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1 **Duffy antigen receptor for chemokines (DARC) nanodisc-**
2 **based biosensor for the detection of staphylococcal**
3 **bicomponent pore-forming leukocidins**

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21 **Abstract**

22 *Staphylococcus aureus* (*S. aureus*) is an opportunistic infectious pathogen, which leads to a
23 high mortality rate during bloodstream infections. The early detection of virulent strains in
24 patient blood samples is of medical interest for rapid diagnosis. One of the main virulent factors
25 identified in patient isolates is the leukocidin gamma hemolysin (HlgAB). HlgAB is a
26 bicomponent pore-forming toxin that binds to specific membrane receptors, such as the Duffy
27 antigen receptor for chemokines (DARC), to lyse immune cells and erythrocytes. Current
28 methods are based on ELISA or bacterial culture which takes hours to days. For detecting HlgAB
29 with faster response and higher sensitivity, we developed a biosensor that combines single-
30 walled carbon nanotube field effect transistors with immobilized DARC receptors as biosensing
31 elements. DARC was purified from a bacterial expression system and successfully
32 reconstituted into nanodiscs that preserve binding capability for HlgAB. Dynamic light
33 scattering (DLS) and scanning electron microscopy (SEM) showed an increase of the DARC-
34 containing nanodiscs size in presence of HlgAB, indicating the formation of HlgAB pre-pore
35 or pore complexes. We demonstrate that this sensor could specifically detect the leukocidins
36 HlgA and HlgAB in buffer from a concentration of 1 fM and in a quantitative manner with a
37 LOD of 0.122 fM and a LOQ of 0.441 fM within the dynamic range of 1 fM to 100 pM. The
38 sensor was challenged with human serum spiked with HlgAB as simulated clinical samples,
39 and after dilution for decreasing nonspecific binding, it selectively detected the toxin with
40 similar method detection level (MDL) and apparent dissociation constant as in buffer. This
41 biosensor was demonstrated with remarkable sensitivity to detect HlgAB rapidly holding
42 potential as a tool for fundamental research and clinical applications.

43

44 **Keywords:** leukocidin, gamma-hemolysin, Duffy antigen receptor for chemokines, nanodisc,

45 swCNT-FET

46

47

48 **1. Introduction**

49 *S. aureus* is a common commensal bacterium that can asymptotically colonize human skin,
50 nose and intestine. ¹ However, in some cases, it becomes an opportunistic pathogen responsible
51 for diverse infections frequently observed worldwide in food poisoning and more critically in
52 hospitals where multiresistant *S. aureus* strains to antibiotics cause severe reduction in
53 therapeutic efficacy. ²⁻⁴ The severity of infection ranges widely from mild skin or soft tissue
54 infections to more severe bacteremia, sepsis, infective endocarditis, osteomyelitis, and
55 pneumonia. ⁵ Especially, mortality rate associated with *S. aureus* bacteremia within 30 days
56 stands at approximately 20%, a statistic that has remained constant since the 1990s. ^{6, 7}
57 Therefore, an invasive infection of *S. aureus* via blood stream clearly leads to serious clinical
58 consequences if prevention or treatment is not initiated in early phases.

59 *S. aureus* is able to secrete numerous virulence factors, including toxins, immune evasion
60 factors, and a plethora of proteinaceous or non-proteinaceous factors that can facilitate host
61 colonization during infections. ⁸ Leukocidins from *S. aureus* are secreted as soluble proteins
62 that can specifically lyse leukocytes, thus weakening host defenses. One strain of *S. aureus*
63 related to human infections is capable of producing some cognate pairs of leukocidins: the
64 gamma-hemolysins HlgAB, the leukotoxins LukAB (also known as LukGH), LukED and the
65 Pantan-Valentine leukotoxins LukSF-PV. ⁸ Each bicomponent toxin targets specific innate and
66 adaptive immune cells through specific interactions with membrane receptors and causes death
67 of these specific cells. Among them, gamma-hemolysin HlgAB is secreted by 99% of *S. aureus*
68 isolates, representing a conserved set of leukocidins present in the core genome of *S. aureus*. ⁸
69 HlgAB not only targets on leukocytes, but also erythrocytes, providing heme molecules as an
70 iron source and leading to growth of bacteria in host blood. ⁹ HlgAB also increases vascular

71 permeability in blood vessels, resulting in vascular injury and fluid extravasation into tissue. ¹⁰
72 Toxicity of HlgAB is determined by their binding capability on several chemokine receptors
73 (such as CCR2, CXCR1, and CXCR2) and DARC present on surfaces of leukocyte,
74 erythrocytes and endothelial cells. ^{11,12} HlgAB undergoes oligomerization and assemble into a
75 heterooctameric pore within the plasma membrane of targeted chemokine receptors, which
76 causes osmotic dysregulation and leakage of ions and essential nutrients, leading to cell death.
77 ¹³⁻¹⁵ Therefore, HlgAB in a patient blood is a critical biomarker for the most frequent infectio ns
78 by *S. aureus* strains and rapid, sensitive and specific biosensing techniques to HlgAB are
79 required for implementing early and appropriate treatments in hospitals and communities.

80 Rapid detection of *S. aureus* blood samples can be performed by real-time PCR and other
81 more recent techniques such as T2Dx and Hybrisep, which does not require blood culture. ¹⁶
82 T2Dx method is based on magnetic resonance, while Hybrisep uses in situ hybridization
83 methods. Both technology provide responses in hours. More specific detection of leukocidins
84 in blood samples is rarer and it is generally based on enzyme-linked immunosorbent assay
85 (ELISA). ¹⁷ However, this method also requires several hours in biomedical laboratories. In
86 addition, it is susceptible to interference by staphylococcal protein A which can lead to false-
87 positive results in immunoassays based on mammalian immunoglobulin G (IgG). ¹⁸ Thus,
88 development of biosensors has gained enormous attention for detecting pathogens, toxins,
89 biomarkers and bioactive molecules. ¹⁹⁻²⁶ Especially, electronic devices with biological
90 elements for detecting virulent factors from *S. aureus* in various samples have gained attention
91 thanks to their real-time measurements and simple operation with high sensitivity and
92 selectivity. ²⁷⁻³¹ However, none of them could detect or identify leukocidins or use specific
93 membrane receptors as immobilized baits.

94 The utilization of membrane receptors such as DARC in biosensors is challenging since they
95 belong to G-protein coupled receptor (GPCR) family. This family is notoriously known to have
96 a propensity to aggregate when overexpressed in *Escherichia coli* (*E. coli*).^{32,33} Nanodiscs
97 have been utilized for the appropriate structures for GPCR reconstitution due to their membrane
98 mimetic and enhanced stability.^{34,35} Nanodiscs are composed of lipids and membrane scaffold
99 proteins (MSP) that can tightly hold lipids and membrane proteins together, forming
100 biomimetic patches of membranes. Therefore, nanodiscs can provide a native and stable lipid
101 membrane structures for GPCR functions in a variety of environments.^{36,37}

102 Herein, we report the development of a bioelectronic sensor of HlgAB using immobilized
103 DARC in nanodiscs. DARC was overexpressed in *E. coli*, embedded into nanodiscs, and
104 immobilized on single-walled carbon nanotube field effect transistors (swCNT-FETs). This
105 sensor device could detect the binding of HlgA and HlgAB with high sensitivity in real-time.
106 This sensor responded to HlgAB with higher signal amplitude, showing that it could detect the
107 complexes of HlgAB. This sensor also detected HlgAB in spiked human serum as a clinical
108 sample with a sensitivity comparable to that observed in a buffer solution. This sensor is
109 promising for studying interactions between membrane proteins and gamma-hemolysins and
110 developing highly sensitive *in vitro* diagnostic systems of HlgAB.

