicles were applied onto the surface of the substrate with preformed patterned polymeric bilayer.^[12]

Adsorption of silica nanoparticles onto the polymeric bilayer

Silica nanoparticles were coated with a lipid bilayer (DOPC/ Biotin-PE (5 mol %)/ TR-PE 1 mol % (or MB-PE or 2 mol %)) for binding to the polymeric bilayer by the biotin-streptavidin linkage. Silica nanoparticles were mixed with sonicated vesicles and incubated for 1 hour.^[13] Unbound vesicles were rinsed off by centrifuging the mixed solution several times and collecting the precipitated nanoparticles. Fluorescence of TR-PE in the precipitate and supernatant was measured after the centrifugation to assess the lipid membrane coverage of nanoparticles (Supporting Information: Figure S1). The lipid-coated nanoparticles were attached onto the surface of polymeric bilayer that had been pre-coated with streptavidin. After incorporating fluid bilayers (DOPC/ G_{M1}/ TR-PE (97:2:1)) into the polymer-free regions between polymeric bilayers, streptavidin and silica nanoparticles were sequentially attached onto the biotinylated polymeric bilayer surface. The nanoparticle suspensions were shaken for 5 hours (200 rpm, IKA, MS1 Minishaker) and kept still for another 1 hour. The substrate was rinsed for removing excess silica nanoparticles. The amount of attached nanoparticles was evaluated by comparing the fluorescence intensities of TR-PE in the polymeric and fluid bilayer regions. The area fraction of polymeric bilayer covered with nanoparticles (X) was estimated from the changes of fluorescence intensities before and after the addition of silica nanoparticles using the following equation.

$$X = \frac{(I_{P1}-I_{P0})-(I_{F1}-I_{F0})}{4 \times I_{F0}} \quad (7)$$

I_{P0} , I_{P1} , I_{F0} , and I_{F1} are intensities in the polymeric and fluid bilayers before and after the addition of nanoparticles, respectively. The denominator includes the factor 4, since the surface area of a spherical particle is four times of the projected area the particle occupies. I_{F0} was used as the standard fluorescence intensity of the planar bilayer membrane. The intensity change in the fluid bilayer ($I_{F1} - I_{F0}$) was subtracted from the intensity increase in the polymeric region ($I_{P1} - I_{P0}$) in order to offset the changes in the background fluorescence after addition of nanoparticles.

Fabrication of PDMS sheet

Polydimethylsiloxane (PDMS) (Silpot 184, Toray Dow Corning) sheets were made by mixing the silicon elastomer and the curing agent at the ratio of 10:1 (w/w). PDMS was degassed for 15 min in a vacuum desiccator, and poured onto a silicon mold having micro-channels on the surface. Then it was cured in an oven (70 °C) for 1 hour. After curing, PDMS was peeled off from the mold and covered with an aluminum foil to avoid the attachment of particles onto the surface. The typical size of PDMS sheet was 38 mm x 26mm. Before use, the PDMS sheet was cut into an appropriate size, and holes for the inlet and outlet (3.5 mm and 2.0 mm) were punched. After peeling off the aluminum foil, the surface of PDMS was coated with BSA and subsequently biotinylated by reacting with NHS-PEG₄-biotin. We then applied streptavidin (10µg/mL) onto the PDMS surface and incubate for 30 min. Unbound streptavidin was subsequently rinsed off from the surface.

Attachment of the polymeric bilayer and PDMS

We attached the polymeric bilayer and PDMS by using the biotin-streptavidin linkage. A patterned bilayer with adsorbed silica nanoparticles was put onto a filter paper. Excess MilliQ water was applied onto the surface to avoid exposure of the membrane to the air. Then, we gently put the streptavidin-coated PDMS sheet onto the substrate. We waited for about 1 hour so that the PDMS sheet sank onto the surface of the patterned bilayer by its weight. Water was occasionally wiped from the side and the outlet with a filter paper to reduce the water layer between PDMS and polymeric bilayer. We also occasionally added MilliQ water into the inlet to avoid complete drying of the gap layer. For evaluating the distance between PDMS and patterned membrane, water soluble dye (calcein, 0.5 μM) was incorporated in the aqueous phase and its fluorescence intensity was measured. We used a PDMS sheet that had a step structure with a defined height (150 nm) as a reference to evaluate the gap thickness. PDMS sheets with a step structure were made with a silicon mold having chromium stripes (height: 150 nm).

