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Novel Alkyl Thioether Cyclodextrin Derivatives Against Bacterial Quorum Sensing in *Aliivibrio fischeri*

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Abstract

Aliivibrio fischeri is a heterotrophic marine bacterium capable of bioluminescence which is controlled by quorum sensing. Quorum sensing is a population density dependent communication method for the bacteria to coordinate their activities by producing and detecting low-molecular-weight signal molecules. The effective infectivity of the bacteria is based on various virulence factors controlled by quorum sensing. Cyclodextrins, a family of toroidal-shaped oligosaccharides, reversibly encapsulate in their hydrophobic interior the fatty acid acyl chain of the signal molecules, thus preventing their binding to the receptors and interrupting the bacterial communication consequently the expression of various properties including different virulence factors.

Short-term tests were performed to examine the potential quorum quenching ability of newly prepared cyclodextrin derivatives: α- and β-cyclodextrins monosubstituted with alkylthio moieties. In these derivatives one of the modifiable hydroxyl groups of the cyclodextrin molecule is substituted with an alkylthio group of six, ten, twelve or sixteen carbon atoms. Cyclodextrins at 1.563–12.5 mM concentrations were added to *A. fischeri* culture in exponential growth phase, then the bioluminescence intensity, the population growth and the cell viability were kept under systematic review.

According to our results, the cyclodextrins have inhibitory effect on the quorum sensing system of *A. fischeri,* the alkylthio-substitution improves this effect compared to the native molecules and the length of the alkyl chain has significant influence on the quorum quenching efficiency.

Keywords

Aliivibrio fischeri; alkyl thioether cyclodextrins; bioluminescence; quorum sensing; quorum quenching

Introduction

Even though, antibiotics are one of the greatest discoveries in medical sciences, the increasing rates of bacterial resistance to current antibiotics and the scarcity of novel antibiotics resulted in considerable interest in novel treatment strategies targeting challenging bacterial infections caused by multidrug-resistant strains [1]. The microbial species that can form biofilms, like *Pseudomonas aeruginosa* [2,3], cause an outstanding problem. In recent years, the repurposing of "old" drugs for new clinical applications has become a major research area in drug discovery [4] as innovative treatment methods targeting bacterial virulence are expected to exert a weaker selection for resistance than conventional antibiotics [1,5].

The virulence ability of bacterial pathogens mainly relies on their capacity to modulate the expression of virulence factors through a complex network [4] based on their cooperative activities through releasing, sensing, and responding to various signal molecules [6]. This mechanism is called quorum sensing (QS), the detection and response to population density by activating different types of genes. QS allows a population of bacteria to behave as a multi-cellular organism in host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments [7, 8].

Quorum sensing was originally defined for the heterotrophic marine bioluminescent bacterium, *Aliivibrio fischeri* in the early 1970s [9]. Since then, the mechanism of bioluminescence has been described in detail, the genes required for their control and the main QS signal, N-3-oxohexanoyl-L-homoserine lactone was determined; *A. fischeri has* become one of the most frequently used model organisms for QS studies [10-12].

Gram-positive and -negative bacteria produce different kinds of signal molecules to induce QS. Gram-positive species usually express autoinducer peptides (AIH), while Gram-negatives mainly produce N-acyl-homoserine lactone (AHL) type signals, where the length of the side chain varies between 4 to 18 carbon atoms, and in the substitution on the β-position [13]. Some examples of AHLs are depicted in Figure 1.


side chain (R)	name
-CH$_2$CO(CH$_2$)$_{10}$CH$_3$	*N*-(3-Oxotetradecanoyl)-L-homoserine lactone
-CH$_2$(CH$_2$)$_3$CH$_3$	*N*-Hexanoyl-L-homoserine lactone
-(CH$_2$)$_2$CH$_3$	*N*-Butyryl-DL-homoserine lactone
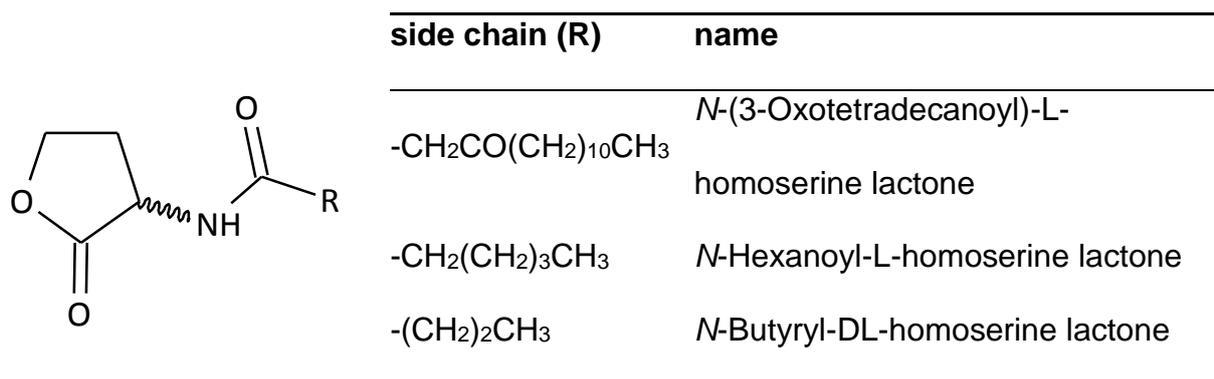
Figure 1: Some examples of AHLs.

Any compound that prevents the production of these signal molecules or the interaction between these molecules and related receptor proteins can block QS and related gene expression. The effects of several potential QS inhibitors (furaneol acetate, DL-pyroglutamic acid, L–prolinol, D-prolinol, sulfamethoxypyridazine, (Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone, sulfonylureas) were investigated in the *A. fischeri* model system applying the bioluminescence inhibition bioassay [14-16]. However, in the case of *A. fischeri,* literature is scarce on the quorum quenching (QQ) effects of cyclodextrins (CDs) that are cyclic oligosaccharides composed of α(1,4)-linked D-(+) glucopyranose units, with a hydrophobic inner cavity and hydrophilic outer shell. Their prominent representatives are the α-, β- and γ-CDs with six, seven or eight glucopyranose units [17]. They can form inclusion complexes with several molecules [18].

The complexation of signal molecules by CDs opens the way for the development of CD-based signal traps to suppress QS [19].

