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Cyclodextrin-immobilized microspheres for uptake of the quorum-sensing signaling molecule *N*-acylhomoserine lactone

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ABSTRACT: β-Cyclodextrins (CDs) were immobilized on microspheres possessing poly(methacrylic acid) shell layers over polystyrene cores to effectively interact with *N*-acylhomoserine lactone (AHL), the main signaling molecule for the bacterial cell-to-cell communication system known as quorum sensing (QS). Furthermore, these CD-immobilized microspheres inhibited the expression of a QS-dependent gene, *pig* cluster, in *Serratia marcescens* AS-1. The AHL-mediated production of prodigiosin was suppressed to approximately 30% when the culture was started in the presence of 9.1% (w/w) core/shell microspheres immobilized with β-CD in a liquid broth, with shaking. This result suggested that the AHL concentration was maintained below the threshold of QS activation because transcription of the target genes would be activated via a stable AHL–receptor complex under high concentrations of AHL. The AHL reporter assay with *Chromobacterium violaceum* CV026 showed that the AHL concentration in the culture broth distinctly decreased after addition of the microspheres, confirming that uptake by the immobilized β-CDs was responsible for the observed QS suppression. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43198.

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INTRODUCTION

Quorum sensing (QS) is a unique cell-to-cell communication system in bacteria; however, much remains to be discovered about QS and the potential for manipulating this system. Although bacteria are single-celled organisms, group behaviors of some bacteria have been recognized. Gram-negative bacteria can produce and secrete *N*-acylhomoserine lactone (AHL) as a signaling molecule to regulate various cell functions, including biofilm formation and bioluminescence.[1–3] Diverse bacterial species produce various AHLs, in which a homoserine lactone ring is amide-bonded to an acyl chain with a diverse chain length.[4] To activate the sequential processes for QS-related gene expression, AHL molecules can form complexes with their respective receptors, which are classified as LuxR family proteins and are homologs of a receptor for *N*-(3-oxo-hexanoyl)homoserine lactone in *Vibrio fischeri*.[5] The LuxR family of proteins can simultaneously recognize AHLs and DNAs at the N-terminal and C-terminal domains, respectively.[6] An increase in AHL concentration can trigger activation of the QS system for bacterial cell-to-cell communication.

This complex formation between AHL and its receptor protein can be exploited for artificial control of communication systems, which shows promise as a valuable approach for preventing bacterial infections and for inhibiting or enhancing biofilm formation. Some strategies have been shown to successfully inhibit AHL-mediated QS. Enzymatic degradation of AHLs is one of the simplest ways to intercept communication between cells.[7,8] For example, homoserine lactone, which is produced by AHL-acylase, intercepts QS signals via the formation of complexes with its receptor protein.[9] In addition, both natural and synthesized antagonists of AHLs have been identified and developed to competitively inhibit the formation of complexes with the receptor protein.[10] As an alternative approach, cyclodextrin (CD) has been utilized for QS inhibition. The acyl chain of AHL can form an inclusion complex with the hydrophobic cavities of α- and β-CD in aqueous media.[11] The CD-immobilized polymer hydrogel sheets can then effectively suppress the production of AHL-mediated prodigiosin in *Serratia marcescens*, as well as production of β-galactosidase, a reporter enzyme in *Pseudomonas aeruginosa*.[12,13] AHL signals diffuse in and out of cells through the cell membrane and accumulate locally around

the cells; thus, AHL concentration increases with increasing cell density due to cell division. The complex between AHL and its receptor protein interacts with the promoter region of the QS target gene, and activates its expression. In the case of AHL-mediated QS in *S. marcescens* AS-1, the SpnR protein, a member of the LuxR protein family, acts as a negative regulator for the *spn* operon.[14] Thus, the interaction between the SpnR and the promoter of the *spnR* gene, the *spn* box, greatly decreases with increasing AHL concentration. Binding of AHL to SpnR can induce a change in the SpnR conformation, which in turn leads to transcriptional activation of the target gene. In addition, multiple QS systems can be associated within the same cell. In *P. aeruginosa*, two independent QS-related gene systems, *lasB* and *rhlA*, are regulated by *N*-(3-oxo-dodecanoyl) homoserine lactone (3oxoC12HSL) and *N*-butanoyl homoserine lactone (C4HSL), respectively.[15] The *lasB* system primary regulates elastase production; however, sufficient elastase expression is enabled only after activation of the *rhlA* system. Thus, the two QS systems control various cell functions in cooperation with each other.