111

112

113 **2. Materials and Methods**

114 *2.1 Protein constructs*

115 For production of gamma-hemolysins in *E. coli*, the genes of *S. aureus* HlgA (UniprotKB-
116 P0A074), HlgB (UniprotKB-P0A077) and HlgC (UniprotKB-Q07227) were synthesized and
117 subcloned into pQE-30 vectors. For expression of DARC in mammalian cells, the full-length
118 of gene of human DARC isoform-2 (UnioprotKB-Q16570) was synthesized and subcloned into
119 modified pXOOM vectors. For production of DARC in *E. coli*, human DARC genes in
120 pXOOM vectors were amplified and subcloned into pET-DEST42 vectors. For production of
121 the membrane scaffold proteins MSP1E3D1 in *E. coli*, the expression vectors for MSP1E3D1
122 were purchased from Addgene.

123

124 *2.2 Expression and purification of leukocidins*

125 M15 *E. coli* strains (Qiagen, Germany) were transformed with the expression plasmids
126 including HlgA or HlgB. These transformed strains were cultured in 2 L of Luria-Bertani broth
127 (LB) supplemented with 100 µg/ml ampicillin and 30 µg/ml kanamycin at 37°C under 150 rpm
128 agitation until reaching an optical density (OD) of 0.3. Prior to induction, the culture was pre-
129 cooled at 15°C for 30 minutes under 150 RPM agitation at 15°C. Isopropyl β-D-1-
130 thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM and the
131 cultures were grown overnight. The induced cultures were centrifuged at 4500 g for 15 minutes
132 and the resulting pellet was resuspended in 40 ml of lysis buffer (20 mM HEPES (pH 7.5), 150
133 mM NaCl, 50 mM imidazole, 1 tablet of cOmplete EDTA-free protease inhibitor (Roche), 10
134 µg/ml DNase I, and 10 µg/ml RNase). The cells were lysed by subjecting them to four cycles
135 using the microfluidizer LM20 (Microfluidics, USA) at 17000 psi while maintaining a

136 temperature of 4°C. Subsequently, the lysate was clarified by centrifugation at 38500 g or
137 relative centrifugal force (RCF) for 20 minutes at 4°C. His-tagged proteins were purified using
138 immobilized metal chelate affinity chromatography on a 1 ml His-Trap HP column (Cytiva).
139 Prior to loading the lysate, the column was equilibrated with 20 mM HEPES (pH 7.5), 150 mM
140 NaCl, and 50 mM imidazole. A stepwise elution was performed using elution buffers (20 mM
141 HEPES (pH 7.5), 150 mM NaCl, and 250 mM or 300 mM imidazole). Following purification,
142 the eluate from the final elution step was dialyzed against the buffer containing 20 mM HEPES
143 (pH 7.5) and 150 mM NaCl overnight at 4°C using a Spectra/Por 4 dialysis membrane.

144

145 *2.3 Cell viability assay with recombinant leukocidins*

146 Human embryonic kidney-293T (HEK-293T) cells were purchased from American Type
147 Culture Collection (ATCC). These cells were cultured at 37°C with 5% CO₂ condition in
148 Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Biowest, USA)
149 supplemented with 10% fetal bovine serum (FBS) and 100 µg/mL of penicillin-streptomycin
150 mix (Sigma-Aldrich, USA). HEK-293T cells were seeded into 96-well plates at a density of
151 2×10^4 cells/well for 24 h. After that, HEK-293T cells were transfected with 0.1 µg/well of
152 DARC-encoding plasmid using Lipofectamine 3000 (Thermo Fisher Scientific, USA) and
153 incubated overnight. The expression of DARC was verified by western blot analysis using
154 rabbit anti-DARC antibody (Santacruz, USA). For western blot analysis, membrane fraction of
155 HEK-293 cells was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific,
156 USA) and each sample were loaded with 20 µg per each lane. The cells were then exposed
157 either to HlgA alone or to a mixture of HlgA and HlgB together with 100 nM concentration for
158 each at 37°C for 30 min. For concentration-effect experiments, concentrations were applied
159 sequentially from 3 nM to 1000 nM. Cell viability was measured with Cell Counting Kit-8

160 (CCK-8) (Dojindo Laboratories, Japan). A volume of 10 μ L of CCK-8 reagent was added into
161 each well. Plates were then incubated at 37°C for 2 h. Absorbance at 450 nm was measured
162 using a Spark™ 10M multimode microplate reader (Tecan, Switzerland).

163

164 *2.4 Cell permeability assay with recombinant leukocidins*

165 For DAPI staining, HEK-293T cells were seeded into 6-well plates at a cell density of 6×10^5
166 cells/well, cultured for 24 h, and then transfected with 2.5 μ g/well of DARC-encoding plasmid
167 using Lipofectamine 3000 (Thermo Fisher Scientific, USA). After incubation at 37°C
168 overnight, recombinant leukocidins HlgA and HlgB were added at a concentration of 100 nM
169 or 1000 nM to the transfected cells in each well and incubated at 37°C for 30 min. Pore
170 formation caused by leukocidins was detected by intracellular staining of DNA with 4',6-
171 diamidino-2-phenylindole (DAPI) at a final concentration 0.1 μ g/mL without any fixation.
172 Positive controls for pore formation were treated with 0.1% Triton X-100 instead of the
173 leukocidins. Images of DAPI-stained cells indicating a permeabilized plasma membrane were
174 acquired using a TE300 microscope (Nikon, Japan) with DAPI filter. DAPI-stained cells were
175 counted using the National Institutes of Health (NIH) ImageJ software. For Ca²⁺ influx assay,
176 HEK-293T cells were plated into 96-well plates at 2×10^4 cells/well, incubated for 24 h, and
177 were transfected with 0.1 μ g/well of DARC-encoding plasmid using Lipofectamine 3000
178 (Thermo Fisher Scientific, USA). After incubation overnight, the medium was replaced with
179 Ringer's solution (10 mM HEPES, 5 mM glucose, 140 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂
180 and 5 mM KCl, pH 7.4) containing 10 mM Fura 2-acetoxymethyl ester (Fura 2-AM) dye
181 (Thermo Fisher Scientific, USA) for 1 h at 37°C. The cells were then washed with Ringer's
182 solution without the dye and incubated at 37°C for 30 min to cleave intracellular AM esters
183 completely. The fluorescence signal upon addition of recombinant leukocidins HlgA and HlgB

184 mix was measured at dual excitation at 340 and 380 nm using a Spark™ 10M multimode
185 microplate reader (Tecan, Switzerland).