The effect of CDs on QS pathways in *Serratia marcescens, Chromobacterium violaceum, Aliivibrio fischeri* or *Pseudomonas aeruginosa* model systems has been studied [20-22] (Ikeda *et al.*, 2002, Morohoshi *et al.*, 2013, Zhang and Li, 2016). The QQ effect of different CD derivatives in the *A. fischeri* bioluminescence model system was first demonstrated by Molnár *et al.* [23]. This systematic study with twelve different CDs revealed high quorum quenching effect (64% inhibition of bioluminescence) for α-CD in 10 mM concentration after 120 min exposure time [23]. Further several α- and β-CD derivatives proved to be efficient CD-based signal traps resulting in quorum quenching [21, 23-31]. The most promising ones are 6-monosubstituted alkylamino CD derivatives, where the association constants increase with an increasing chain length of the alkylamino moiety [21]. These molecules, however, have low water solubility due to their alkyl side chains.

The present work aimed to develop water-soluble CD derivatives with a higher complexation rate to AHLs, and an easier synthetic approach than the preparation of the alkylamino derivatives. CDs were monosubstituted with thioalkyl moieties applying good leaving groups such as bromide for α- and γ-CDs and tosyl for β-CD following the synthetic strategies for the preparation of 6-monosubstituted derivatives reviewed by Kasal and Jindrich [32]. CDs were further substituted randomly with quaternary amino (QA) moieties for solubility enhancement. Bacterial cell walls contain different types of negatively charged molecules due to the dissociation of acidic groups such as carboxyl and phosphate, so compounds with positive charges may penetrate the cell wall disturbing its function [33, 34].

Thus, the QA-substitution of CDs can not only modify their pharmacokinetic properties but also provide a new possible attacking point against bacteria.

Here we report the design, synthesis, and evaluation of a series of α-, β- and γ-CD derivatives containing each only one alkylthio ether side chain and several quaternary amino moieties. Their structure is demonstrated in the example of *N,N,N*-trimethyl-aminopropyl-(6-monodeoxy-6-monodecylthio-ether)-β-CD in Figure 2.
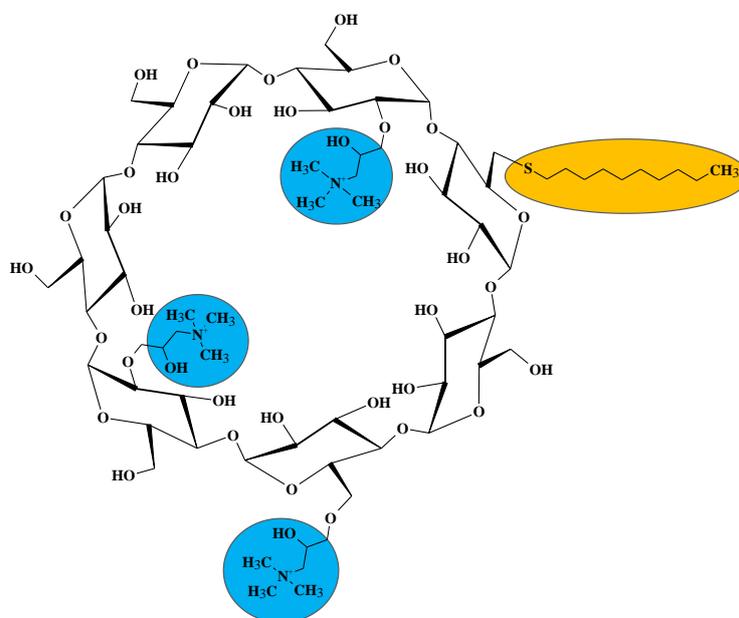

Figure 2: Structural formula of QA-(6-monodeoxy-6-monodecylthio ether)-β-CD.

The potential concentration- and time-dependent quorum quenching (QQ) ability of selected cyclodextrin molecules was also investigated, as these CD derivatives may interfere with the control mechanisms of bioluminescence by *A. fischeri* without directly affecting bacterial viability. In support of this concept, the cytotoxic effect was also monitored in addition to the effect of the studied CDs on the QS system. Modulation of the QS control mechanisms of bioluminescence in *A. fischeri* by thioalkyl and quaternary amino moieties substituted CDs compared to native α-, β-CDs and a systematic, comprehensive approach is unique in the scientific literature.

Results and Discussion

Synthesis of random quaternary amino 6-monodeoxy-6-monoalkylthio CD derivatives

The following products were synthesized: 6-monotosyl-beta-CD was reacted with 1-decanthiol in *N,N*-dimethylformamide using sodium methoxide under inert atmosphere to get 6-monodeoxy-6-mono-decylthio-β-CD. Similar reactions were performed to obtain 6-monodeoxy-6-mono-hexylthio-, 6-monodeoxy-6-mono-dodecylthio- and 6-monodeoxy-6-mono-hexadecylthio-β-CD. The products were purified by chromatography when necessary and reacted with glycidyltrimethylammonium chloride to incorporate positive charge (quaternary amino group = QA) into the molecule to get QA-6S-C6-BCD, QA-6S-C10-BCD, QA-6S-C12BCD and QA-6S-C16-BCD (Figure 3). For comparison QABCD was also prepared.
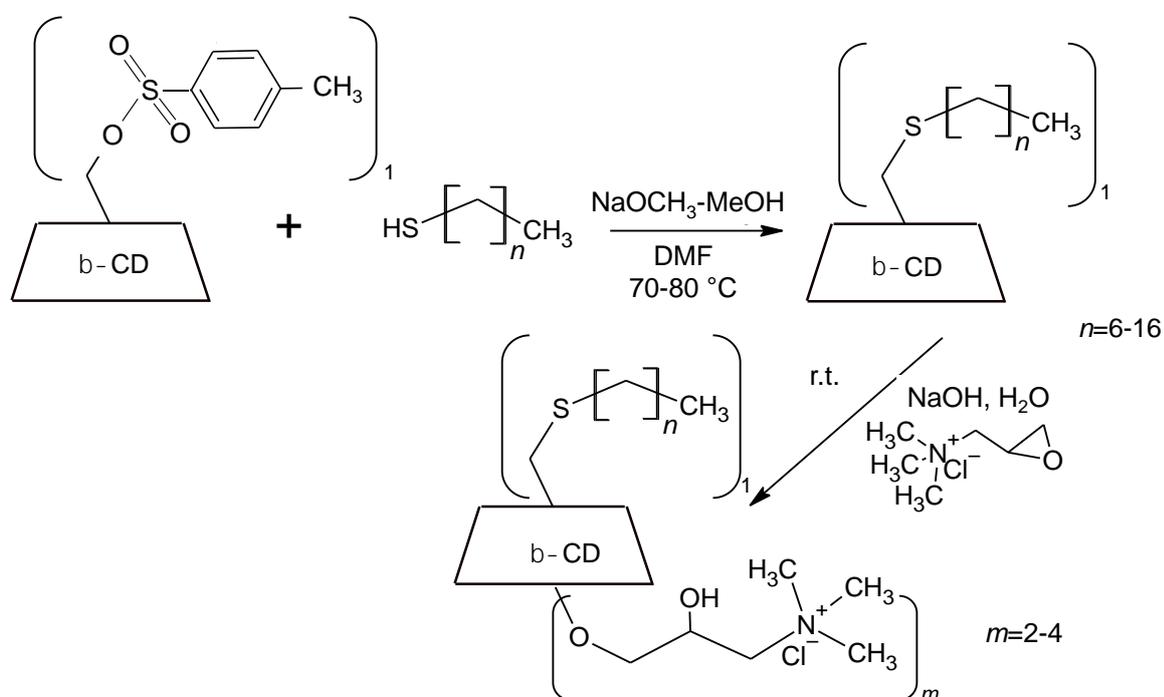

