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Correspondence and requests for materials should be addressed to Y.-O.K. (yokim@kiost.ac) or H.-J.S. (shinhj@chosun.ac.kr)

Utilizing the algicidal activity of aminoclay as a practical treatment for toxic red tides

Young-Chul Lee[1], EonSeon Jin[2], Seung Won Jung[3], Yeon-Mi Kim[2], Kwang Suk Chang[2], Ji-Won Yang[4], Si-Wouk Kim[5], Young-Ok Kim[3] & Hyun-Jae Shin[6]

[1]Department of Chemical and Biomolecular Engineering (BK21 program), KAIST, 291 Daehakno, Yuseong-gu, Daejeon, 305-701, Republic of Korea, [2]Department of Life Science and Research Institute for Natural Sciences, Hanyang University, Seoul, 133-791, Republic of Korea, [3]Korea Institute of Ocean Science & Technology, South Sea Institute, Geoje, 656-830, Republic of Korea, [4]Advanced Biomass R&D Center, KAIST, 291 Daehakno, Yuseong-gu, Daejeon, 305-701, Republic of Korea, [5]Department of Environmental Engineering, Chosun University, Seosuk-dong, Dong-gu, Gwangju, 501-759, Republic of Korea, [6]Department of Chemical & Biochemical Engineering, Chosun University, Seosuk-dong, Dong-gu, Gwangju, 501-759, Republic of Korea.

In recent decades, harmful algal blooms (HABs) – commonly known as red tides – have increasingly impacted human health, caused significant economic losses to fisheries and damaged coastal environments and ecosystems. Here, we demonstrate a method to control and suppress HABs through selective algal lysis. The approach harnesses the algicidal effects of aminoclays, which are comprised of a high density of primary amine groups covalently bonded by metal cation backbones. Positively charged colloidals of aminoclays induce cell lysis in HABs within several minutes exposure but have negligible impact on non-harmful phytoplankton, zooplankton and farmed fish. This selective lysis is due to the ammonium characteristics of the aminoclay and the electrostatic attraction between the clay nanoparticles and the algal cells. In contrast, yellow loess clay, a recognized treatment for HABs, causes algal flocs with little cell lysis. Thus, the aminoclay loading can be effective for the mitigation of HABs.

Harmful algal blooms (HABs) include red tides caused by microscopic algae and macroalgae (seaweeds) that can disturb benthic systems by impacting indigenous species. Red tides of microalgae also disrupt marine ecosystems, often causing catastrophic mass mortality of fish in open water and in near-shore fish farms. HABs deplete oxygen[1,2], clog the gills of fish, generate damaging reactive oxygen substances (ROS)[1,3–5] and produce toxins. Red tides can cause illness and death in humans and in fish, shellfish and seabirds. Losses to the fisheries industry by HABs have been estimated at US$1 million per year for South Korea alone, and more than US$1 billion per year for Japan[4]. With the red tides now frequent in many coastal areas around the world[1,2,6–9], the global economic impact of HABs is enormous.

Management of HABs has focused on the use of algicides for control and suppression. The most widely used strategy today is the direct application of chemicals, especially copper sulfate, to blooms. This approach has serious drawbacks, including the non-specific killing of marine organisms and the recurrence of blooms because the effectiveness of Cu^{2+} ions is impaired by precipitation as $Cu(OH)_2$. Other management strategies include the application of zooplankton to graze on bloom species – a form of top-down control. This biological approach also faces obstacles: It is complicated by the need for mass culturing of zooplankton and the substantial cost of field trials[10–14], and so far large-scaled applicability of this method has been limited[15,16].

A promising alternative approach is mechanical treatment of HABs using non-toxic natural clay, generally readily available locally and similar to suspended particles in ambient waters. Clay sprayed on the water surface causes flocculation (which is comparable to coagulation) of algal cells. While the formation of clay/algal flocs involves only a minority of algal cells, most of the cells are quickly transported to the sea bed[4]. However, many of the cells escape and survive[1]. Almost 60,000 tons of yellow loess clay (at approximately 400 gm^{-2}) have been applied to control HABs in a coastal aquaculture area off the coast of Korea, most notably in the treatment of *Cochlodinium* red tides. Application has been undertaken several times since 1990, utilizing the optimal loading of clay, which is 0.25 gL^{-1} [17–19]. Although these treatments were considered to be broadly successful, they did not save fish in near-shore fish farms, required massive amounts of clay and had very high costs, all of which severely limit this approach. In addition, the flocs resulting from treatment could not prevent HABs completely, and the

resulting limited algal cell disruption was associated with disturbances in benthic communities and substantial economic loss[20].

In recent years, clay-mimicking materials with unique properties have been developed. Called aminoclays, they are composed of metal cations (for example, Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Al^{3+}, and Fe^{3+}) at the center, sandwiched by (2 : 1 trioctahedral structure) or paired with (1 : 1 dioctahedral) amino functional groups (i.e., 3-aminopropyltriethoxysilane precursor) linked via covalent bonding (Supplementary Table S1). These structures form delaminated (cationic) dispersions of clay sheets in aqueous media, due to the repulsion of protonated amino ($-NH_3^+$) active sites[21,22]. The development of aminoclays has focused mostly on creating structures that wrap biomaterials such as DNA, enzymes, and bacteriorhodopsin[21,23], enhancing their mechanical and thermal properties.

The hydrodynamic size distribution of aminoclay particles created in a one-pot sol-gel reaction under ambient conditions depends on the metal cations selected[22]. We recently described the antimicrobial function and low cytotoxicity toward animal cells of magnesium- and calcium-centered aminoclays[24,25]. In the present study, we demonstrate the algicidal activity of these magnesium- and calcium-centered aminoclays (denoted as Mg aminoclay and Ca aminoclay, respectively). These materials selectively inhibit HABs, indicating that they may be effective, and ecologically and environmentally safe, new treatments for HABs in coastal systems.