186

187 2.5 Preparation of DARC-containing nanodiscs

188 The DARC-containing nanodiscs were prepared as previously described.²² Briefly, the
189 plasmid containing the DARC-encoding sequence was incorporated in *E. coli* and synthesized
190 by the bacterial protein expression system in a form of inclusion body. The proteins were
191 solubilized, dialyzed and then purified by immobilized metal chelate affinity chromatography
192 using HisTrap HP column (Cytiva). Following purification, DARC-containing nanodiscs were
193 reconstituted using purified DARC, membrane scaffold proteins MSP1E3D1 and 1,2-
194 dimyristoyl-sn-glycero-3-phosphocholine (DMPC (, Avanti Polar Lipids, USA) with the molar
195 ratio 1:10:800. The nanodiscs were applied to a size exclusion chromatography (SEC) column
196 Superdex 200 Increase 10/300 GL (Cytiva) to remove unassembled proteins. The concentration
197 of purified nanodiscs was calculated by absorbance value at 280 nm and the Beer-Lambert
198 equation as follows: $A = \epsilon cL$, where A, ϵ , c and L were absorbance, molar extinction coefficient,
199 protein concentration and path length, respectively. The molar extinction coefficient of the
200 entire complex at 280 nm absorbance was estimated from the amino acid sequence as follows:
201 $\epsilon_{\text{nanodisc}} (121810 \text{ M}^{-1} \text{ cm}^{-1}) = \epsilon_{\text{DARC}} (67950 \text{ M}^{-1} \text{ cm}^{-1}) + 2 \times \epsilon_{\text{MSP1E3D1}} (26930 \text{ M}^{-1} \text{ cm}^{-1})$. For
202 identification of DARC-containing nanodiscs, sodium dodecyl sulfate-polyacrylamide gel
203 electrophoresis (SDS-PAGE) and Western blot analysis were performed. Nanodisc samples
204 were migrated on a polyacrylamide gel, which was then stained with Coomassie Blue solution
205 (Coomassie Blue 0.5 g/L, acetic acid 7% (v/v), methanol 40% (v/v)) for 1 h at 25°C and
206 destained in acetic acid 7% (v/v) and methanol 5% (v/v) overnight at 25°C. Western blot was
207 then performed using rabbit anti-DARC antibody (Santacruz, USA) and mouse anti-His tag

208 antibody (Santacruz, USA) as primary antibody at 1:1000 dilution. Goat anti-rabbit IgG-
209 horseradish peroxidase (HRP) antibody and goat anti-mouse IgG-HRP antibody were used as
210 secondary antibody at 1:5000 dilution.

211

212 *2.6 Size analysis of DARC-containing nanodiscs*

213 The size of purified DARC-containing nanodiscs was analyzed by a DLS spectrophotometer
214 DLS-7000 (Otsuka Electronics, Japan) under the following measurement conditions:
215 temperature (25°C), refractive index (1.3315), and viscosity (0.891). A disposable, small
216 volume cuvette (Malvern Panalytical, Marvern, UK) was used to measure size distribution. For
217 measurements in the presence of leukocidins, DARC-containing nanodiscs were prepared at a
218 concentration of 1 nM and mixed with bovine serum albumin (BSA) (negative control) or with
219 the combinations of gamma-hemolysins at a final concentration of 1 nM for each added protein.
220 Prepared samples were incubated at 37°C for 30 min. The size of DARC-containing nanodiscs
221 in presence of leukocidins or BSA was analyzed with the same DLS method as mentioned
222 before.

223

224 *2.7 Fabrication of swCNT-FET*

225 swCNTs were dispersed in 1,2-dichlorobenzene by applying ultrasonication for 4 h to final
226 concentration of 50 µg/mL. For selective adsorption of swCNT assembly, an
227 octadecyltrichlorosilane (OTS) monolayer with hydrophobic terminal groups was patterned on
228 the SiO₂ substrate via photolithography. The patterned substrate with an OTS monolayer was
229 immersed in the swCNT solution for 20 s. Then, swCNTs were selectively adsorbed on polar

230 SiO₂ region avoiding non-polar OTS monolayer patterns. Excess swCNT suspension was
231 washed out with 1,2-dichlorobenzene. Source and drain electrodes were fabricated on the
232 substrate via photolithography and thermal evaporation. Exposed swCNT channels were 3 μm
233 wide and 170 μm long.

234

235 *2.8 Functionalization of swCNT-FET with DARC-containing nanodiscs*

236 For immobilization of DARC-containing nanodiscs, 1 mM of 1-pyrenebutanoic acid
237 succinimidyl ester (1-PSE) in methanol was used as a linker. First, 1-PSE was used to treat
238 channel regions followed by incubated at room temperature (RT) for 1 h. Channel regions were
239 rinsed with double distilled water (DDW) three times. The solution of DARC-containing
240 nanodiscs was applied to the same regions followed by incubation at RT for 1 h. All
241 immobilization processes were performed under humidified conditions to prevent evaporation
242 of solutions. Functionalized sensors were rinsed with 0.1× Dulbecco's Phosphate Buffered
243 Saline (dPBS) three times. For verifying the immobilization of DARC-containing nanodiscs
244 on a swCNT-FET, field emission scanning electron microscopy (FE-SEM, Auriga Zeiss,
245 Germany) images were captured. After the devices were completely rinsed with 0.1× dPBS 3
246 times, DARC-containing nanodiscs were fixed with OsO₄ 1% in 0.1× dPBS buffer at RT for 1
247 h. FET devices were then rinsed using DDW 3 times and dehydrated with sequential 30%, 60%,
248 90% and 100% ethanol (EtOH) incubation for 10 min each. Samples were freeze-dried
249 overnight and stored at -80°C before SEM analysis. All samples were sputter-coated by Pt at a
250 thickness of 2 ~ 10 nm before SEM images were captured.

251

252 *2.9 Sample preparations*

253 All tested leukocidins were prepared by diluting samples with 0.1× dPBS (pH 7.4) for the
254 real-time detections of leukocidins. Human serum was purchased from Sigma-Aldrich (USA)
255 to prepare simulated clinical samples. Human serum was thawed at 37°C and centrifuged at
256 3000 g or RCF for 15 min at 4°C. Supernatants were filtered using a 0.22 μm syringe filter
257 (Sartorius, Germany) and stored at -80°C until used. Prepared human serum was diluted to a
258 concentration of 1/10³ with 0.1× dPBS for treating to the sensors with a concentration of 1/10⁴
259 and spiked with leukocidin HlgAB at various concentrations. For preparations of bacterial
260 supernatants, *S. aureus* ATCC 6358 (Korean Collection for Type Cultures (KCTC), Republic
261 of Korea) and *E. coli* DH5α (Thermo Fisher Scientific, USA) were used. *S. aureus* was streaked
262 from the frozen stock on from a brain heart infusion (BHI) media agar plate and single colony
263 from the BHI plate was inoculated into 50 mL BHI broth media. Cells were incubated in a
264 shaking incubator at 150 rpm at 37 °C overnight. Cells were centrifuged at 3000 g for 5 min
265 and supernatants were harvested and filtered using a 0.22 μm syringe filter. For the control
266 tests, *E. coli* DH5α was used and supernatants of *E. coli* were harvested under the same
267 procedure of supernatants. Each supernatant was diluted with 0.1 × dPBS including 1/10³-
268 dilluted human serum for a simulated clinical sample.

269

270 2.10 Real-time electrical measurements

271 For real-time monitoring of leukocidin interactions with DARC on FET devices, leukocidin
272 samples were prepared with 0.1× dPBS for dilutions. Current changes between source and drain
273 electrodes were measured in real time using a Keithley 2636 A source meter (Keithley, USA)
274 and a MST 8000 probe station (MS Tech, South Korea). Voltage between source and drain

275 electrode was maintained at 0.1 V. A 20 μ L droplet of 0.1 \times dPBS was placed on channel
276 regions and 2 μ L of diluted samples was injected sequentially into the droplet of buffer.
277 Dilution factor ranged from 10⁻¹⁶ to 10⁻⁸.