Figure 3: Reaction route for preparing β-CD alkyl thioether derivatives and their substitution with *N,N,N*-trimethylpropyl moieties.

The preparation of the α-/γ-CD series followed different reaction route (Figure 4): Native CDs were reacted with *N*-bromosuccinimide and triphenylphosphine to obtain 6-monobrominated CDs. The strict control of the temperature allows the introduction of one single unit of halogen on the primary rim of the CDs. The 6-monodeoxy-6-monoalkylthio CD derivatives were obtained by reacting 6-monobromo-CDs with the proper alkanethiol with sodium methoxide in dimethylsulfoxide (DMSO). By using an excess of thiol, the conversion was quantitative and the reaction crudes could be purified by crystallization from methanol. This way, 6-monodeoxy-6-monohexylthio, 6-monodeoxy-6-monododecylthio and 6-monodeoxy-6-monohexadecylthio ACD and GCD were prepared. Permanent positive charges (quaternary amino, QA groups) were randomly installed on the 6-monodeoxy-6-monoalkylthioether CD derivatives in aqueous alkaline conditions with glycidyltrimethylammonium chloride.

The insertion of positive charges on the CD scaffolds increased remarkably the aqueous solubility of the small library of CD derivatives thus allowing an exhaustive purification by dialysis. QA-(6-monodeoxy-6-monohexylthio), QA-(6-monodeoxy-6-monododecylthio) and QA-(6-monodeoxy-6-monohexadecylthio) ACD and GCD (QA-6S-C6-ACD, QA-6S-C6-GCD, QA-6S-C12-ACD, QA-6S-C12-GCD, QA-6S-C16-ACD, QA-6S-C16-GCD, 5 g of each) were obtained and their structures were elucidated by NMR and MS. For comparison QAACD was also prepared.
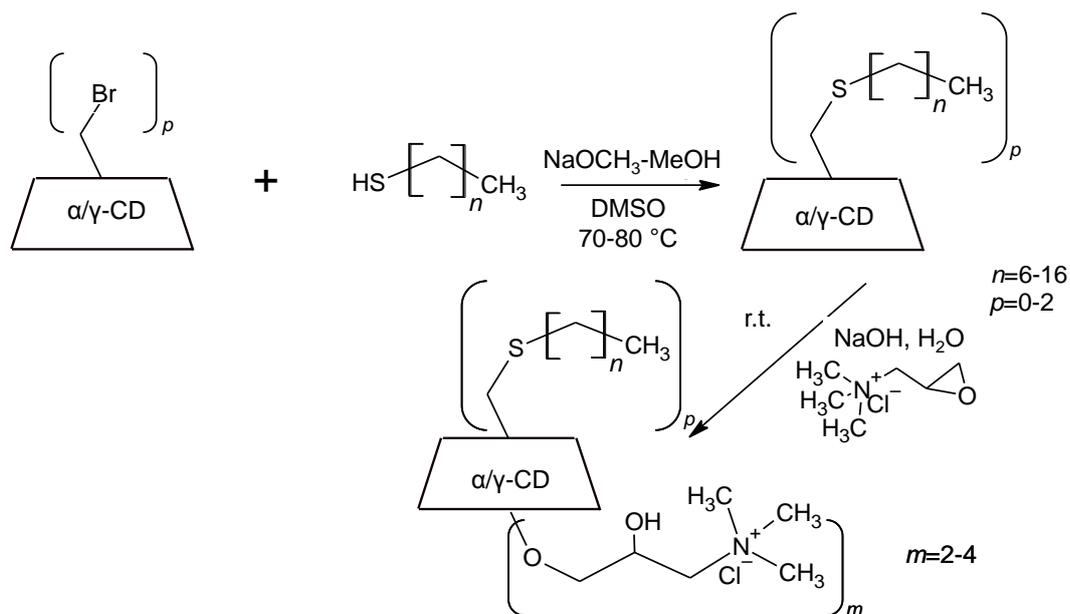
Figure 4: Reaction route for preparing α- and γ-CD alkyl thioether derivatives substituted with *N,N,N*-trimethylpropyl moieties.

Structure elucidation by NMR

As an example, the NMR study of QA-6S-C6-ACD can be seen in Figures 5-6. In the ^{1}H NMR spectrum five well defined regions can be detected:

- At around 5.2 ppm the signals of the anomeric protons can be seen.

- At around 4.5 ppm are the methine signals of the quaternary amino groups.

- Between 4.2 and 3.4 ppm the core region of the cyclodextrin ring, the methylene signals of the quaternary amino side chains and the two methylene protons, which are in the vicinity of the sulfur atom, can be detected.

- At around 3.3 ppm the high intensity peaks of the methyl groups composing the quaternary amino moieties can be seen.