Results

Structural characterization of aminoclays. Powder X-ray diffraction (XRD) profiles indicated that a 2 : 1 trioctahedral phyllosilicate series (Fig. 1a i) comprised the ideal unit structure, with aminopropyl side chains (Fig. 1a ii). The basal spacing (d-spacing) of Mg and Ca aminoclay powders revealed low-angle interlayer d_{001} reflections at 1.60 nm and 1.40 nm, respectively, in agreement with results reported previously[21,22]. In addition, the broad peaks at d_{002}=0.82 nm (2θ=10.81), $d_{020,110}$=0.40 nm (2θ=22.33), $d_{130,200}$=0.25 nm (2θ=35.37), and $d_{060,330}$=0.16 nm (2θ=59.29), with an intra-plane $d_{060,330}$ smectite reflection at 2θ= 59.0°, indicated that the basic unit of 2 : 1 trioctahedral smectite was retained. If the broad higher-angle regions at 31.7° and 50.1° are assigned to (130, 200) and (060, 330), respectively, Ca aminoclay can be considered to exhibit the characteristics of 2 : 1 trioctahedral phyllosilicate except for the disorder of the inorganic portion of the compound. This finding is based on the weak in-plane reflections of Ca aminoclay. The FT-IR spectra of Mg and Ca aminoclays (Supplementary Fig. S1) exhibited vibration modes for Mg-O (559 cm^{-1}) and Ca-O (589 cm^{-1}), respectively. The peaks observed for CH_2 (3,000 cm^{-1}), OH (3,384 cm^{-1}), Mg-O-Si (849 cm^{-1}), Si-O-C (849 cm^{-1}), Si-OH (1,034 cm^{-1}), and Si-O-Si (1,130 cm^{-1}) were in good agreement with the unit structure of aminoclays with central Mg^{2+} or Ca^{2+} ions exhibiting coordination (covalent) bonding with silica and sandwiched $-(CH_2)_3NH_2$ functionalized groups (Fig. 1a ii).

Hydrodynamic behavior of aminoclays in aqueous solution. TEM micrographs of cationic dispersions of Mg and Ca aminoclays revealed particle diameters of 20–150 nm and 10–200 nm respectively (Fig. 1a iii and iv). Because the TEM images of aminoclay particles would be different for dried sections in the samples[22], aqueous particle distributions were characterized by measuring the distribution of the hydrodynamic radius of the delaminated sheets (Fig. 1b). These results, obtained in deionized (DI) water, showed that the cumulative particle sizes at 10%, 50%, and 99% ($X_{10}/X_{50}/X_{99}$) of Mg aminoclay were 29.17, 36.21, and 54.13 nm, respectively, and those of Ca aminoclay were 348.22, 424.96, and 562.46 nm, respectively (Fig. 1b ii). The mean surface and volume diameters of Mg aminoclay were 35.72 nm and 36.76 nm, respectively, and those of Ca aminoclay were 418.59 nm and 427.46 nm, respectively. These measurements allow estimates of specific surface area ($m^2\ g^{-1}$) and specific volume ($m^3\ g^{-1}$), which in turn yield estimates of surface-to-volume ratios of 168.00 m^2cm^{-3} for Mg aminoclay and 14.33 $m^2\ cm^{-3}$ for Ca aminoclay. These values indicate that a higher surface-to-volume ratio results in the formation of more spherical particles by the exfoliated sheets[24].

To further investigate the long-term stability of aminoclay in freshwater and sea water, the average particle diameters were determined after 1 and 15 days of storage. The particle diameters in freshwater for Mg aminoclay were 204.2 nm on Day 1 and 332.3 nm on Day 15 (Fig. 2b iii), while the corresponding values in sea water were 305.8 nm on Day 1 and 2,300 nm on Day 15 (Fig. 2b iv). For Ca aminoclay, the particle diameters in freshwater were 585.5 nm on Day 1 and 3,100 nm on Day 15, and the corresponding values in sea water were 615.5 nm on Day 1 and 3,400 nm on Day 15 (Fig. 2b v and vi). The particle diameters for Mg aminoclay in DI water were 51.29 nm on Day 1 and 217.1 nm on Day 15 days, and for Ca aminoclay in DI water they were 435.6 nm on Day 1 and 470.6 nm on Day 15. The differences, most notably in sea water, are due to the increase in charge-screening effects by ionic strength. Thus, this aggregation behavior should be considered for real field applications.

The surface charge potentials of the aminoclays at concentrations of 1.0 to 5.0 mg mL^{-1} in DI water were +20 to +30 mV for Mg aminoclay (Fig. 1c i) and +1 to +7 mV for Ca aminoclay (Fig. 1c ii). The reason for this difference may be that the size distribution (degree of delamination) of Mg aminoclay dispersions is smaller than that of Ca aminoclay dispersions. In contrast, the surface charge potentials of yellow loess with the composition indicated in Supplementary Table S2 corresponded to a negatively charged state at neutral pH, approximately -6 mV (Supplementary Fig. S2).

Mechanism of disruption of HABs by aminoclays. Figure 2 shows Mg aminoclay-mediated mortality of single and mixed HAB organisms in a laboratory test. Cell lysis occurred over a 20 minute period in single-species cultures of HAB organisms *Cochlodinium polykrikoides* (Fig. 2a and Supplementary Movie 1), *Chattonella marina* (Fig. 2b and Supplementary Movie 2), and *Heterosigma akashiwo* (Fig. 2c and Supplementary Movie 3) following treatment with 0.01% (w/v) Mg aminoclay. In order to examine quantitatively and kinetically for selective algicidal activity by clays, Mg and Ca aminoclays, and yellow loess were evaluated. Fig. 3a and b show data on the algicidal activity of Mg aminoclay, illustrating the specific effect on HABs (Fig. 3a) and the impact of treatment over time (Fig. 3b). The results of the corresponding treatment with Ca aminoclay were consistent with the results of the treatment with Mg aminoclay (Fig. 3c and Supplementary Fig. S3). In contrast, yellow loess, used for comparison as a clay material with a negatively charged surface, showed a significantly poorer level of algicidal selectivity and lower inhibition effects on HABs in mixed-cell culture (Fig. 3d). Cationic dispersions of Mg aminoclay were surrounded by the plasma membranes of disrupted cells (Supplementary Fig. S4 and Movie 4).

In experiments conducted with mixtures of *C. marina* and other species (*Phaeodactylum* sp., *Amphidinium* sp., *Navicula pelliculosa*, and *Nannochloropsis* sp.), specific lysis of *C. marina* cells occurred (Fig. 2d, g and Fig. 3a, c, and Supplementary Movie 5).