Fluorescence microscopy observation

Fluorescence microscopy observation was performed using an inverted microscope (IX-71, Olympus, Tokyo, Japan) equipped with a xenon lamp (UXL-75XB, Olympus), 20x objective (NA 0.75) or 100x objective (NA 1.40), a CMOS camera (Orca 4.0, Hamamatsu Photonics, Hamamatsu, Japan), and an image intensifier (C9016-05, Hamamatsu Photonics, Hamamatsu, Japan). Four types of filter sets were used: (1) excitation 330-385 nm/ emission >420 nm (UV fluorescence), (2) excitation 470-490

nm/ emission 510-550 nm (green fluorescence), (3) excitation 545-580 nm/ emission >610 nm (red fluorescence), (4) excitation 690-730 nm/ emission 750-800 nm (far-red fluorescence).

Supporting Information. Additional information about adsorption of lipid bilayer onto silica nanoparticles and comparison of the experimentally obtained S/B ratios for 30 nm and 100 nm silica nanoparticles and the theoretical predicted values from Equation (4).

Acknowledgements. This work was supported by Grant-in-Aid for Scientific research from Japan Society for the Promotion of Science (#23106714, #15K14489, #17H03666), Nippon Sheet Glass Foundation, and Tokyo Ohka Foundation for the Promotion of Science and Technology. We thank Dr. Hideto Matsuyama and Dr. Daisuke Saeki (Kobe University) for allowing us to use the dynamic light scattering instrument.

References:

- [1] J. J. Gooding, K. Gaus, *Angew. Chem. Int. Ed.* **2016**, 55, 11354-11366.
- [2] a) D. Axelrod, *J. Cell Biol.* **1981**, 89, 141-145; b) T. Funatsu, Y. Harada, M. Tokunaga, K. Saito, T. Yanagida, *Nature* **1995**, 374, 555-559; c) H. Noji, R. Yasuda, M. Yoshida, K. Kinoshita Jr., *Nature* **1997**, 386, 299-302.
- [3] a) M. J. Levene, J. Korlach, S. W. Turner, M. Foquet, H. G. Craighead, W. W. Webb, *Science* **2003**, 299, 682-686; b) J. Eid, A. Fehr, J. Gray, K. Luong, J. Lyle, G. Otto, P. Peluso, D. Rank, P. Baybayan, B. Bettman, A. Bibillo, K. Bjornson, B. Chaudhuri, F. Christians, R. Cicero, S. Clark, R. Dalal, A. deWinter, J. Dixon, M. Foquet, A. Gaertner, P. Hardenbol, Cheryl Heiner, K. Hester, D. Holden, G. Kearns, X. Kong, R. Kuse, Y. Lacroix, S. Lin, P. Lundquist, C. Ma, P. Marks, M. Maxham, D. Murphy, I. Park, T. Pham, M. Phillips, J. Roy, R. Sebra, G. Shen, Jon Sorenson, A. Tomaney, K. Travers, M. Trulson, J. Vieceli, J. Wegener, D. Wu, A. Yang, D. Zaccarin, P. Zhao, F. Zhong, J. Korlach, S. Turner, *Science* **2009**, 323, 133-138.
- [4] a) M. Krishnan, I. Mönch, P. Schwille, *Nano Lett.* **2007**, 7, 1270-1275; b) P. Abgrall, N. T. Nguyen, *Anal. Chem.* **2008**, 80, 2326-2341; c) K. Mawatari, Y. Kazoe, H. Shimizu, Y. Pihosh, T. Kitamori, *Anal. Chem.* **2014**, 86, 4068-4077.
- [5] a) T. A. Dickinson, J. White, J. S. Kauer, D. R. Walt, *Nature* **1996**, 382, 697-700; b) Y. Rondelez, G. Tresset, K. V. Tabata, H. Arata, H. Fujita, S. Takeuchi, H. Noji, *Nat. Biotechnol.* **2005**, 23, 361-365; c) D. M. Rissin, C. W. Kan, T. G. Campbell, S. C. Howes, D. R. Fournier, L. Song, T. Piech, P. P. Patel, L. Chang, A. J. Rivnak, E. P. Ferrell, J. D. Randall, G. K.