- Between 1.6 and 0.9 ppm the proton signals of the aliphatic side chain are promptly assigned.

- In the ^{1}H NMR spectrum three residual solvent peaks can be distinguished: at 4.76 ppm is the HDO (used as internal standard), at 2.85 ppm DMSO and at 2.22 ppm the residual signal of acetone can be seen.

Figures 5 and 6 show the full assignment of the QA-6S-C6-ACD. The chemical shifts are listed in S1 Table in Supplementary Information.
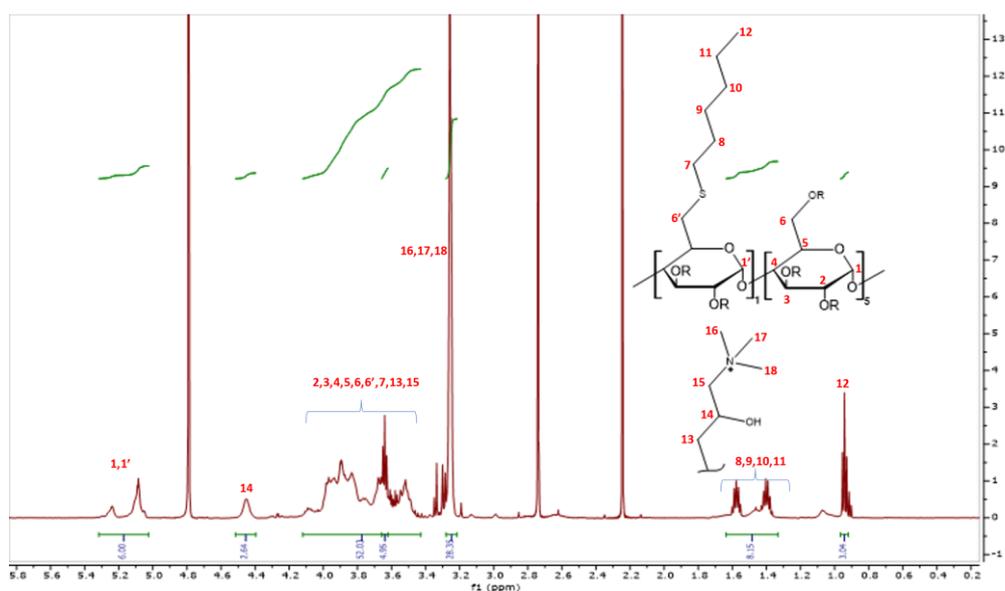

**Figure 5:** ^{1}H NMR spectrum of QA-6S-C6-ACD (D$_2$O, 600 MHz)
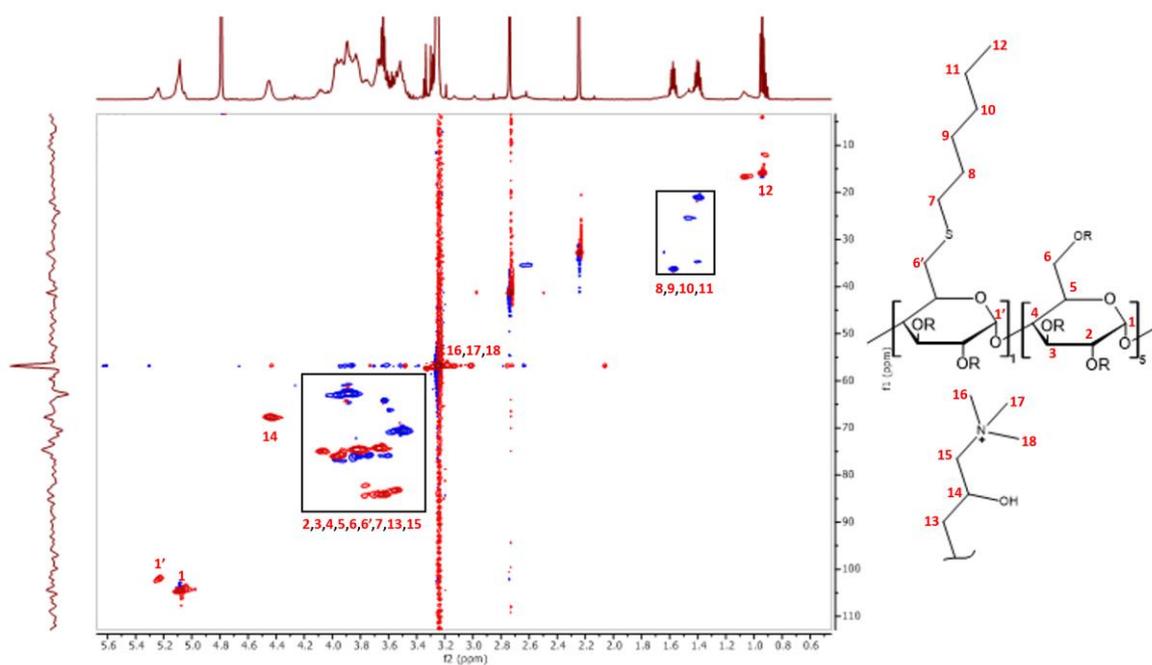
Figure 6: DEPT-edited HSQC spectrum of QA-6S-C6-ACD (D$_2$O, 600 MHz)