Confocal microscopy was used to study the effect on another HAB organism, *C. polykrikoides*, in more detail. After adsorption of the Mg aminoclay, the cell chloroplasts took on a shrunken appearance (Fig. 2e and Supplementary Movie 1 for 20 min). With *C. marina*, cell shape changed from a droplet (fusiform) to a sphere within 30 min (Fig. 2 b and f), a result of the effect of the positively charged Mg aminoclay sheets on the plasma membrane of the cells. Subsequently, the lipid bilayer was disrupted, releasing intracellular material from the cells (Supplementary Fig. S5).

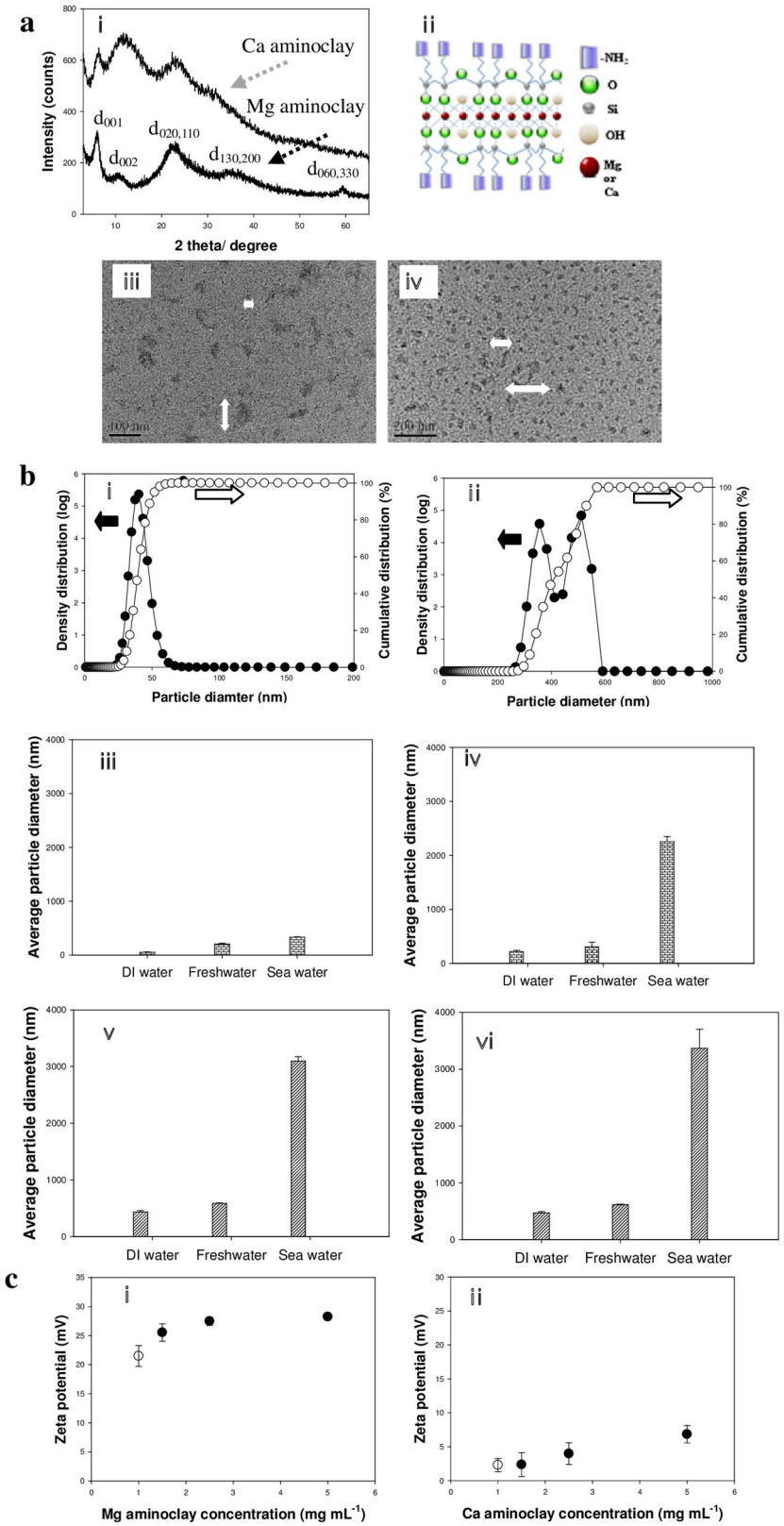

Figure 1 | Characterization of aminoclays. (a) Powder X-ray diffraction (XRD) patterns (i), ideal unit structure (ii), TEM micrographs of Mg aminoclay (iii) and Ca aminoclay (iv) dispersed in DI water. (b) Hydrodynamic particle size distributions of aminoclays in aqueous media by dynamic light scattering (DLS) analysis. Mg aminoclay (i) and Ca aminoclay (ii) dispersed in DI water. Note: black-filled and empty arrows indicate the density distribution (log) and the cumulative probability distribution of particle sizes, respectively. Average particle diameters of Mg aminoclay (iii, iv) and Ca aminoclay (v,vi) at 1 mg mL^{-1} in DI water, freshwater, and filtered sea water at Day 1 and Day 15, respectively. (c) Zeta potential values of Mg aminoclay (i) and Ca aminoclay (ii) as a function of the loading of aminoclay in DI water. Values in bar graphs are mean ± s.d. of three independent experiments.