The uptake of AHL signals on some matrices is responsible for maintaining AHL concentration below the QS activation threshold, as well as for prolonging the inactive status of the QS target gene. In this study, a CD-immobilized core/shell-type microsphere was designed as a novel polymer matrix for AHL uptake. β-CD was immobilized on hydrophilic poly(methacrylic acid) (polyMA) shell layers around the polystyrene (PSt) cores because highly mechanical PSt particles of relatively uniform size could be easily synthesized by emulsion polymerization. The use of renewable CD-immobilized matrices could allow for repeated use, which would reduce the required amount of CD and, consequently, its cost.

Pressure-resistant, mechanically tough microspheres can be utilized for preprocessing in water treatment systems because some water treatment plants use reverse osmosis (RO) under high pressure to separate treated water from sewage prior to chlorine sterilization.[16] Biofilm formation is a critical factor in membrane fouling that results in the deterioration of filtration performance.[17] Such suppression of biofilm formation can be achieved via AHL-dependent QS inhibition, by trapping AHL. Pressure-resistant, CD-modified microspheres are suitable for trapping AHL and thereby suppressing AHL-mediated biofilm formation in the membrane separation reactor for activated sludge. Although the hydrophobic cavity of CD can effectively interact with AHL, in the case of a microbial filtration system using an RO membrane, the addition of free CD has the potential to cause problems. Specifically, addition of a low-molecular-weight oligosaccharide occasionally causes clogging of the RO membrane, which may contribute to a drastic decrease in the filtration rate. One of the potential applications of CDs is to prevent biofilm formation and bacterial colonization of storage water containing living organisms. Thus, immobilization of CDs could be a valid approach for inhibiting QS owing to its ability to form a complex with AHLs; as the water-soluble CD is degraded by living organisms, the content of AHL could become reduced. Note that the use of CD as an additive to drugs and food has been approved by US Food and Drug Administration and other countries, and its safe properties for organisms were confirmed.

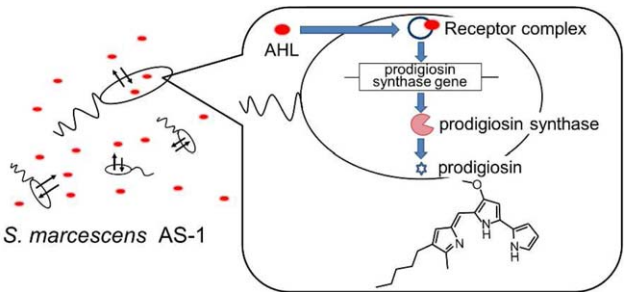

Figure 1. A schematic illustration of AHL-dependent prodigiosin production through the quorum sensing system in *S. marcescens* AS-1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Thus, in this study, the effects of the synthesized polymeric microspheres on QS were investigated in *S. marcescens* AS-1 as the model bacterium (Figure 1). The properties of this strain enable clear visualization of the degree of QS activation. This is because *S. marcescens* produces *N*-hexanoylhomoserine lactone (C6HSL) and *N*-(3-oxo-hexanoyl)homoserine lactone (3oxoC6HSL), which regulate the expression of *pig* gene clusters encoding the synthase of 4-methoxy-5-[(*Z*)-(5-methyl-4-pentyl-2*H*-pyrrol-2-ylidene)methyl]-1*H*,1′*H*−2,2′-bipyrrole, termed prodigiosin. When the QS system is normally activated, the color of the culture broth turns red as the red pigment prodigiosin accumulates inside the cells. Therefore, interactions between AHL signals and immobilized CDs at the surface of the core/shell microspheres can be detected by monitoring the color change. In addition, QS suppression via the interaction between the immobilized CDs and AHLs was tested in *P. aeruginosa* AS-3, another bacterium that uses AHL-mediated QS.

The objectives of this study were to characterize the β-CD-modified core/shell microspheres, and to demonstrate whether QS could be suppressed via AHL uptake by the microspheres when dispersed in a culture broth. QS inhibition by CD-modified microspheres was determined based on monitoring of AHL-mediated prodigiosin and pyocyanin production in *S. marcescens* AS-1 and *P. aeruginosa* AS-3, respectively. The CD-modified microspheres possessing a hard core were expected to serve as AHL-trapping matrices, which could be utilized in high-pressure systems such as membrane separation processes.