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In Figure 7, the MALDI spectrum of QA-6S-C6-ACD is shown. The main peak can be assigned to the sodium adduct of 6-monodeoxy-6-monohexyl-ACD substituted with 3 QA moieties.
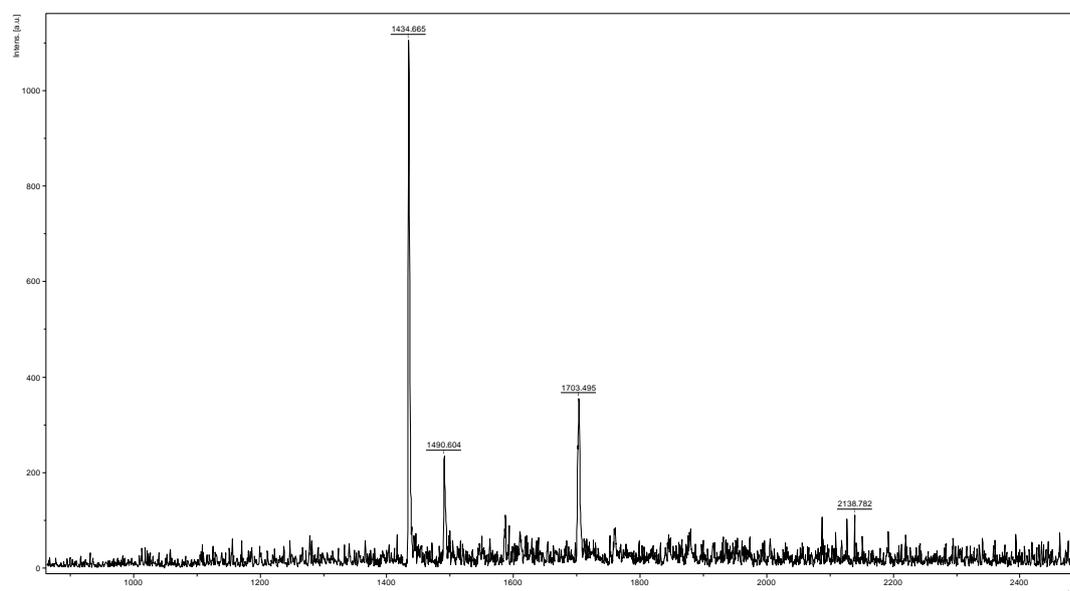
Figure 7: MALDI spectrum of QA-6S-C12-ACD

Aggregation behavior

Aggregation behavior of CDs is an important factor in their mechanism of binding to guest molecules [35] (Loftsson 2014). The volume particle size distribution curves of an ACD and 2 BCD derivatives are illustrated in Fig. 8. The size (i.e., hydrodynamic equivalent spherical diameter) of the non-aggregated CDs is approx. 1.5 nm. In the solutions of the QA-alkylthio-CD derivatives only aggregated species can be identified. The alkylthio derivatives show a tendency for aggregation in accordance with the size of the alkyl chains. The QA-6S-C10-ACD formed small-sized aggregates of 8-10 nm (average 8.2 nm). The QA-6S-C10-BCD showed bimodal distribution of aggregates: in addition to the small particles of 8–10 nm average diameter also larger particles of approx. 50–500 nm size (average 163 nm) can be found.

The QA-6S-C16-BCD sample exhibited even more pronounced self-association: only large-sized aggregates of 50–500 nm (average 111 nm) can be found. The GCD series showed similar behavior: increasing affinity to aggregation with increasing alkyl chain length.
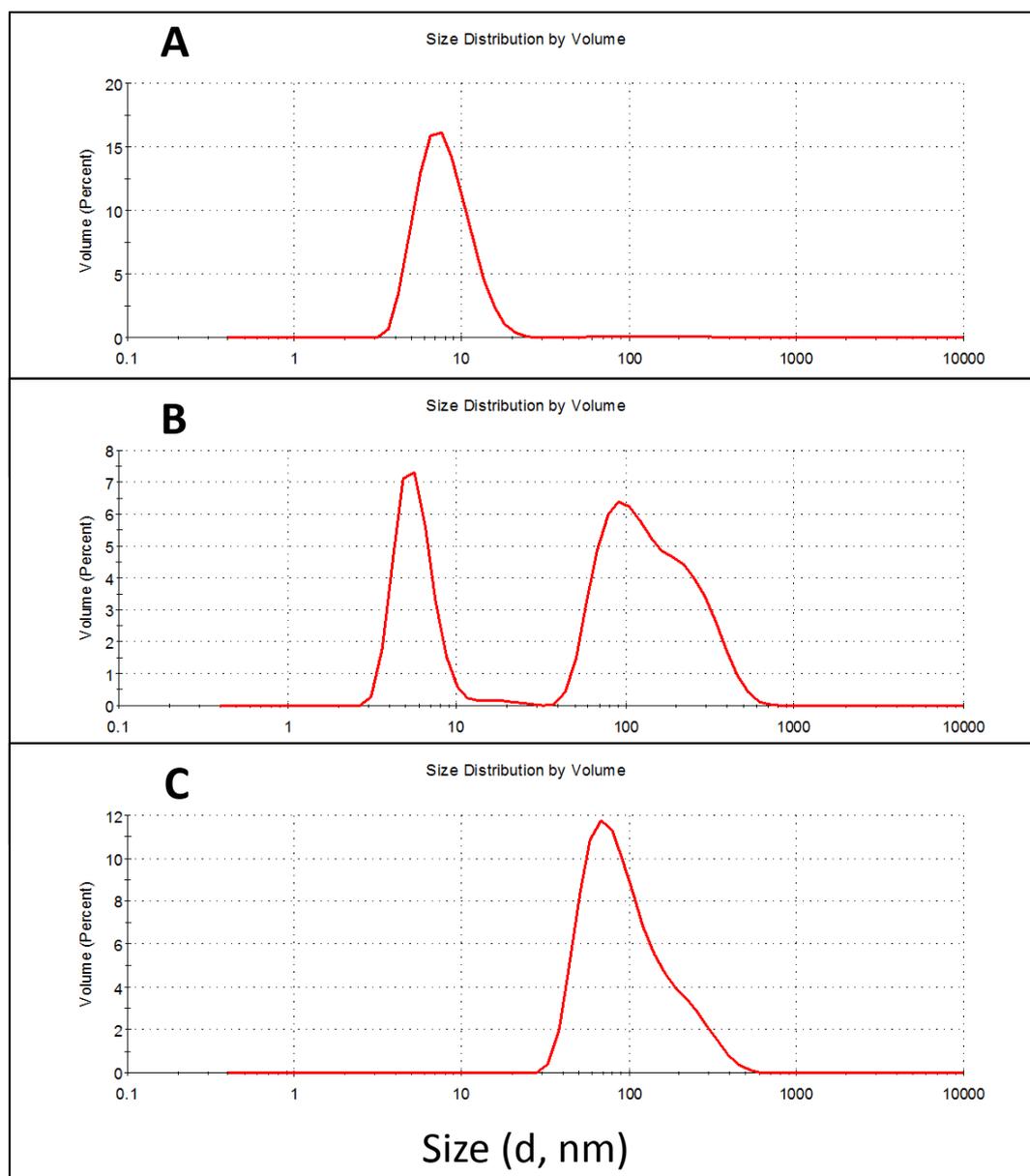

Figure 8: Size distribution of aggregates in 1% solutions of the new BCD derivatives (average of 5 parallel measurements) A: QA-6S-C10-ACD; B: QA-6S-C10-BCD; C: QA-6S-C16-BCD.