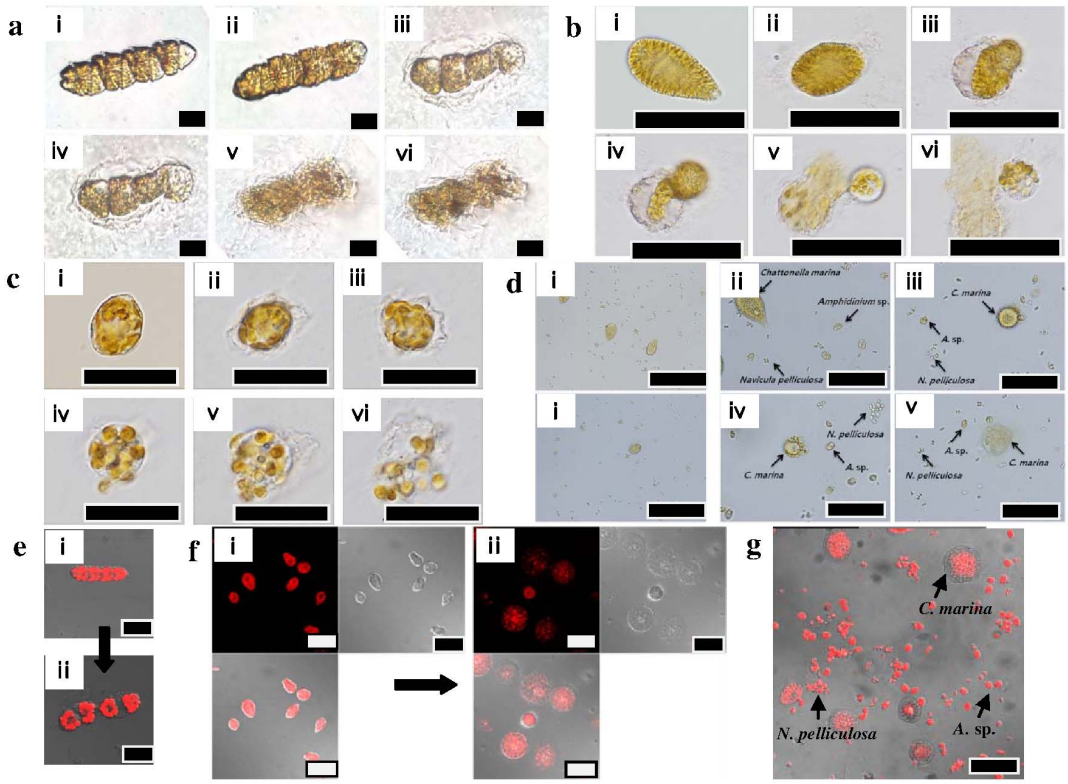

Figure 2 | Microscopic observations of time-dependent algicidal activity produced by aminoclay (0.01%, w/v) loading. Light microscopy images of (a) *Cochlodinium polykrikoides*, (b) *Chattonella marina*, (c) *Heterosigma akashiwo*, and (d) *Chattonella marina* with other phytoplanktons (*Amphidinium* sp. and *Navicula pelliculosa*): (i) control without aminoclay treatment, (ii) 1 min, (iii) 5 min, (iv) 10 min, (v) 15 min, and (vi) 20 min. Scale bars in a,b, and d are 10 μm. Scale bar in c is 5 μm. Confocal microscopy images of (e) *Cochlodinium polykrikoides* and (f) *Chattonella marina*: (i) control without aminoclay treatment, (ii) 5 min after aminoclay treatment. Scale bars are 20 μm. In (f) the confocal microscopy image is the left panel, light microscopy image is the right panel and merged image is the vertical panel. Scale bars are 50 μm. (g) Confocal microscopy image of mixture of *Chattonella marina* with other phytoplankton (*Amphidinium* sp. and *Navicula pelliculosa*). Scale bars are 50 μm. Abbreviations: *C. marina* (*Chattonella marina*), *A.* sp. (*Amphidinium* sp.), *N. pelliculosa* (*Navicula pelliculosa*).

Ecological risk assessment of aminoclay. Mg aminoclay was used in natural sea water microcosm experiments because the turbidity and zeta potential of Mg aminoclay in sea water were more suitable than those of Ca aminoclay (Fig. 1b iii –vi and c, Supplementary Fig. S6) and because in cytotoxicity assays using human cells, Mg aminoclay had less of an effect than Ca aminoclay[24].

Compared to the control, Mg aminoclay loading had a 100% algicidal activity against *C. polykrikoides*. The algicidal effect on *C. marina* and *H. akashiwo* occurred only above 0.001% Mg aminoclay loading (Fig. 4a).

The effect of predators on HABs was also assessed in Fig. 4b. Algicidal activity occurred in all HABs when predators were excluded. Furthermore, Mg aminoclay loading did not disrupt a healthy phytoplankton ecosystem, (Fig. 4c i). A non-HAB species, *Skeletonema costatum*, dominated the phytoplankton community and remained abundant following Mg aminoclay loading (Fig. 4c ii).

Zooplankton were killed by applications of 0.1% and 0.01% (w/v) Mg aminoclay, but 0.001% and 0.0001% applications had negligible impact (Fig. 4c iii). These findings suggest that a Mg aminoclay concentration of 0.001% would be suitable for controlling HABs. During the experiment, specific conditions in the microcosm, including the water temperature, salinity, dissolved oxygen, and pH were monitored (Supplementary Fig. S7). These were stable throughout, with the exception of pH, which rose abruptly before normalizing (< 0.03%, w/v).

In HAB outbreaks in Korea and Japan, red seabream (*Pagrus major*) and olive flounder (*Paralichthys olivaceus*) in cage cultures have suffered mass mortality. We assessed the lethal concentration of Mg aminoclay in water that kills 50% of the test fishes in a given time (LC_{50}). The LC_{50} (95% confidence limits) was expressed in terms of the No Observed Effect Concentration (NOEC) values for two fish species to get the safe treatment concentration of Mg aminoclay for caged fish. The LC_{50} values, expressed as NOEC values/(confidence limit) for *Pagrus major* and *Paralichthys olivaceus* were 0.050/(0.043)% and 0.080/(0.056)%, respectively. Thus, a concentration of Mg aminoclay of 0.001% (w/v), which we recommend for control of HABs, is fully tolerated. As the Mg aminoclay loading (>0.03%. w/v) increased, it resulted in fish deaths. This death is related to the adsorption of Mg aminoclay onto the gills and the resulting interference with the supply of O_2 from the water to the gills. Mucous cells located in the external layer of the primary lamella secrete mucous to protect the gill epithelium[26]. The stimulation of these cells induces the production of a mucous-like substance (Supplementary Fig. S8).

Discussion

Algicidal strategies are required that selectively control HABs, have negligible impacts on other marine organisms, are safe for humans and are environmentally friendly. These are demanding requirements and, despite many research trials, no satisfactory methodology has yet been developed. Ammonium compounds and their derivatives showed some promise within certain applied concentration ranges but their mechanism of selective cell death has remained unclear[27,28] and there are issues of ammonium toxicity.