EXPERIMENTAL

Materials

β-CD, 4-toluenesulfonyl chloride, ethylenediamine, styrene (St), ethylenediaminetetraacetic acid (EDTA), and sodium formaldehydesulfoxylate (SFS) were purchased from Kanto Chemical. 2,2′-azobis(2-methylbutyronitrile) and cumyl hydroperoxide (CHPO) were purchased from Wako Chemicals. Polyvinylpyrrolidone and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich and Peptide Institute, respectively. All the commercial reagents were used without further purification.

Core/Shell Polymeric Microspheres

Mono(6-deoxy-6-*N*, *N*-diethylamino)-β-CD was synthesized according to standard methods.[18,19] Briefly, 6-*O*-monotosyl-6-

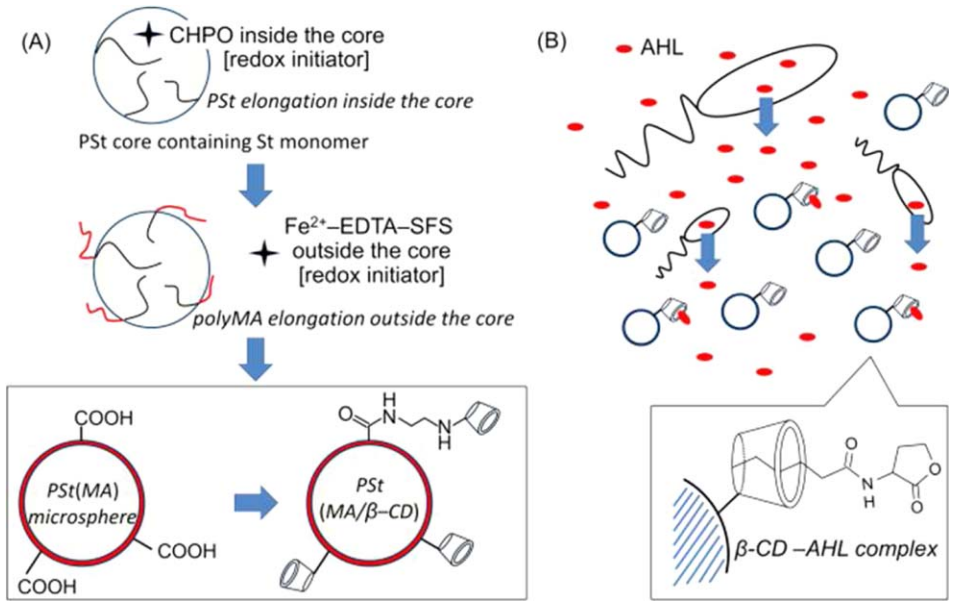

Figure 2. (A) Fabrication of a hydrophilic layer on the PSt core and immobilization of β-CD. (B) Formation of the inclusion complex between the immobilized β-CD and AHLs released from bacterial cells in the culture medium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

deoxy-β-CD was synthesized by adding 4-toluenesulfonyl chloride into an aqueous alkaline solution of β-CD. The crude crystal of 6-*O*-monotosyl-6-deoxy-β-CD was dissolved in ethylenediamine and then heated to 50°C.

The core/shell polymeric microspheres were synthesized through a two-step emulsion polymerization.[20,21] The first step was the nucleation of PSt by free-radical polymerization. St (25.0 g), polyvinylpyrrolidone (4.0 g), 2,2′-azo-bis(2-methylbutyronitrile) (1.0 g), and Triton X-305 (1.4 g) in ethanol (75 g) were stirred at 300 rpm in a 250-mL three-neck reaction flask under a flow of nitrogen gas at 70°C. After 24 h, in the second step, the shell layer was fabricated on the PSt core. An aqueous solution (10 mL), including $FeSO_4{\cdot}7H_2O$ (40 mg), EDTA (30 mg), SFS (20 mg), and MA (0.9 mL), was gradually added to the ethanol solution (50 mL) of the PSt cores that were swollen with St (0.1 g) containing CHPO (30 mg) as the initiator. CHPO, which remained inside the PSt cores because of its hydrophobicity, was reduced by Fe^{2+} on the surface of the PSt core. The carboxylic acid groups of MA were anchored to the surface of the PSt core by the CHPO radicals at the surface. To exchange the ethanol for water, the suspension was centrifuged and the resulting pellet was resuspended in water. To determine the amount of the carboxylic acid groups on the PSt microspheres, the PSt suspension was titrated with an aqueous NaOH solution. Mono(6-deoxy-6-*N*, *N*-diethylamino)-β-CD (0.50 g) was coupled with the carboxylic acid groups of polyMA in the presence of EDC. The amount of CD immobilized on the microspheres was determined by measuring the concentration of the remaining CD by using high-performance liquid chromatography equipped with a refractive index detector.