The C16 derivatives of ACD and GCD aggregated so profoundly that no clear solutions could be obtained in water. The other derivatives showed similar size distribution curves. It should be noted, however, that the derivatives with longer alkyl chain often gave hazy solutions due to formation of large aggregates.
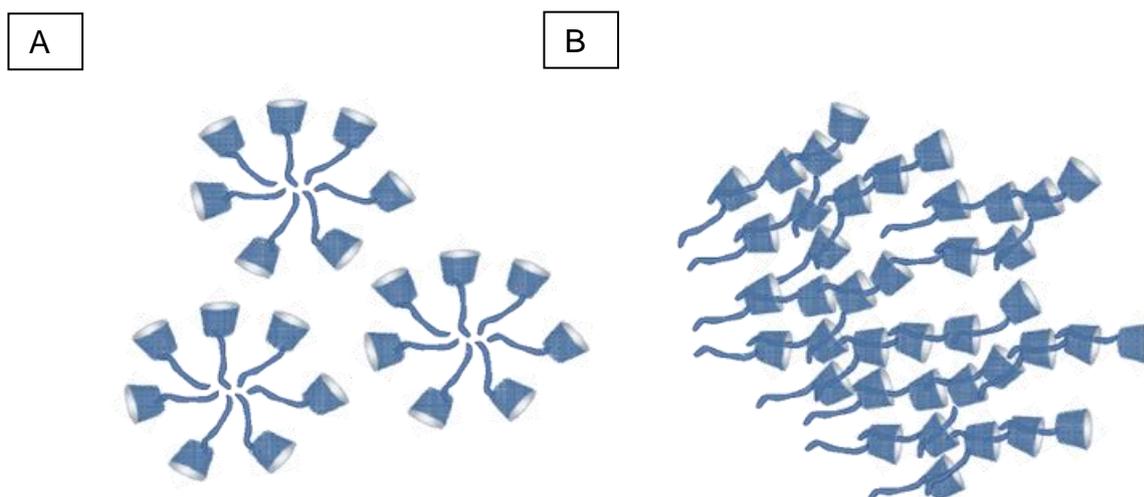
Figure 9: Self-assembly of 6-monodeoxy-6-monoalkylthio derivatives into small micelle like aggregates (A) and into larger sized aggregates (B) *via* supramolecular interactions.

Bioluminescence Inhibition Assay

Effect of α-cyclodextrins on the *Aliivibrio fischeri* model system

The potential quorum quenching (QQ) effect of ACD, QAACD, and their alkyl thioether derivatives (6S-C12-ACD, QA-6S-C10-ACD) on the *A. fischeri* model organism was determined through the measurement of the bioluminescence intensity, the population growth, and the viability, applying transparent 96-well microtiter plates.

The increasing concentrations of ACD, QAACD, 6S-C12-ACD, and QA-6S-C10-ACD affected the bioluminescence, as it is presented in Figure 10.
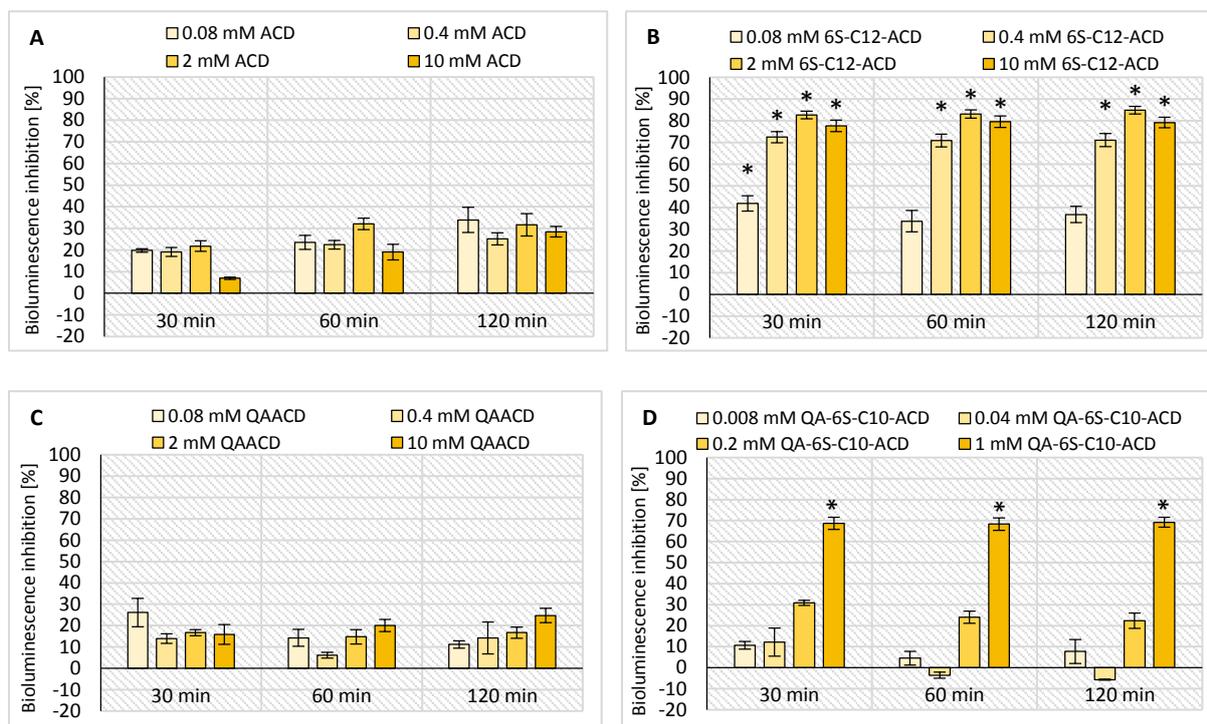
Figure 10. Effect of ACD (A), 6S-C12-ACD (B), QAACD (C), and QA-6S-C10-ACD (D) on the bioluminescence emission after 30, 60, and 120 minutes of exposure time. Significant inhibition compared to control is marked by an asterisk (*) ($p < 0.05$). Data represent averages of four replicates.