278

279 **3. Results and Discussion**

280 *3.1 Schematic of a bioelectronic sensor for detecting bicomponent pore-forming toxin HlgAB*

281 **Figure 1** shows a schematic diagram depicting the detection of pore-forming activity of
282 HlgAB using a swCNT-FET device functionalized with DARC-containing nanodiscs. Here,
283 swCNT-FET device was fabricated and DARC-containing nanodiscs were immobilized on
284 CNT channel regions with 1-PSE as the linker. Leukocidins bound to DARC in nanodiscs.
285 HlgAB is known as a bicomponent pore-forming toxin. Its targeting capability is derived from
286 S component, HlgA. ^{38,39} Firstly, HlgA targets and binds to DARC. Then HlgB, F component,
287 is assembled with HlgA and forms a pore. HlgAB may form intermediate pre-pore assemblies
288 on the extracellular side of DARC or form pores in DARC-containing nanodiscs. ⁴⁰ These
289 binding events will lead to changes of net charges in sensing areas. Since HlgAB proteins have
290 positive charges (+23.63) in the buffer at pH 7.4 due to their isoelectric point (pI) values (9.53
291 for HlgA and 9.18 for HlgB), the conductance of p-type semiconducting CNT channel will
292 decrease after HlgAB binding. ^{41,42}

293 *3.2 Cell lytic activity of HlgAB depends on DARC expression*

294 To assess cell lytic activity of gamma-hemolysins on DARC-expressing cells, HEK-293T

295 cells were transiently transfected with DARC-encoding plasmid. Western blot with anti-DARC
296 antibody demonstrated the expression of DARC in HEK-293T cells with a band at an expected
297 size of 35.5 kDa (**Figure S1**, red arrow). These cells were then used for *in vitro* assay of cell
298 lytic activity of recombinant leukocidins. **Figure 2a** shows cell viability test for assessing
299 cytotoxicities of various combinations of gamma-hemolysins. Equimolar concentrations of
300 two-component gamma-hemolysins (HlgAB, HlgCB and HlgAC) as well as single gamma-
301 hemolysins were added at 100 nM to DARC-expressing cells. Only HlgAB significantly
302 decreased cell viability. These results confirmed the specific cytotoxicity of HlgAB on cells
303 expressing DARC as previously reported.¹²

304 **Figure 2b** shows concentration-dependent cytotoxicity of HlgAB specifically on cells
305 expressing DARC. The cytotoxic effect on DARC-expressing cells was significant for HlgAB
306 at concentrations equal to or higher than 100 nM compared to mock-transfected cells. Cell
307 viability decreased to ~50% at 100 nM and to ~30% at 1 μ M. These results showed that HlgAB
308 were functional and generated a cytotoxic effect specifically on HEK-293T cells expressing
309 DARC as previously reported.¹⁰

310 In physiological conditions, HlgAB can bind to DARC and form a pore, leading to leakage
311 of small molecules through the plasma membrane. To assess if HlgAB formed pores, DAPI
312 staining and Ca^{2+} influx assay were performed. **Figure 2c** shows DAPI staining images after
313 treating cells with 1000 nM of HlgAB. Statistics are shown in **Figure 2d**. DAPI staining was
314 used as a standard assay for cell permeability based on its membrane impermeability and its
315 ability to stain nucleus of permeabilized cells. Positive controls were performed by
316 permeabilizing cells with 0.1% Triton X-100. While DAPI could not pass through cell
317 membrane in mock-transfected cells regardless of the existence of HlgAB, DAPI significantly
318 passed through the cell membrane of DARC-expressing cells when 1000 nM HlgAB was added

319 and incubated for 30 min. A second method of assessing cell permeabilization was measuring
320 Ca^{2+} influx with fluorescent Fura-2 AM probe accumulated intracellularly. When cytosolic
321 concentration of Ca^{2+} increased, the fluorescence signal proportionally increased. **Figure 2e**
322 shows a clear increase of the fluorescence when DARC-expressing cells are incubated with
323 100 nM of HlgAB, indicating a Ca^{2+} influx induced by the toxins. No Ca^{2+} influx was observed
324 for non-transfected-cells, confirming that Ca^{2+} influx induced by HlgAB was DARC-
325 dependent. These results of DAPI staining and Ca^{2+} influx support the ability of HlgAB to
326 permeabilize cells by interacting specifically with DARC to form pores that lead to cell death.
327

328 3.3. Characterization of DARC-containing nanodiscs

329 DARC was cloned into *E. coli* expression vector pET-DEST42 and produced from *E. coli*
330 BL21 strains. It is known that GPCRs overexpressed in *E. coli* are insoluble inclusion bodies
331 in the cytosol.^{32,33} Under our experimental conditions, DARC was also mainly expressed as
332 inclusion bodies. Its solubilization was achieved using harsh detergents such as SDS. The
333 detergents were then removed by dialysis and Bio-Beads. Membrane scaffold protein
334 MSP1E3D1 was also expressed in *E. coli* but in a soluble form. During the reconstitution of
335 DARC into nanodiscs, the molar ratio of DARC, MSP1E3D1, and DMPC was set as 1:10:800
336 for optimizing the size distribution of nanodiscs under 20 nm. After self-assembled DARC-
337 containing nanodiscs were purified by size exclusion chromatography, they were subjected to
338 SDS-PAGE, Western blot, and DLS analyses for quality control.

339 **Figure 3a** shows SDS-PAGE gel loaded with DARC-containing nanodiscs and stained by
340 Coomassie blue. Two bands (red arrows) with molecular weights similar to theoretical
341 molecular weights of 39 kDa and 33 kDa for DARC and MSP1E3D1, respectively, were

342 observed. Western blot analysis confirmed the nature of these two bands using anti-DARC
343 antibody (**Figure 3b**) and anti-His antibody (**Figure 3c**). These results indicated that DARC
344 was successfully reconstituted into nanodiscs.

345 **Figure 3d** shows DLS profile of purified DARC-containing nanodiscs, revealing an average
346 diameter of 15.9 nm. These nanodiscs showed one sharp peak, inferring that purified nanodiscs
347 had a homogeneous size distribution. DLS can be utilized as a tool for characterizing protein
348 aggregation or protein-protein interactions.^{43, 44} Therefore, we used DLS to assess the
349 interaction of HlgAB with DARC-containing nanodiscs since the binding event of HlgAB (~35
350 + ~37 kDa for HlgA and HlgB, respectively) on DARC (~39 kDa)-containing nanodiscs should
351 be detectable. **Figure 3e** shows DLS profiles of nanodiscs treated with various gamma-
352 hemolysins. Bovine serum albumin (BSA, negative control) (1 nM) or various gamma-
353 hemolysin combinations was incubated with DARC-containing nanodiscs and size
354 distributions of samples were then measured by DLS. They are summarized in the legend of
355 **Figure 3e**. DARC-containing nanodiscs alone had a size of 15.9 nm. The average size of BSA
356 or leukocidins alone was 3-5 nm (Data not shown). The average size of DARC-containing
357 nanodiscs treated with BSA and all combinations of leukocidins was between 16.8 nm and 18.9
358 nm except for HlgAB, which had a ~2.5-fold larger average size (40.2 nm). These results
359 confirmed that HlgAB could specifically bind on DARC-containing nanodiscs and
360 significantly increase the hydrodynamic radius of these nanodiscs. Two hypotheses could
361 explain such size increase: 1) HlgAB could bind to DARC and form pre-pore or pore structures
362 in the lipid bilayer surrounding DARC in nanodiscs. However, the diameter of the nanodiscs
363 was constrained by the length of the surrounding MSP protein, which could hinder insertion of
364 such large pores; 2) HlgAB could bind to DARC and remain attached as a dimer or higher
365 orders of oligomers to the extracellular side of DARC. The increase of the height would also