Characterization of the Core/Shell Polymeric Microspheres

The morphology of the synthesized microspheres was observed using a digital microscope (MS-200, Asahi Kogakuki, Japan). The size distribution of the microspheres, in the range of 0.1–600 μm, was determined using a laser diffraction particle size distribution analyzer (LA-300, Horiba, Japan). After the dispersion in water, the 10%, 50%, and 90% diameter [d10, d50 (median diameter), and d90, respectively] of the particle diameter distribution and specific surface area were determined. The turbidity of the microsphere suspension in a 5 mL quartz batch cell was optimally adjusted before laser irradiation. The zeta-potential of the microspheres was determined using the laser-Doppler velocimetry technique (SZ-100-Z, Horiba, Japan).

Suppressive Effects on QS in *S. marcescens*

Serratia marcescens AS-1 was grown in Luria–Bertani (LB) medium (4 mL) at 25°C for 15 h with the desired amount (C_m) of PSt(MA/β-CD) microspheres. After sedimentation of most of the microspheres was achieved by leaving the culture broth to stand at 4°C for 24 h, the apparent turbidity of the cell suspension was determined at 600 nm (OD_{600}). The suppressive effects on QS-dependent production were determined based on the monitoring of the production of the red pigment prodigiosin after extraction. Cell pellets obtained by centrifugation were resuspended in a 4% (v/v) hydrochloric acid ethanol solution for cell lysis. After removing cell debris, the amount of prodigiosin was measured as the absorbance of the supernatant at 534 nm (A_{534}).[22] The amount of prodigiosin produced per cell unit was calculated by dividing the A_{534} value by the OD_{600} value of the culture broth. The relative prodigiosin production level, normalized to the A_{534}/OD_{600} ratio of the control without any microspheres, was used to determine the overall suppressive effects on QS. The coefficient of variation (CV %) of three measurements for relative prodigiosin production was calculated to be 7.7%, irrespective of additives.

Suppressive Effects on QS in *P. aeruginosa*

Pseudomonas aeruginosa AS-3 was grown in LB medium (4 mL) at 30°C for 18 h in the presence of 2.5% (w/w) PSt or PSt(MA/*β*-CD). After centrifugation to remove any cells and microspheres, the pyocyanin and other solutes were extracted by mixing chloroform with the supernatant because pyocyanin accumulates in the extracellular pigment. The pyocyanin turned from green to red because it was re-extracted in a diluted hydrochloric acid solution. The pyocyanin concentration was determined by measuring the absorbance at 520 nm (A_{520}).[23] The relative pyocyanin production level normalized to the A_{520}/OD_{600} ratio of the control without any microspheres was used to determine the suppressive effects on QS. The coefficient of variation (CV %) of three measurements for relative pyocyanin production was calculated to be 7.2%, irrespective of additives.

Bioassay for AHL Detection

The culture broth of *P. aeruginosa* AS-3 was centrifuged to remove the cells and microspheres. The AHLs and others were extracted from the supernatant with ethyl acetate. After the extracts were dried with a rotary evaporator, they were redispersed in dimethyl sulfoxide (DMSO).

Chromobacterium violaceum CV026 was used as the AHL reporter, which was precultured in LB medium (4 mL) containing 0.1% (w/w) kanamycin at 30°C.[24,25] The cell suspension was mixed with an autoclaved pre-gel solution of the LB agar medium just before gelation, and the CV026 agar gel plates were cooled in preparation to detect C4-C8 AHLs. Then, the extracts in DMSO solution were spotted onto paper disks on the CV026 plates, followed by incubation at 30°C for 10 h. AHL was detected according to the appearance of a purple spot on the plate, signifying that the purple pigment violacein was induced by AHL-meditated QS in CV026 cells. The diameter of the purple spot that appeared was considered to be proportional to the AHL concentration in the sample.