As illustrated in Figure 10, the CD concentration, the exposure time, and the alkyl thioether substitution significantly influenced the QQ effect of the tested α-CDs. The highest inhibition (85%) was exerted by 2 mM 6S-C12-ACD after 120 minutes, but this CD proved very effective after 30 and 60 minutes of exposure (in the 0.4–10 mM concentration range), too. The inhibition was also significant at 1 mM QA-6S-C10-ACD concentration (68%) at each time point, but no significant inhibition was observed in the case of the ACD and QAACD in the examined period. In the case of the alkyl thioether derivatives, increasing inhibition in proportion to time was measured,

suggesting the contribution of other influencing parameters in the test system and the decreased availability of the signal(s).

The results of repeated measures variance analysis (RM ANOVA) of ACD, 6S-C12-ACD, QAACD, and QA-6S-C10-ACD (Table 1) also demonstrated the significant QQ efficiency of these CDs.

Table 1. RM ANOVA results over time to evaluate the effect of ACD, QAACD, and their alkylthio-substituted derivatives on the bioluminescence intensity. Bold numbers indicate significant differences at $p < 0.05$.

Source of Variation	Df[1]	MS[2]	F[3]	p[4]
ACD				
ACD treatment	4	7.94×10^7	0.51	0.73
Time	**2**	**7.57×10^9**	**570.07**	**0.00**
Time x ACD treatment	**8**	**8.12×10^7**	**6.12**	**0.00**
6S-C12-ACD				
6S-C12-ACD treatment	**4**	**7.75×10^9**	**88.86**	**0.00**
Time	**2**	**1.54×10^9**	**186.96**	**0.00**
Time x 6S-C12-ACD treatment	**8**	**1.43×10^8**	**17.37**	**0.00**
QAACD				
QAACD treatment	4	1.02×10^8	0.62	0.65
Time	**2**	**4.48×10^9**	**322.40**	**0.00**
Time x QAACD treatment	**8**	**4.27×10^7**	**3.07**	**0.00**
QA-6S-C10-ACD				
QA-6S-C10-ACD treatment	**4**	**2.60×10^9**	**22.00**	**0.00**
Time	**2**	**2.87×10^9**	**353.13**	**0.00**
Time x QA-6S-C10-ACD treatment	**8**	**5.78×10^7**	**7.10**	**0.00**

[1] Degree of freedom; [2] mean square; [3] F-ratio, [4] *p*-value.

In the case of the alkyl thioether derivatives, both the contact time and the CD treatment affected the bioluminescence of the bacteria. In the case of the ACD and the QAACD, the exposure time and the combined effect of the exposure time and the CD treatment also significantly affected the bioluminescence intensity.

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Effect of α-cyclodextrins on cell viability

The optical density values (from which we deduced the cell growth in the population) were measured spectrophotometrically after 30, 60, and 120 minutes of incubation time. Regarding the results (data not shown), ACD, QAACD, and QA-6S-C10-ACD caused a maximum of 28% inhibition (regardless of the tested concentration) of the optical density of the cell suspension after 30 and 60 minutes. In contrast, 6S-C12-ACD caused a maximum of 50, 54, and 59% inhibition (after 30, 60, and 120 minutes of exposure, respectively), increasing proportionally with concentration. This result draws attention to the potentially detrimental effect of CDs on the viability of the cells, which might influence the QS process. For this reason, the possible cytotoxic effect of CDs was also determined using the cell viability assay.

The cell viability assay characterizing enzyme activity is based on the spectrophotometric measurement of a formazan product after 120 minutes of exposure, whose absorbance is directly proportional to the metabolic activity of the cells. According to the results, 10 mM of ACD and 2 mM of QAACD resulted in 29 and 21% inhibition in the viability of *A. fischeri*. However, in the case of 6S-C12-ACD, we measured a maximum 40% (at 10 mM) decrease in cell viability, increasing proportionally with the concentration; and QA-C10-ACD caused 24-27% inhibition of the enzyme activity, regardless of concentration. These results (population growth and enzyme activity) indicated that the outstanding QQ effect of the novel alkyl thioether derivatives is partly due to their cytotoxic nature. Further studies are necessary to assess the extent and nature of the cytotoxic effect, including examining the reactive oxygen species and the integrity of the cell membrane.

Effect of β-cyclodextrins on the *Aliivibrio fischeri* model system

Similarly to the α-CDs, to examine the potential QQ effect of BCD, QABCD, 6S-C12-BCD, QA-6S-C10-BCD, and QA-6S-C16-BCD on *A. fischeri*, the bioluminescence intensity, the population growth, and the viability were measured after 30, 60, and 120 minutes of exposure time. The increasing concentrations of BCD, QABCD, 6S-C12-BCD, QA-6S-C10-BCD, and QA-6S-C16-BCD affected the bioluminescence, as it is presented in Figure 11.

In the case of the studied β-CDs, overall, it can also be stated that the QQ effect was significantly affected by the cyclodextrin concentration, exposure time, and alkyl thioether substitution. However, the degree of inhibition was generally lower than that of the α-CDs.