Here, we introduce a novel approach using aminoclays. These have some of the desirable functional characteristics of ammonium without the detrimental impact on phytoplankton growth (Fig. 4).

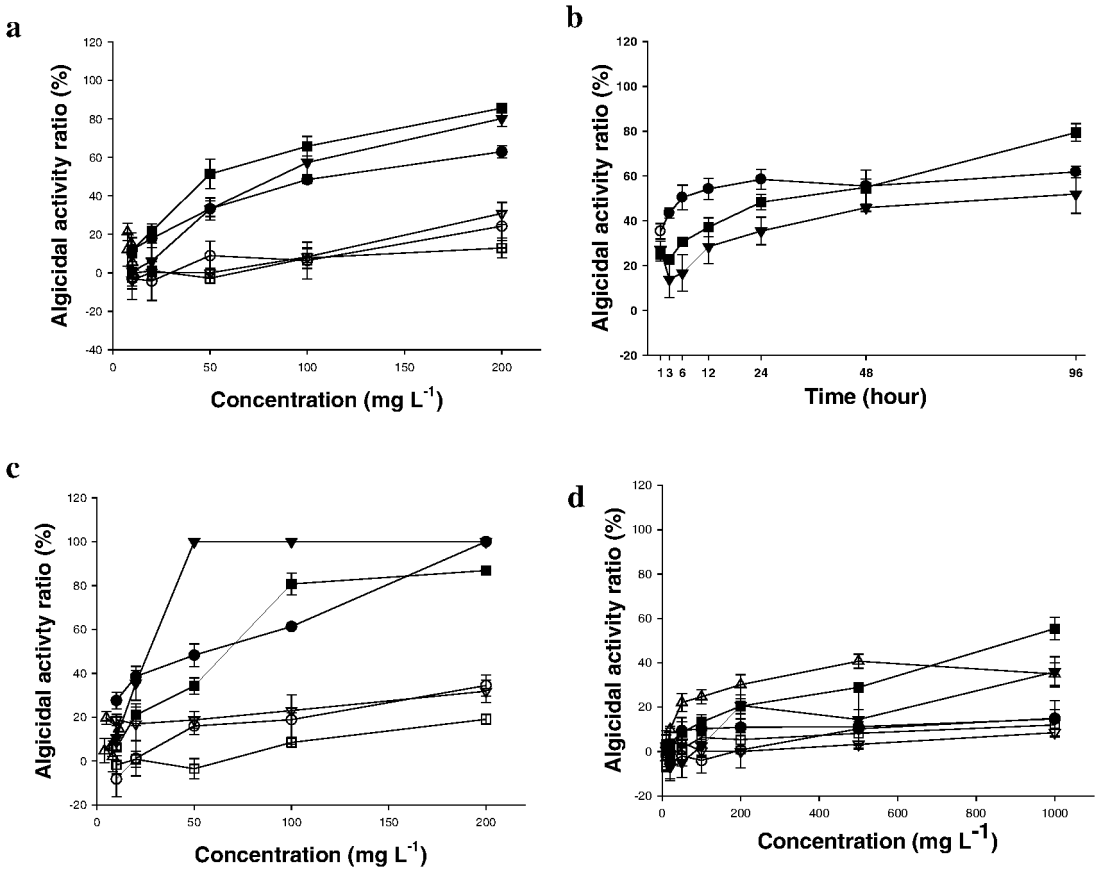

Figure 3 | Algicidal activity assays. (a) Mg aminoclay loading in mixed phytoplankton culture. (b) Time-dependent assay of algicidal activity for HABs using Mg aminoclay loading at 0.01% (w/v). (c) Ca aminoclay loading in mixed phytoplankton culture. (d) Algicidal activity assay of yellow loess in mixed phytoplankton culture. Symbols: *C. polykrikoides* (■), *C. marina* (●), *H. akashiwo* (▼), *Phaeodactylum* sp. (▽), *Amphidinium* sp. (○), *Navicula pelliculosa* (△), *Nannochloropsis* sp. (□). Values in line graphs are mean ± s.d. of three independent experiments.

Our favored explanation of their mechanism of action is that the Mg aminoclay (including the organic portion of the ammonium sites) adheres to the cell surface, enhancing its stickiness in mucilage on most of cell surface, which triggers cell-cell adhersion[29]. Because cell lysis occurred quickly, we investigated the mechanism using a green microalgae with a thick cell wall (Supplementary Fig. S9). Mg aminoclay caused flocculations of green microalgae within five minutes, trapping the cells (see Fig. 2e–g and Supplementary Movies 1–5). Subsequent cell lysis may be due to disruption of the internal environment caused by surface interactions of the ammonium sites within the Mg aminoclay. We conclude that both Mg aminoclay and Ca aminoclay use the same mechanism of action to disrupt HABs. Ca aminoclay has slightly superior algicidal activity against HABs but the difference is not significant ($P < 0.05$) which is focused on harmful algae against non-harmful algae, resulting in *Heterosigma akashiwo* was sensitive to be disrupted ~100% of algicidal activity at 50 mg/L of Ca aminoclay, compared to ~50% of that at the same loading of Mg aminoclay. However, as increase in both aminoclays loading, the algicidal activity ratio (%) reached ~100% in all HABs (Fig. 3a,c).

Aminoclays offer several advantages as practical treatments. Their syntheses use a one-pot sol-gel reaction under ambient conditions. In detail, cationic metals (Mg or Ca) and aminosilane precursors were simply mixed in solvent media, for example, ethanol solution, which produced white slurries (aminoclays). Those are composed of coordination bonding of aminosilane onto metal backbone via hydrolysis and condensation at room temperature and atomospheric environment[21–23]. It is indicated that mass-production to selectively control and manage HABs is feasible. They form cationic dispersions of highly delaminated sheets, which have favorable optical transparency properties. And they are colloids[21–23,30], ranging in size from 20 to 600 nm in diameter: the average size of aminoclay particles in aqueous solution is smaller than those of conventional inorganic clays such as kaolinite, montmorillonite, and yellow loess. Finally, the modification step with alkylammonium cationic surfactants to produce a hydrophobic (organophilic) and positive surface charge is not needed[30]. In general, to prepare conventional organoclay[31], after making a slurry type in aqueous solution using natural inorganic clays, the alkylammonium cationic surfactants solution was added into the clay slurry. And then, further mechanically mixed surfactants monomers adsorbed or intercalated into the clay minerals. Thus, without alkylammonium cationic surfactant modifications, the synthesized aminoclay itself maintains an ammonium property persistently with a superior water-dispersibility. Taken together, these properties suggest that relatively small amounts of aminoclay may provide effective control of HABs, The suitable loading of aminoclay for HABs control is suggested ~10 mg/L but the reported amounts of bentonite or phosphatic clay were 100–4000 mg/L (Supplementary Table S3)[1,4,18].