366 increase the hydrodynamic radius as observed. Thus, it was likely that this size increase of the
367 nanodiscs was mainly due to the formation of large HlgAB complexes on the extracellular side
368 of DARC or potentially due to formation of pore structures by displacement of lipid molecules.
369 The structure of the HlgAB pore (PDB: 3B07) indicates a diameter of 11.4 nm of the complex.¹⁵
370 The size of the DARC-containing nanodiscs being ~16 nm, the increase of size to ~40 nm in
371 presence of HlgAB suggests that two complexes (pre-pore or pore) are bound to the DARC-
372 nanodiscs: 16 nm (DARC-containing nanodisc) + 2×11.4 nm (HlgAB octameric complex) =
373 38.8 nm. Dimers of DARC have been reported and could explain the potential presence of two
374 HlgAB complexes.⁴⁵ While the size of nanodiscs is relatively homogenous and constrained by
375 the length of the surrounding MSP, nanodiscs composed of non-circularized MSP (like
376 MSP1E3D1 used in this study) can fused and form very large nanodiscs (≤ 90 nm) when they
377 are close to each other and additional molecules are inserted in the lipid bilayer.⁴⁶ These
378 observations raise the hypothesis that HlgAB binding on DARC could bring together two
379 DARC-containing nanodiscs during the formation of pre-pore complex and that the insertion
380 of pores in the lipid bilayer could induce the fusion of nanodiscs up to a size of 40 nm.

381

382 *3.4. Real-time detection of bicomponent pore-forming toxin HlgAB by swCNT-FET devices* 383 *functionalized with DARC-containing nanodiscs*

384 **Figure 4a** depicts a schematic diagram of two different responses of swCNT-FET devices
385 functionalized with DARC-containing nanodiscs upon introduction of single- or two-
386 component leukocidins. When only single-component HlgA was added, HlgA molecules
387 recognized DARC receptors on the sensor device and bound to the nanodiscs without forming
388 pores or complexes. On the other hand, when two-component HlgA and HlgB were added,

389 receptor-bounded HlgA molecules could recruit HlgB molecules to form octameric complexes.
390 These different responses resulted in different changes in charge distribution near the sensing
391 area. Since HlgA and HlgB proteins have positive net charges as discussed in **Figure 1**, two-
392 component HlgAB could form complexes or pore structures and induce more accumulation of
393 positive charges than single-component HlgA, leading to different signal amplitudes of the
394 sensor device.

395 **Figure 4b** shows field-emission scanning electron microscopy (FE-SEM) images of DARC-
396 containing nanodiscs immobilized on the channel region before and after HlgAB binding. The
397 FE-SEM samples were prepared with paraformaldehyde and osmium tetroxide solution for
398 fixation of protein and lipids in the nanodiscs, respectively. The surface of nanodisc-
399 immobilized swCNT-FET device was coated with a 3 nm Pt layer for a high electron scattering
400 rate. The images showed attachment of nanodiscs onto CNT network lines clearly. The average
401 diameter of DARC-containing nanodiscs was estimated as 20 nm before HlgAB binding. After
402 HlgAB binding, diameters of DARC-containing nanodiscs were increased about 40 nm up to
403 80 nm (right image in **Figure 4b**), consistent with the broader size distribution of DARC-
404 containing nanodiscs after HlgAB binding estimated by DLS measurement as shown in **Figure**
405 **3e**. Also, these results indicated that these swCNT-FET devices were successfully
406 functionalized with DARC-containing nanodiscs. Size increase of DARC-containing
407 nanodiscs based on complex formation of HlgAB also occurred after immobilizing these
408 nanodiscs on the channel region of the devices. On the other hands, **Figure S2** shows FE-SEM
409 image of a pore structure of HlgAB bound to DARC-containing nanodiscs on swCNT-FET.
410 This result demonstrates that HlgAB bound to DARC formed not only a pre-pore complex but
411 pore structure, indicating this device could have a potential to measure a pore-forming activity
412 of HlgAB to DARC.

413 **Figure 4c** shows the real-time monitoring on a swCNT-FET device functionalized with
414 DARC-containing nanodiscs to HlgAB solutions. Decrease of source-drain currents were
415 observed as HlgAB concentrations increase. The current change was detected at first when
416 treated with 1 fM of HlgAB in this experiment. Limit of detection (LOD) was calculated as the
417 concentration that corresponds to the signal-to-noise (S/N) ratio of 3. The LOD of the device
418 for HlgAB was 0.122 fM.⁴⁷ Limit of quantitation (LOQ) was calculated as the concentration
419 that corresponds to the S/N ratio of 10, and the LOQ of the device for HlgAB was 0.441 fM.⁴⁸
420 In this buffer (0.1×dPBS at pH 7.4), HlgAB had a net positive charge since their pI was 8.94
421 (calculated). The swCNT used in this study was a p-type semiconductor. Thus, binding of
422 positively charged HlgAB to DARC could lead to a decrease of source-drain current.
423 Responses were slower than the ones from volatile compounds due to much higher molecular
424 weights of leukocidins (~35 kDa) that could slow down the diffusion speed of molecules after
425 adding toxins into the buffer.^{49,50} A similar response of the swCNT-FET device functionalized
426 with DARC-containing nanodiscs to HlgA solutions was provided in **Figure S3**. Surface
427 plasmon resonance has previously reported the binding of HlgA alone on purified and
428 immobilized DARC.¹¹ Other gamma-hemolysin HlgB or HlgC alone cannot make any change
429 of currents in **Figure S4**, showing that responses of the sensor require binding to DARC in the
430 nanodiscs.

431 Next, we used empty nanodiscs composed of DMPC and MSP1E3D1 only for confirming
432 the role of DARC in the biosensor. We functionalized the swCNT-FET with empty nanodiscs
433 under the same conditions and procedures, and treated with HlgAB to the biosensor. Figure S5
434 shows the real-time monitoring on the swCNT-FET device functionalized with empty
435 nanodiscs to HlgAB. This device also responded to HlgAB, but higher concentration of HlgAB
436 at 10 pM was required to induce current change compared to when functionalized with DARC-

437 containing nanodiscs. Also, the current change was smaller than the change with DARC-
438 containing nanodiscs. We supposed it was because HlgA could bind to lipid membrane non-
439 specifically, but HlgAB couldn't form a pore structure on the DMPC membrane.^{51, 52}
440 Consequently, this result confirmed that DARC is a key element for sensitivity and selectivity
441 to HlgAB in the fabricated sensor.