- Provuncher, D. R. Walt, D. C. Duffy, *Nat. Biotechnol.* **2010**, *28*, 595-599; d) S. H. Kim, S. Iwai, S. Araki, S. Sakakihara, R. Iino, H. Noji, *Lab Chip* **2012**, *12*, 4986-4991.
- [6] a) J. J. Kasianowicz, E. Brandin, D. Branton, D. W. Deamer, *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 13770-13773; b) D. Branton, D. W. Deamer, A. Marziali, H. Bayley, S. A. Benner, T. Butler, M. D. Ventra, S. Garaj, A. Hibbs, X. Huang, S. B. Jovanovich, P. S. Krstic, S. Lindsay, X. S. Ling, C. H. Mastrangelo, A. Meller, J. S. Oliver, Y. V. Pershin, J. M. Ramsey, R. Riehn, G. V. Soni, V. Tabard-Cossa, M. Wanunu, M. Wiggin, J. A. Schloss, *Nat. Biotechnol.* **2008**, *26*, 1146-1153; c) E. C. Yusko, J. M. Johnson, S. Majd, P. Prangkio, R. C. Rollings, J. Li, J. Yang, M. Mayer, *Nat. Nanotechnol.* **2011**, *6*, 253-260; d) E. C. Yusko, B. R. Bruhn, O. M. Eggenberger, J. Houghtaling, R. C. Rollings, N. C. Walsh, S. Nandivada, M. Pindrus, A. R. Hall, D. Sept, J. Li, D. S. Kalonia, M. Mayer, *Nat. Nanotechnol.* **2017**, *12*, 360-367.
- [7] a) S. J. Singer, G. L. Nicolson, *Science* **1972**, *175*, 720-731; b) K. Simons, D. Toomre, *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 31-41; c) G. Vereb, J. Szöllosi, J. Matkó, P. Nagy, T. Farkas, L. Vigh, L. Mátyus, T. A. Waldmann, S. Damjanovich, *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 8053-8058; d) D. J. Irvine, M. A. Purbhoo, M. Krogsgaard, M. M. Davis, *Nature* **2002**, *419*, 845-849; e) T. Doan, A. Mendez, P. B. Detwiler, J. Chen, F. Rieke, *Science* **2006**, *313*, 530-533; f) D. Chapman, *Langmuir* **1993**, *9*, 39-45; g) A. Kusumi, T. K. Fujiwara, R. Chadda, M. Xie, T. A. Tsunoyama, Z. Kalay, R. S. Kasai, K. G. N. Suzuki, *Annu. Rev. Cell Dev. Biol.* **2012**, *28*, 215-250; h) K. G. N. Suzuki, in *Int. Rev. Cell Mol. Biol.*, Vol. 317 (Ed.: K. W. Jeon), Elsevier, **2015**, pp. 67-96.

- [8] a) K. Morigaki, T. Baumgart, A. Offenhäusser, W. Knoll, *Angew. Chem., Int. Ed.* **2001**, *40*, 172-174; b) K. Morigaki, K. Mizutani, M. Saito, T. Okazaki, Y. Nakajima, Y. Tatsu, H. Imaishi, *Langmuir* **2013**, *29*, 2722-2730.
- [9] K. Ando, M. Tanabe, K. Morigaki, *Langmuir* **2016**, *32*, 7958-7964.
- [10] a) R. Wang, J. Shi, A. N. Parikh, A. P. Shreve, L. Chen, B. I. Swanson, *Colloids Surf. B* **2004**, *33*, 45-51; b) V. Yamazaki, O. Sirenko, R. J. Schafer, J. T. Groves, *J. Am. Chem. Soc.* **2005**, *127*, 2826-2827.
- [11] T. Okazaki, T. Inaba, Y. Tatsu, R. Tero, T. Urisu, K. Morigaki, *Langmuir* **2009**, *25*, 345-351.
- [12] T. Okazaki, K. Morigaki, T. Taguchi, *Biophys. J.* **2006**, *91*, 1757-1766.
- [13] S. Mornet, O. Lambert, E. Duguet, A. Brisson, *Nano Lett.* **2005**, *5*, 281-285.