The critical challenge is selective killing of HAB organisms in a mixed culture containing non-harmful phytoplankton. Do aminoclays represent a suitable chemical treatment that should progress to field applications of biological risk? We believe so, and cite the following evidence to substantiate the case for aminoclays. First, *C. polykrikoides*, *C. marina*, and *H. akashiwo* cells were lysed within 20 minutes of aminoclay treatment whereas other phytoplankton species, such as *Nannochlopsis* sp., *Phaeodactylum* sp., and *Amphidinium* sp. survived with only slightly reduced cell mobil-

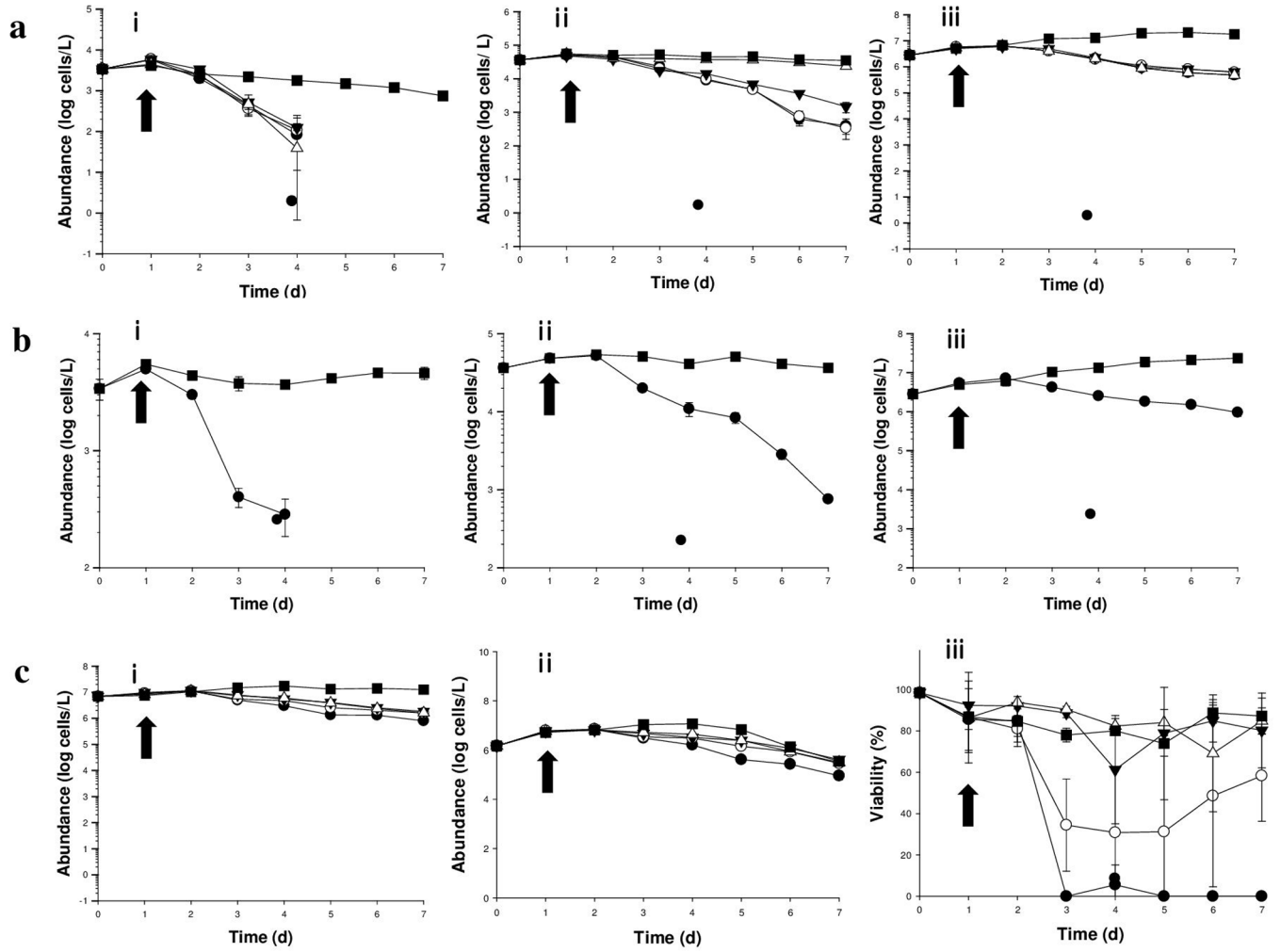

Figure 4 | Algicidal activity of Mg aminoclay in an indoor microcosm (10 L). (a) HAB survival as a function of Mg aminoclay loading: (i) *C. polykrikoides*, (ii) *C. marina*, (iii) *H. akashiwo*. (b) HAB survival in the absence of predators and 0.1% (w/v) Mg aminoclay loading: (i) *C. polykrikoides*, (ii) *C. marina*, (iii) *H. akashiwo*. (c) Ecological evaluation of phytoplankton and zooplankton systems: (i) Total abundance of phytoplankton communities excluding HABs; (ii) the abundance of *Skeletonema costatum* like species (non-HABs); (iii) the abundance of zooplankton communities. Note: all black arrows indicate the time at which Mg aminoclay was introduced. Symbols: control without Mg aminoclay treatment (■), 0.1% (w/v) Mg aminoclay (●), 0.01% (w/v) Mg aminoclay (○), 0.001% (w/v) Mg aminoclay (▼), 0.0001% (w/v) Mg aminoclay (△). Values in line graphs are mean ± s.d. of three independent experiments.

ity due to the viscosity of the aminoclay (1.18 mPa•s, measured viscosity value of 10 mg L^{-1} Mg aminoclay in DI water at 18°C). Second, ecological risk assessment in microcosm enclosures demonstrated that aminoclays served as an algicidal agent for HAB species at 0.001% (by weight, i.e., 10 mg L^{-1}) loading for input conditions. These results were supported by accompanying acute ecotoxicologic results for species cultured in fish farms, namely *Pagrus major* and *Paralichthys olivaceus*.