442 **Figure 4d** shows concentration-dependent responses of CNT-FET devices functionalized
443 with DARC-containing nanodiscs to single-component HlgA and two-component HlgAB
444 solutions. Here, detection signals were defined as relative changes of CNT channel
445 conductance with respect to the initial value ($\Delta G/G_0$) at a given leukocidin concentration.
446 Detection signals at a single concentration were repeatedly measured with three devices to
447 acquire average values and standard errors. Our device responded to leukocidin solutions from
448 a concentration of 1 fM. The response was saturated at a concentration of around 100 pM,
449 indicating the dynamic range of our sensor device. Its dose-dependent responses could be fitted
450 by the Hill equation written as $\Delta G/G_0 = C^n/(K_d^n + C^n)$, where C , K_d , and n were the concentration
451 of leukocidin, the dissociation constant for the binding of leukocidins to DARC-containing
452 nanodiscs, and Hill's coefficient, respectively.^{53, 54} Calculated dissociation constants of HlgA
453 and HlgAB to DARC-containing nanodiscs were 5.94×10^{-14} M and 6.96×10^{-14} M, respectively.
454 These two values were similar, inferring that binding affinity of HlgAB was similar to that of
455 HlgA in the fabricated sensor platform, which is in agreement with the consensual mechanism
456 of binding of the S component (HlgA) on the receptor, while HlgB is recruited for pore
457 formation. Thus, these similar values of K_d between HlgA and HlgAB suggest that HlgB does
458 not affect the apparent affinity in this sensor, consistent with the role of F component.
459 Importantly, the amplitudes of maximal signal responses to two-component HlgAB (~40%)
460 were more than twice as large as those to single-component HlgA (~20%), which could be

461 interpreted as a higher density of positive charges of HlgAB in CNT channel region than that
462 of HlgA. This increase of signal amplitude was consistent with the size increase of DARC-
463 containing nanodiscs after HlgAB binding estimated by DLS measurement as shown in **Figure**
464 **3d** and **3e**. This size increasing behavior was not observed in the case of HlgA binding. These
465 results showed that our sensor device could be used to detect bicomponent pore-forming
466 HlgAB.

467 Next, we assessed the biosensor's ability to detect HlgAB in human serum mimicking
468 clinical samples from infected patients. Detecting HlgAB with high sensitivity in blood
469 samples appears to be relevant for diagnosis of bacteremia caused by *S. aureus*.^{55, 56} To
470 determine optimal conditions for avoiding false-positive responses, serial dilutions of human
471 serum were applied on bare swCNT-FET and conductance changes were observed for dilutions
472 up to 1/10³, but not to 1/10⁴ or higher dilutions (**Figure S6**). **Figure 4e** shows dose-dependent
473 responses of swCNT-FET devices functionalized with DARC-containing nanodiscs to HlgAB
474 diluted in human serum compared to dilutions in buffer. Concentration-dependent responses to
475 HlgAB diluted in human serum were also analyzed by fitting them with the Hill equation. The
476 fitted response curve of human serum case showed almost similar behavior to that of the buffer
477 case. The K_d value of HlgAB in human serum was 3.57×10^{-14} M, similar to the K_d value of
478 HlgAB in buffer solution (6.96×10^{-14} M). Moreover, amplitudes of maximal signal responses
479 to HlgAB in human serum were about 40%, consistent with those in buffer solution. These
480 results showed that human serum did not affect the dose-dependent response of the sensor
481 device to HlgAB, indicating that our sensor device could be used to detect pore-forming toxin
482 HlgAB even in clinical samples from infected patients.

483 Finally, we used the culture of *S. aureus* to verify the medical applications of the biosensor. *S.*
484 *aureus* ATCC 6538 was used as a model of *S. aureus* due to its expression of gamma-

485 hemolysins, while *E. coli* DH5 α was used as a negative control^{57, 58}. **Figure S7** shows the real-
486 time monitoring on a swCNT-FET device functionalized with DARC-containing nanodiscs to
487 the bacterial supernatants. The current decrease was detected at first when treated with 1/10⁷-
488 diluted *S. aureus* supernatants and the change increased as the higher concentration of *S. aureus*
489 supernatants was treated. While, there was no response to *E. coli* supernatants, which showed
490 selectivity in our sensor devices. The concentrations of gamma-hemolysins in *S. aureus*
491 supernatants were estimated as 1 nM to 100 nM^{59,60}. Thus, the concentration of HlgAB in 1/10⁷-
492 diluted supernatants was expected to be 0.1 fM to 10 fM, which was consistent with a limit of
493 detection (LOD) of 0.122 fM in our devices. These results show that our devices could detect
494 *S. aureus* supernatants in simulated clinical samples, indicating our sensor device could have a
495 potential to be applied in the clinical field.

496

497 **4. Conclusion**

498 Herein, we developed a biosensor for rapid and sensitive detection of leukocidins HlgAB
499 using swCNT-FETs functionalized with DARC-containing nanodiscs. In the present study, we
500 validated the activity of purified recombinant HlgAB using cell viability and permeability tests
501 with a human cell line HEK-293T transiently transfected with DARC-encoding plasmid. A
502 bioelectronic sensor was then designed for detecting HlgAB using DARC-containing
503 nanodiscs as a bioelement. DARC was successfully expressed in *E. coli*, purified, and
504 reconstituted into nanodiscs of 15.9 nm in size. DLS measurements in the presence of various
505 compositions of single- or two-component gamma-hemolysins interestingly showed a large
506 increase in the average size of DARC-containing nanodiscs only in the presence of HlgAB.
507 This result indicated that HlgAB was bound to DARC incorporated in the nanodiscs and
508 formed large complexes or pore structures on DARC. DARC-containing nanodiscs were
509 immobilized on the channel region of the swCNT-FET device with 1-PSE as a linker. This was
510 confirmed by FE-SEM. The fabricated biosensors detected HlgA alone and HlgAB with a LoD
511 of 1 fM and showed concentration-dependent response which was saturated at 100 pM. This
512 biosensor detected HlgA and HlgAB with similar apparent dissociation constants, but with
513 higher signal amplitudes (~2-fold). Thus, this sensor could detect the complex formation of
514 HlgAB in response on DARC-containing nanodisc compared to HlgA alone. Medical
515 applications of this biosensor are envisioned for blood testing of HlgAB contamination due to
516 *S. aureus* strains expressing these virulent factors in the bloodstream. Due to false-positive
517 signals in diluted serum until 1/10³, measurements with HlgAB were performed in diluted
518 serum at 1/10⁴. Results confirmed the ability of this DARC-based biosensor to detect HlgAB
519 in diluted human serum with similar apparent K_d and LoD than in buffer.

520 *S. aureus* strains can secrete different leukocidins, including LukED or non-cognate pairs of
521 HlgA/LukD or Luke/HlgB that are also able to bind to DARC and form pores. The DARC-
522 based biosensor will detect all of them since the specificity of the binding depends on the S
523 component (HlgA or Luke). Thanks to the multiplex capabilities of on-chip transistors and the
524 specificity of interaction of leukocidins with other receptors (e.g. HlgA on CCR2, Luke on
525 CCR5), it is conceivable to develop biosensors composed of different receptors to identify the
526 leukocidins present in samples. ⁶¹

527 These biosensors have numerous advantages: 1) rapid response (seconds to min) compared
528 to ELISA (> 1 h); 2) suitable for microelectronics; 3) label-free; 4) high sensitivity (fM)
529 compared to ELISA (10 pM) ⁶²; 5) high specificity for *S. aureus* leukocidins; 6) stability of the
530 receptor in nanodiscs; 7) multiplexing capacities for *in situ* controls and leukotoxin
531 identification with various receptors; 8) potential for miniaturization; 9) low volume of samples.
532 On the other hand, the disadvantages are: 1) it requires membrane protein purification and
533 reconstitution in nanodiscs, which could be tricky for some receptors. However, the bacterial
534 expression system that is used in this study, is the fastest and the cheapest one, and the amount
535 of functional receptor required for immobilization is low.; 2) it is a single-use test when positive.
536 However, medical *in vitro* diagnostic systems are single-use, and recycling of chips are
537 theoretically possible by replacing the immobilized nanodiscs.; 3) tested blood samples had to
538 be diluted $1/10^4$ due to non-specific binding. However, improvements could be explored with
539 saturation of the non-specific binding surface.