Supporting Information

Nanofluidic Biosensor Created by Bonding Patterned Model Cell Membrane and Silicone Elastomer with Silica Nanoparticles

Masashi Tanabe¹, Koji Ando¹, Ryota Komatsu¹, Kenichi Morigaki^{1, 2 *}

1: Graduate School of Agricultural Science, Kobe University, Rokkodaicho 1-1, Nada, Kobe 657-8501, Japan

2: Biosignal Research Center, Kobe University, Rokkodaicho 1-1, Nada, Kobe 657-8501, Japan

*Corresponding author:

Kenichi Morigaki: E-mail: morigaki@port.kobe-u.ac.jp, Fax: +81-78-803-5941

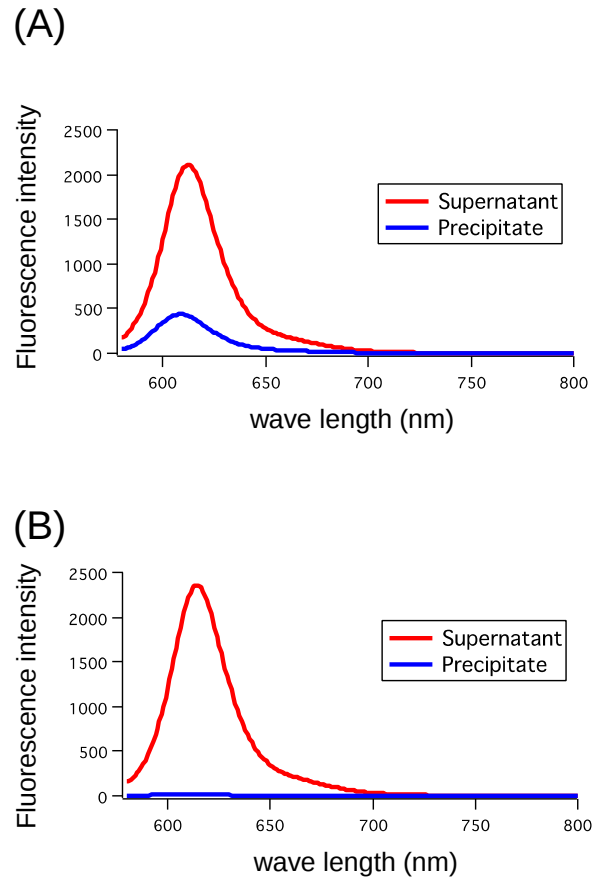


Figure S1

Adsorption of lipid bilayer onto silica nanoparticles: Silica nanoparticles were coated with a lipid bilayer (DOPC/ Biotin-PE (5 mol %)/ TR-PE (1 mol %)) for binding to the polymeric bilayer by biotin-streptavidin linkage. Silica nanoparticles were mixed with sonicated vesicles and incubated for 1 hour. Afterwards, the nanoparticles were collected by the centrifugation. (A) Fluorescence of TR-PE in the precipitate and supernatant after the centrifugation. (B) Fluorescence of TR-PE in the precipitate and supernatant after the centrifugation without silica nanoparticles.

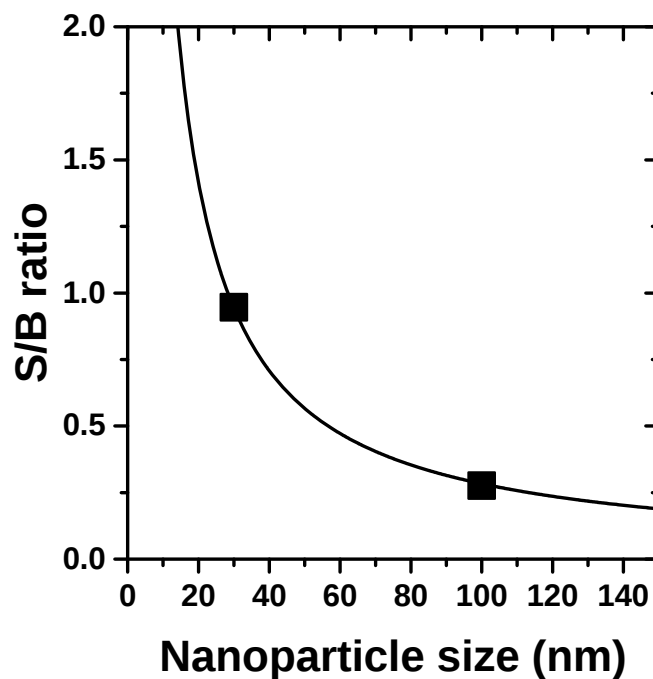


Figure S2

The dependency of the S/B ratio on the nanogap thickness was assessed by fitting the experimentally obtained S/B ratios for 30 nm and 100 nm silica nanoparticles (square points) to Equation (4) (black line), assuming that the gap thickness is proportional to the nanoparticle size as suggested in Figure 3. The fitting demonstrates that the S/B ratio was inversely proportional to the nanoparticle size, in agreement with the prediction of model calculation.