Aminoclays thus represent an important step toward the direct control of HABs. Nevertheless, a few challenges remain. Chronic toxicological studies, with a focus on the biodegradability of the organic portion of aminoclays, are needed to measure the half-life of these organic components. Prior to field application, mesocosm studies are underway to gain an in-depth understanding of possible adverse environmental impacts of aminoclays[32]. During algicidal tests, ammonium production, dissolved oxygen (DO), dissolved organic carbon (DOC), and other bacteria growth are monitored, along with nutrient cycles.

Reductions in the amount of exotic (artificial) aminoclay loading may be achieved through a combination of aminoclay libraries and input (spraying) engineering methods. While the present study presents the utility of a single aminoclay, combinations of aminoclays and the development of multi-functionalized hybrid clays may increase efficiency and reduce the cost of controlling and managing a broad spectrum of HAB species in coastal marine systems. If the approach proves damaging to coastal resources[28], the immobilization of organosilicon quaternary ammonium on a substrate or a process of mixing selected agents with clay, sediment, and humic acid to reduce the toxicity of the agent in marine ecosystems may be pursued although efficiency of cell lysis can be reduced[32–35]. Furthermore, such methods can be used in pretreatment of red tide conditions in the Arabian Gulf to prevent membrane fouling problems in desalination facilities[36,37].

Methods

Algal cultures, culture media, and condition. The red-tide-producing marine strain *C. polykrikoides* was obtained from the algal culture collection of the National Fisheries Research & Development Institute (NFRDI). The microalga *C. marina* was provided by Dr. Y.H. Yoon at the Faculty of Marine Technology, Chonnam National University, Korea. *H. akashiwo*, CCMP 452 was obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP) in East Boothbay, Maine,

USA. *Amphidinium* sp., *Navicula pelliculosa*, *Nannochlopsis oculata* and *Phaeodactylum* sp. were provided by Professor M.-S. Han, Department of Life Science, Hanyang University, Korea.

Cultures of *C. polykrikoides*, *C. marina, H. akashiwo*, and non-harmful algae were grown in culture flasks (Becton Dickinson Labware, Franklin Lakes, New Jersey, USA) at 20°C under constant light in Guillard's f/2 medium with filtered sea water, as reported previously (Guillard and Keller, 1984). The f/2 medium was prepared by sterile filtering sea water with 0.22 μm filtration units (Nalgene, Rochester, New York, USA) and enriched aseptically using nutrients and vitamins purchased from Sigma (St. Louis, Missouri, USA). Inhibition experiments were conducted when the cultures achieved exponential growth. The concentrations of *C. polykrikoides*, *C. marina*, and *H. akashiwo* used in the experiments were approximately 2.4×10^3, 1.2×10^4, and 3.2×10^5 cells mL^{-1}, respectively.

Assay for algicidal activity assay of Mg and Ca aminoclays, and yellow loess. Algicidal activity against *C. polykrikoides*, *C. marina*, and *H. akashiwo* was examined at various concentrations. Each experiment was carried out in 24 well tissue culture test plates with 1 mL total volume per well. Test compounds were introduced during the exponential growth phase at final concentrations of 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 mg mL^{-1}. Control cultures were maintained without aminoclays but enriched with sea water used in dispersing the aminoclays. Algal cell density was counted three days after inoculation using a Burker Turk hemocytometer (Fujirika, Osaka, Japan) with a Sedgwick–Rafter counting chamber under an Olympus light microscope with ×40 or ×100 magnification (Olympus Co., Tokyo, Japan). The algicidal activity (%) was determined using the following equation: Algicidal activity (%) = $(1-T_t/C_t) \times 100$, where T (treatment) and C (control) are the cell densities and t is the inoculation time (day). The experiments were performed at least three times. The data are expressed as the mean ± SD. All statistical analyses were performed with SPSS 17.0 software (SPSS, USA). The statistical significance of the differences between the mean values was determined by a one-way analysis of variance (ANOVA) followed by a Tukey HSD *post hoc* test. A p value < 0.05 was considered significant.

Observations of the algicidal process in HABs. Changes in the cell shape and mobility of the HAB organisms were observed with microscopes, and digital photos were taken. Changes in the morphological characteristics were further monitored with a light microscope (Olympus model DP71) at a magnification of ×600 for *H. akashiwo* and *C. marina*, and with an Olympus model IX71 at a magnification of ×640 for *C. polykrikoides*, or with a confocal microscope (Carl Zeiss, LSM510 Meta NLO).

For the mixed cell culture, 6-well tissue culture test plates with 6 mL total volume per well were used (f/2 media 3 mL, *C. marina* 1 mL, *Amphidinium* sp. 1 mL, and *Navicula pelliculosa* 1 mL). Algal lysis was monitored by changes in morphology following the addition of Mg aminoclay to a final concentration of 0.1 mg mL^{-1} (0.01%). The morphology of the algal cells was observed, recorded and photographed when significant changes appeared in each species during the experimental period of 30 min. The control group without aminoclays was observed at the same time for comparison.

Preparation of hydrophilic Mg and Ca aminoclays, and fluorescein isothiocyanate (FITC) functionalized Mg aminoclay. Aminopropyl functionalized magnesium or calcium phyllosilicate clays (Mg aminoclay or Ca aminoclay) were prepared as previously reported in the literature[22,24,33]. 3-aminopropyltriethoxysilane (APTES, 2.6 mL) was added dropwise to magnesium chloride hexahydrate or calcium chloride dihydrate (1.68 g) in ethanol (40 mL) at room temperature and a white slurry formed after 5 min. The molar ratio of Mg to Si was approximately 0.75. The reaction vessel was stirred overnight, then the precipitate was isolated by centrifugation, washed with ethanol (50 mL) to remove excess metal chloride, and dried at 40°C. The synthesis of FITC functionalized Mg aminoclay followed the literature[30]. Briefly, after dissolving Mg aminoclay in DI water (2.6 mg mL^{-1}), it was added to FITC ethanolic solution (2.6×10^{-4} mmol mL^{-1}) and stirred overnight with minimal light exposure. Unreacted chemicals were washed out twice using ethanol and the final product dried at 40°C in oven.