RESULTS AND DISCUSSION

Characterization of the Core/Shell Microspheres Possessing *β*-CD

Various applications of core/shell microspheres have been reported because the desired shell layers can be easily fabricated on the cores. Moreover, the cores have additional functionalities such as uniform size distribution, mechanical toughness, and magnetic properties. Carboxyl groups and their derivations are functional groups well suited for surface modifications. In this study, a polyMA shell layer was fabricated on the PSt core [Figure 2(A)]. For the preparation of PSt cores, polyvinylpyrrolidone and Triton X-305 were used as the stabilizer and co-stabilizer, respectively.[21] In the PSt cores, the polymerization of St was induced using CHPO as the redox initiator. The surrounding aqueous solution contained hydrophilic components of the redox initiators (Fe^{2+}/EDTA/SFS), allowing for interfacial-initiated seed emulsion polymerization of MA and St to proceed. To modify the hydrophilic shell layers possessing COOH groups, mono(6-deoxy-6-*N*, *N*-diethylamino)-*β*-CD was reacted in the presence of water-soluble carbodiimide. After washing, the obtained microspheres containing *β*-CD at the hydrophilic surface were added to the liquid medium of *S. marcescens* AS-1 in preparation for culturing [Figure 2(B)]. Accumulation of the QS signal AHL was detected according to the increasing number of cells in the culture broth. CD-AHL complex formation is expected to remove AHL signals from the culture broth, which should maintain QS in the inactive state even after a high cell density is reached.

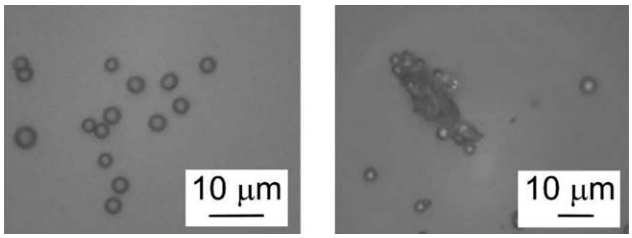

Figure 3. Digital microscope images of the PSt (MA/*β*-CD). The microspheres were suspended in water at 25°C.

The morphology of the microspheres suspended in aqueous solution was investigated. Figure 3 shows the digital microscope images of the PSt (MA/*β*-CD). The primary particles of PSt (MA/*β*-CD) was approximately 1–3 *μ*m in diameter, whereas few microspheres formed tens of micrometers sized secondary particles. A laser diffraction particle size distribution analyzer equipped with a 650-nm laser (5.0 mW) was used to determine the aggregation of the microspheres in water. The laser diffraction method principally determines the volume distribution of the particles. Figure 4 shows that the mode diameter of the PSt core was 4.2 *μ*m, and the particle size clearly increased after fabrication of the polyMA layer (12.3 *μ*m) and subsequent CD-immobilization (24.3 *μ*m). The difference in the broad distribution curves suggested a difference in the degree of aggregation in water, resulting from a difference in the zeta-potential. The

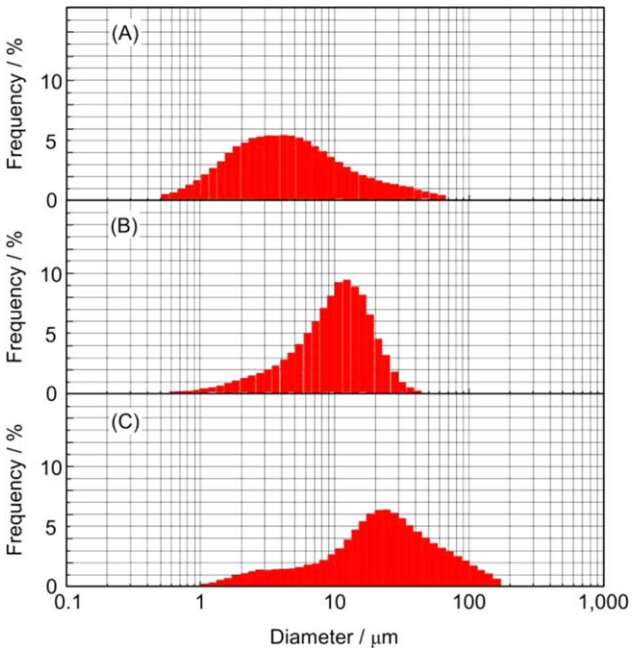

Figure 4. Size distribution of synthesized microspheres in water. (A) PSt, (B) PSt microspheres with polyMA shell layers [PSt (MA)], and (C) *β*-CD-modified core/shell microspheres [PSt (MA/*β*-CD)]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Physical Properties of Polymeric Microspheres in Water