540 This technology has promising applications in basic research for studying leukocidins
541 binding on membrane receptors and in the clinical field for early detection of gamma-
542 hemolysins in blood samples and diagnosis of virulent toxins from *S. aureus* in the bloodstream.
543

544 **Associated Contents**

545 **Supporting Information**

546 Figure S1: Identification of DARC expression in HEK-293T. Figure S2: SEM image of a pore
547 structure of HlgAB bound to DARC-containing nanodiscs. Figure S3: Real-time responses of
548 the fabricated device to HlgA only. Figure S4: Real-time responses of the fabricated device to
549 HlgC, HlgB and HlgA consecutively. Figure S5: Real-time responses of the device
550 functionalized with empty nanodiscs to HlgAB. Figure S6: Control test of the swCNT-FET to
551 diluted human serum. Figure S7: Real-time responses of the fabricated device to supernatants
552 from *S. aureus* and *E. coli* in simulated clinical samples.

553

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563 **Figure captions**

564 **Figure 1.** Diagram of the swCNT-FET functionalized with DARC-containing nanodiscs.
565 DARC-containing nanodiscs were immobilized on swCNT with 1-pyrenebutanoic acid
566 succinimidyl ester (1-PSE) as the linker. gamma-hemolysin HlgAB binds to DARC and forms
567 pore structures. The fabricated sensor is expected to decrease channel conductance.

568
569 **Figure 2.** Cell viability test and permeability test for leukocidins from *S. aureus*. (a) Cell
570 viability test of HEK293T transiently transfected with DARC followed by treatment with
571 various combinations of leukocidins at 100 nM. (b) Cell viability relying on HlgAB
572 concentrations in cells with or without the expression of DARC. (c) Fluorescence images after
573 DAPI staining and applications of 100 or 1000 nM of HlgAB for cell permeability test. (d)
574 Statistical analysis of cell permeability test results using the relative number of fluorescent cells
575 as permeabilized cells. (e) Statistical analysis of calcium influx assay using Fura-2 AM as an
576 intracellular probe. Results are presented as percentages of intracellular Ca^{2+} -dependent
577 fluorescence induced by 100 nM HlgAB. All data are expressed as mean \pm standard deviation
578 ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in the t-test).

579
580 **Figure 3.** Production, reconstitution, and characterization of DARC-containing nanodiscs. (a)
581 SDS-PAGE gel with Coomassie blue staining for DARC-containing nanodiscs. (b) Western
582 blot analysis of DARC-containing nanodiscs with anti-DARC antibody. (c) Western blot
583 analysis of DARC-containing nanodiscs with anti-His tag antibody. (d) Dynamic light
584 scattering profile of purified DARC-containing nanodiscs. (e) Dynamic light scattering profiles

585 of DARC-containing nanodiscs treated with various compositions of gamma-hemolysins.

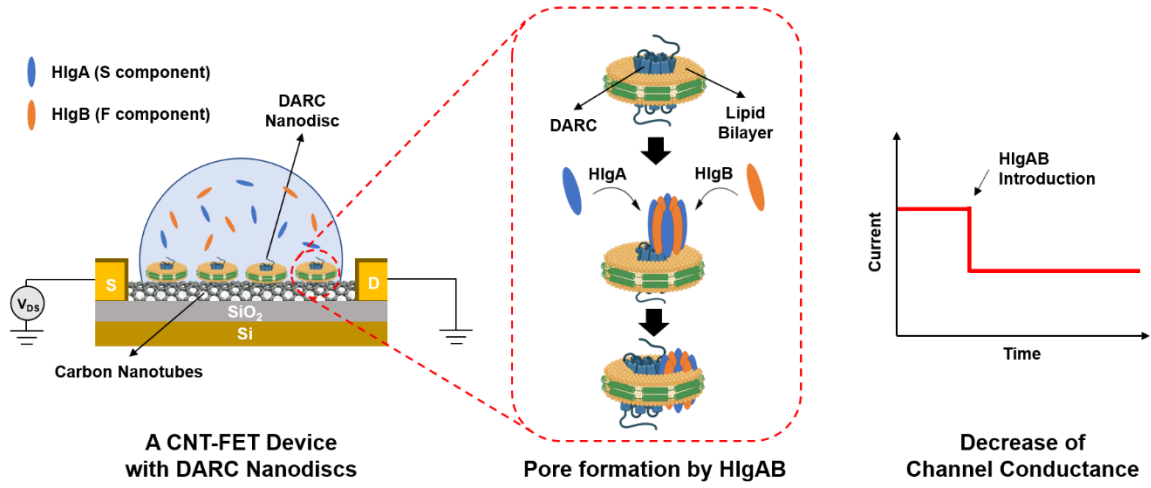
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587 **Figure 4.** Detection of leukocidin HlgA and HlgAB using bioelectronics based on swCNT-FET
588 functionalized with DARC-containing nanodiscs. (a) Diagrams of possible responses after
589 treating fabricated bioelectronics with leukocidins. (b) SEM images of immobilized DARC-
590 nanodiscs on swCNT-FET before and after treating with HlgAB. (c) Real-time detection of
591 HlgAB in the sensor with increasing concentrations of HlgAB. (d) Concentration-response
592 curves of HlgA and HlgAB. (e) Concentration-response curves of HlgAB diluted in 0.1×dPBS
593 and human serum. Data are expressed as mean ± standard deviation (n = 3)

594 Figure 1.

595

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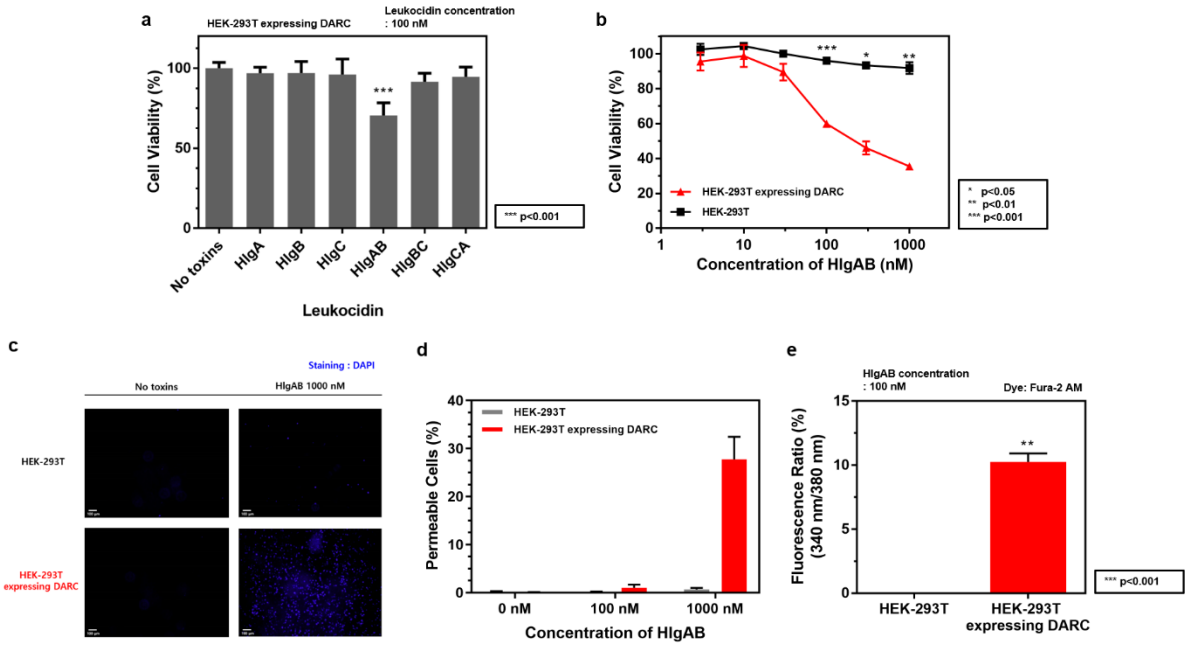
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600 Figure 2.