Characterization of aminoclays and yellow loess. The morphological characterization of the aminoclays was performed by transmission electron microscopy (TEM; JEM-2100F, JEOL LTD, Japan) with energy-dispersive X-ray (EDX, JED-2300T, Japan) analysis. Samples for TEM were dispersed in DI water by sonication for 5 minutes then dropped onto a carbon-coated copper grid and dried overnight prior to injection into the microscopy sample container. XRD patterns were obtained on a Rigaku D/max IIIC (3 kW) with a θ/θ goniometer and a CuKα radiation generator operated at 40 kV and 45 mA. The scan range was 3° to 65°, and the scan rate was 1.2° 2θ/min with a step size of 0.01. Each of the clay powders (approximately 0.5 g) was prepared with a mechanical mortar and placed evenly in the sample container. Fourier transform infrared (FT-IR) spectra of KBr pellets (FTIR 4100, Jasco, Japan) were collected from 4000 cm^{-1} to 400 cm^{-1}. Each spectrum was calculated as the average of 32 scans with a resolution of 4 cm^{-1}. The pellet disks prepared for the analysis were composed of 10% clay powder and 90% KBr by weight. Zeta potential measurements for the aminoclays in an aqueous solution (1.5 mg mL^{-1}) were conducted with a Zetasizer Nano-ZS particle analyzer (Malvern, UK). The viscosity of Mg aminoclay in DI water was measured with SV-10 VIBRO Viscometer (Japan). The pH was determined using an Orion pH meter (Thermo Orion, model 710, USA). Deionized water (>18 mΩ, DI water) was used in all experiments.

Microcosm experiment for Mg aminoclay. To evaluate the algicidal activity of the Mg aminoclay against three types of HAB organisms (*C. polykrikoides*, *C. marina*, and *H. akashiwo*) and to assess the marine ecological risk resulting from the introduction of Mg aminoclay, an indoor microcosm was established in an experimental culture room. The microcosm was formed of 12 L enclosures containing 10 L sea water in which the HAB organisms were cultured. The room was maintained at 20 ± 2°C and 40 μE m^{-2} s^{-1} under 16 : 8 (light : dark) cycles for 24 h. The microcosms were constructed from transparent plastic materials (30 cm L × 20 cm W × 20 cm H) (Supplementary Fig. S10). The sea water in the microcosms was filtered through a 100 μm mesh net to eliminate large organisms, and partitioned. Zooplankton (> 200 μm in size) were inoculated into each microcosm at a density of 50 individuals L^{-1} using a 200 μm mesh net. Copepods predominated in the microcosms, representing 80% of the total number of zooplankton individuals identified. The cultures of *C. polykrikoides*, *C. marina*, and *H. akashiwo* were inoculated at final densities of 3.6×10^4, 3.4×10^3, and 2.8×10^6 cells L^{-1} in sea water, respectively. The experimental algicidal compound, aminoclay, was added to four treatment groups at concentrations of 0.1%, 0.01%, 0.001%, and 0.0001%. A control microcosm (no addition of aminoclay, but with dispersal sea water) was also prepared. Experimental groups with and without zooplankton communities were also prepared to evaluate the possible effects of direct feeding by predators. The water in the microcosms was mixed gently for 1 min twice per day. The experiment was performed in triplicate over a period of seven days from December 10 through 16, 2009.

Subsamples were taken daily from each microcosm at 9:00 AM. The water temperature, pH, salinity, and dissolved oxygen content were measured using a portable meter (556 MPS; YSI, USA). To analyze the phytoplankton, a 30 mL sample was collected in a 50 mL sterilized polyethylene bottle and fixed immediately with glutaraldehyde at a final concentration of 2%. The sample was stored in the dark at 4°C until analysis. The cell counts and identification of phytoplankton were performed for at least 500 cells per sample with a Sedgwick-Rafter counting chamber under a light microscope (Axioplan; Zeiss, Germany) at ×400 magnification. To determine the viability of the copepods, a 150 mL sample was collected in a 200 mL acid-cleaned PE bottle and examined immediately under a dissecting microscope (Discovery V8; Zeiss, Germany). Copepods were considered dead if they exhibited no movement after being touched with a needle.

Ecotoxicological assay for Mg aminoclay using cage-culture fishes (*Pagrus major* and *Paralichthys olivaceus*)[26,38]. The aquatic toxicity of Mg aminoclay against fishes (*Pagrus major* and *Paralichthys olivaceus*) was performed in indoor microcosms (Supplementary Fig. S11). The materials and culture conditions of the microcosm were as described above. Sea water was filtered through a 0.2 μm polycarbonate membrane to eliminate all organic compounds. *P. major* (lengths of 4.8–5.2 cm) and *P. olivaceus* (lengths of 9.6–10.3 cm) were added to each microcosm at 15 and 10 individuals, respectively. The aminoclay was added at concentrations of 0.1%, 0.07%, 0.03%, and 0.01% along with a control microcosm (no addition of aminoclay but with dispersal sea water). Dissolved oxygen in the water was supplied at 6–8 mg L^{-1} (approximately 100 bubbles min^{-1}). The experiment was carried out in triplicate for a period of 96 hours. The fish were observed four times per a day at intervals of six hours. Fishes were considered dead if they exhibited no movement after being touched with a needle. The water temperature, pH, salinity, and dissolved oxygen content were measured using a portable meter (556 MPS; YSI, USA). To identify toxicity of aminoclay, gills of dead and live fishes were observed by using a dissecting microscope (Discovery V8; Zeiss, Germany) and scanning electron microscope (JSM 5600-LV; JEOL, Japan).

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Author contributions

Y.-C.L. and E.S.J. contributed equally to this work. Y.-C.L., E.S.J., S.W.J., Y.-O.K., J.-W.Y. and H.-J.S. designed the research plan; Y.-C.L., Y.-M.K., K.S.C. and S.W.J. performed the experiments; Y.-C.L., E.S.J., J.-W.Y. S.-W.K., Y.-O.K. and H.-J.S. discussed the results; Y.-C.L., S.W.J., Y.-O.K. and H.-J.S. wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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