	Volume distribution				Number distribution				
	*d*10 (μm)	Median diameter (μm)	*d*90 (μm)	Specific surface area (cm^2/cm^3)	*d*10 (μm)	Median diameter (μm)	*d*90 (μm)	Specific surface area (cm^2/cm^3)	Zeta potential (mV)
Pst	1.4	4.2	18.1	2.0×10^4	0.56	0.88	1.9	2.7×10^4	–
PSt (MA)	3.5	10.5	20.3	8.7×10^3	0.64	1.08	2.9	1.1×10^4	−72
PSt (MA/β-CD)	4.5	22.7	73.5	5.3×10^3	1.1	1.7	3.2	1.1×10^4	−52

zeta-potential of PSt(MA) and PSt(MA/β-CD) was approximately −72 and −52 mV, respectively. The distribution of the number of particles was recalculated according to the particle volume distribution. Table I shows the 10%, 50%, and 90% diameter [d10, d50 (median diameter), and d90] of the particle diameter distribution as well as the zeta-potential of the particles, in volume- and number-based cumulative fractions. The 50% and 90% diameter of PSt(MA/β-CD) in number-based distribution were 1.7 and 3.2 μm, respectively. These results suggested that the number of the aggregated microspheres were <10%.

To determine the amounts of the carboxyl groups, potentiometric titration was carried out for the aqueous suspension of PSt(MA). The titration curve for the PSt(MA) suspension in NaOH solution was obtained from the automatic titrator. The titration fraction (F_t) value at a given pH is expressed as,

$$\mathrm{pH} = \mathrm{p}K_a + \log\left(\frac{F_t}{1-F_t}\right) \quad (1)$$

where the acid dissociation constant (pK_a) of polyMA is equal to the pH at $F_t = 0.5$. The equivalent point ($F_t = 1$) and half-equivalent point ($F_t = 0.5$) were determined from the titration curve. The immobilized MA per unit weight and the pK_a of polyMA were 4.2×10^2 μmol/g-dry PSt(MA) and $pK_a = 7.3$, respectively. Thereafter, the β-CDs possessing ethylenediamine moieties were coupled with the carboxyl groups via amide bonding. The amount of the immobilized β-CD was calculated to be 192 μmol/g-dry PSt(MA/β-CD).

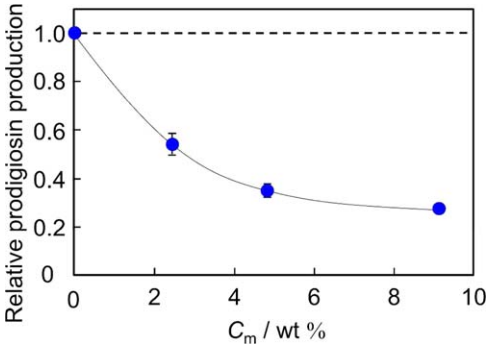

Figure 5. Suppression of prodigiosin production in *S. marcescens* AS-1 using PSt(MA/β-CD). Cells were grown at 25°C for 15 h with shaking. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Suppressive Effects of Immobilized CDs on QS in *S. marcescens* AS-1

The synthesized microspheres were dispersed in the culture medium when *S. marcescens* AS-1 was inoculated. *Serratia marcescens* is a bacterium indigenous to soil and the environment. The activation of QS could be visually monitored because AHL-mediated QS induces production of the antibacterial pigment prodigiosin, which causes the culture broth to turn red. AHL is a common signaling molecule in QS and is produced in many Gram-negative bacteria. Accordingly, polymer matrices showing high affinity to AHL are expected to be highly versatile materials that can be applied to various bacterial species.

Figure 5 shows that the immobilized β-CDs had suppressive effects on prodigiosin production. Compared with the control (without any microspheres), the relative level of prodigiosin production effectively decreased with increasing amounts of CD. Production of prodigiosin was reduced to approximately 0.28 in the presence of PSt(MA/β-CD).