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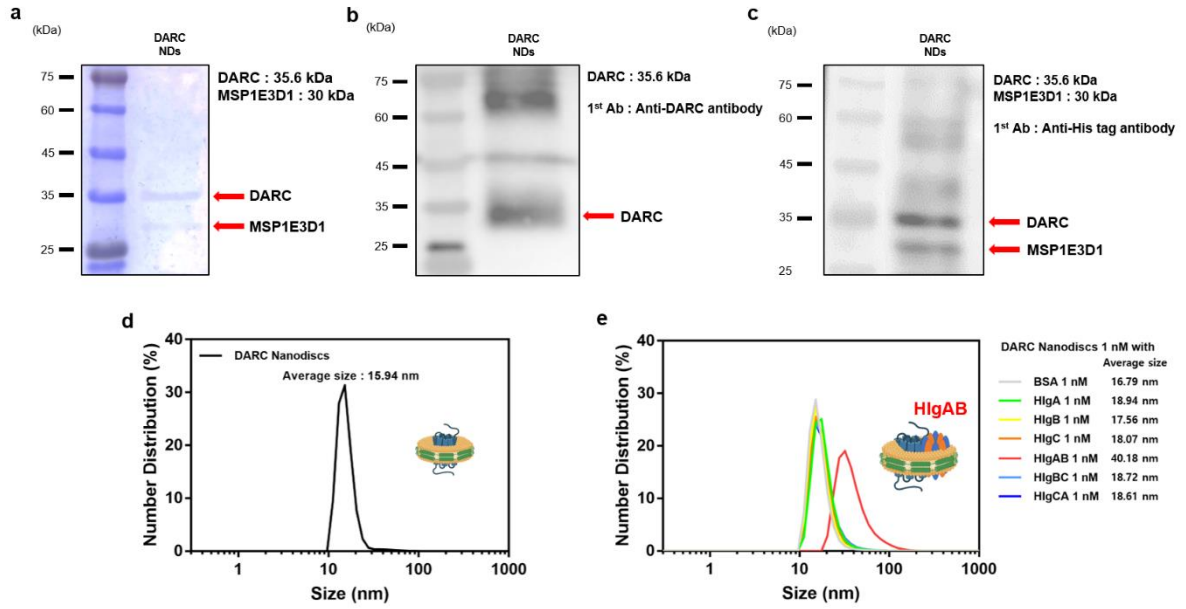
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605 Figure 3.

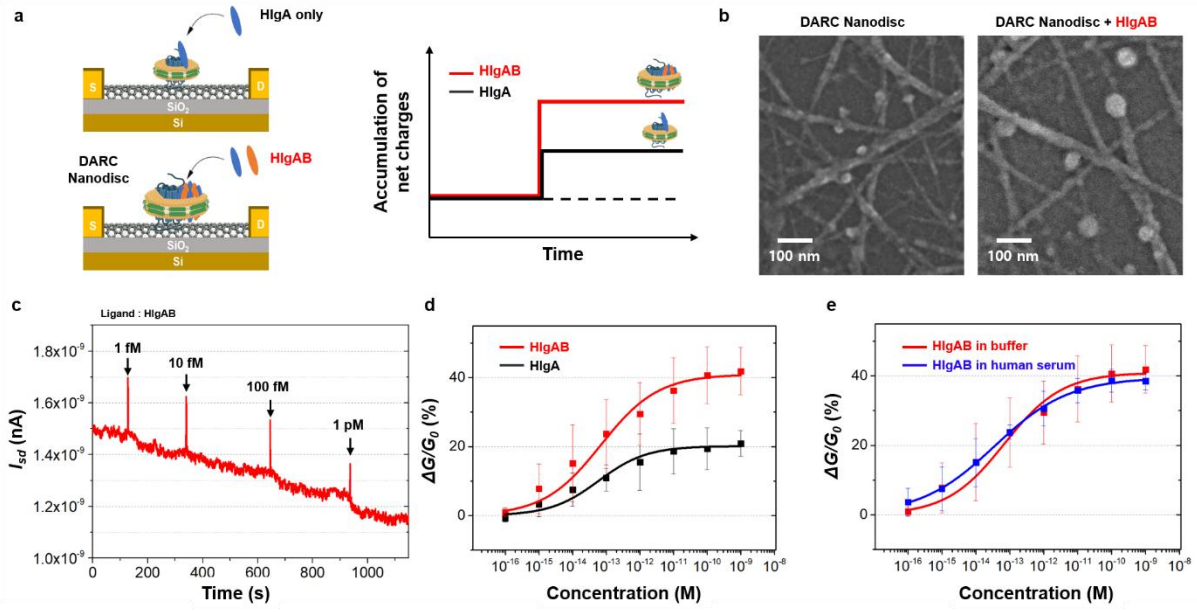
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609 Figure 4.



610

611

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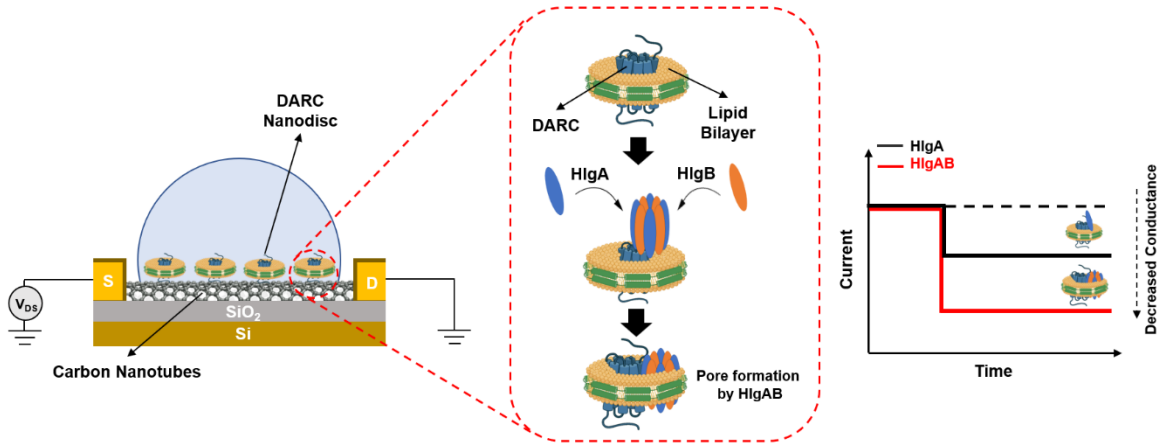
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