Suppressive Effects of Immobilized CDs on QS in *P. aeruginosa*

Pseudomonas aeruginosa possesses two different QS systems (*rhlA* and *lasB*), which are activated by different AHL molecules.[15] The production of the green-pigmented pyocyanin is regulated by the *rhl*-QS system and is mediated by C4HSL. To investigate the interaction of immobilized β-CDs with more hydrophilic AHL signals, 2.5% (w/w) PSt(MA/β-CD) was dispersed in the LB medium to adjust the β-CD concentration to 5 mM, as in the case of *P. aeruginosa*. After an 18 h culture at

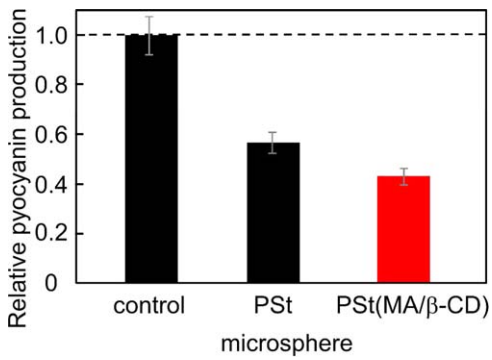

Figure 6. Suppression of pyocyanin production in *P. aeruginosa* AS-3 in the presence of 2.5% (w/w) microspheres. The concentration of the immobilized β-CD corresponded to 5 mM. Cells were grown at 30°C for 18 h with shaking. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

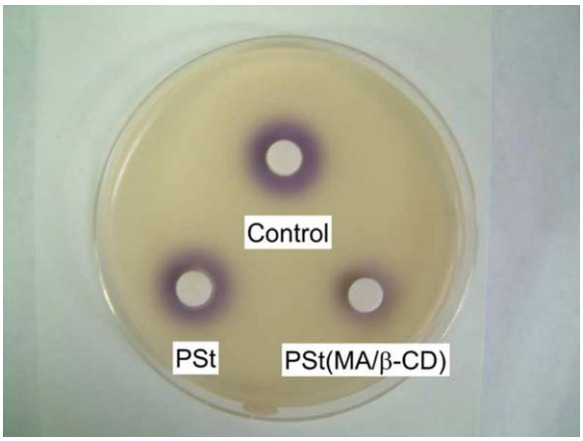

Figure 7. Bioassay for AHL extracts from the culture broth of *P. aeruginosa* AS-3 with or without (control) microspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

30°C, the production of pyocyanin was determined by measuring the absorbance of the acidified supernatant (Figure 6). Relative pyocyanin production was found to decrease with the addition of PSt(MA/β-CD); however, suppressive effects were also observed for PSt without CDs. These results suggested the possibility of a nonspecific adsorption of C4HSL on the PSt surface. A previous study showed that 5 mM free β-CD was required to suppress the relative pyocyanin production to approximately 0.6.[12] This result suggests that the complexation ability of β-CD was not greatly affected by immobilization on the hydrophilic shell layer of the core/shell particles. The stability constant (K) of C6HSL with immobilized β-CD was previously studied using the quartz crystal microbalance (QCM).[26] A drastic decrease in the resonance frequency on the CD-modified QCM electrode under alternating voltage was observed on adding C6HSL, and the association constant of the immobilized β-CD with C6HSL was determined to be approximately $(7 \pm 2) \times 10^2$ [M^{-1}]. It is plausible that the weaker hydrophobic interactions of C4HSL were responsible for the smaller QS suppression effects observed. However, the decrease in pyocyanin observed in this study suggested that immobilized β-CD and C4HSL signals interacted in the culture broth.

Change in AHL Content

To detect the difference in AHL concentration in the presence of microspheres before and after immobilization of CDs, we used the CV026 assay. The AHL extracts from the culture broth were spotted onto the agar gel plates containing AHL-reporter CV026 cells. Different amounts of AHLs result in differences in pyocyanin production, which are displayed as shades of purple on gel plates. Figure 7 clearly shows that small purple spots appeared with PSt(MA/β-CD). Thus, the AHL bioassay confirmed the effective interaction between the added β-CDs in the culture broth and the AHLs produced by cells.

CONCLUSIONS

β-CD-modified core/shell type microspheres were successfully synthesized to suppress AHL-mediated cell-to-cell communication in bacterial cells. The β-CD-immobilized polymeric microspheres show great potential to suppress virulence genes in various Gram-negative bacteria possessing an AHL-mediated QS system. Quenching of the QS signal by effectively trapping AHLs on CDs was confirmed in a bioassay with the AHL-negative mutant, *C. violaceum* CV026.

ACKNOWLEDGMENTS

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