Among the studied β-CDs, QA-6S-C10-BCD exhibited the highest significant QQ effect (86%) at 5 mM concentration after 120 min contact time. However, at lower concentrations (in the 0.04–1 mM concentration range), this alkyl thioether derivative had a slight (statistically not significant) stimulatory effect (a maximum of 21%) on the bioluminescence emission after 30 and 60 minutes of contact time. In addition, the 6S-C12-BCD derivative also exerted an outstanding, concentration-proportionally increasing inhibition at all three examined timepoint, with a maximum of 77% (after 30 minutes of exposure, at 2 mM concentration). However, the inhibitory effect did not exceed 35% for BCD and QABCD in any case, and these slight effects were not statistically significant.
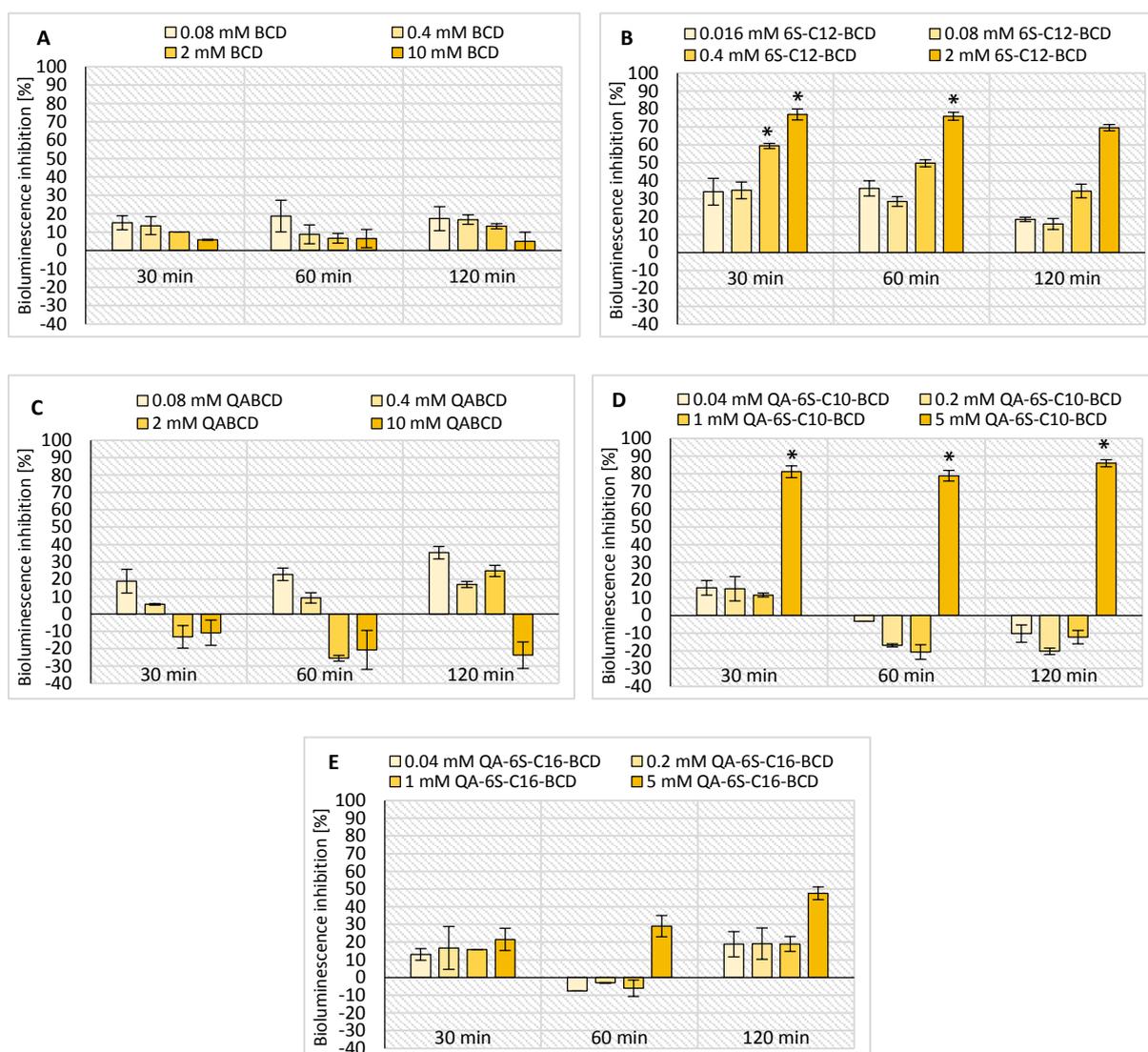
Figure 11. Effect of BCD (A), 6S-C12-BCD (B), QABCD (C), QA-6S-C10-BCD (D), and QA-6S-C16-BCD (E) on the bioluminescence emission after 30, 60, and 120 minutes of exposure time. Significant inhibition compared to control is marked by an asterisk (*) ($p < 0.05$). Data represent averages of four replicates.

As illustrated, the longer contact time (120 min) and the higher concentrations (0.4–5 mM) resulted in significant bioluminescence inhibition. It is also presented that the alkyl substitution increases the QQ effect in the case of the β-CDs as well, but this improving effect does not increase proportionally to the length of the alkyl chain.

This is strongly supported by comparing the effect of QA-6S-C10-BCD and QA-6S-C16-BCD derivatives on the bioluminescence of *A. fischeri*.

The results of the RM ANOVA analysis of BCD and its derivatives (Table 2) suggest that in the case of the 6S-C12-BCD, QABCD, and QA-6S-C10-BCD, the CD treatments significantly influenced the bacterial communication.

Table 2. RM ANOVA results over time to evaluate the effect of BCD, QABCD, and their alkyl-substituted derivatives on the bioluminescence intensity. Bold numbers indicate significant differences at $p < 0.05$.

Source of Variation	Df[1]	MS[2]	F[3]	*p*[4]
BCD				
BCD treatment	4	3.70×10^9	2.59	0.05
Time	**2**	**8.94×10^9**	**48.18**	**0.00**
Time x BCD treatment	8	9.67×10^7	0.52	0.84
6S-C12-BCD				
6S-C12-BCD treatment	**4**	**2.38×10^9**	**22.35**	**0.00**
Time	2	5.74×10^6	0.68	0.51
Time x 6S-C12-BCD treatment	**8**	**1.50×10^8**	**17.60**	**0.00**
QABCD				
QABCD treatment	**4**	**7.68×10^9**	**5.01**	**0.00**
Time	**2**	**6.25×10^9**	**40.00**	**0.00**
Time x QABCD treatment	8	1.89×10^8	1.21	0.31
QA-6S-C10-BCD				
QA-6S-C10-BCD treatment	**4**	**2.80×10^{10}**	**33.18**	**0.00**
Time	**2**	**2.53×10^9**	**15.48**	**0.00**
Time x QA-6S-C10-BCD treatment	8	2.59×10^8	1.58	0.15
QA-6S-C16-BCD				
QA-6S-C16-BCD treatment	4	2.85×10^9	2.44	0.07
Time	**2**	**5.32×10^9**	**33.89**	**0.00**
Time x QA-6S-C16-BCD treatment	**8**	**6.49×10^8**	**4.13**	**0.00**

[1] Degree of freedom; [2] mean square; [3] F-ratio, [4] *p*-value.

The exposure time also proved to be a significant influencing factor (except for 6S-C12-BCD), but at the same time, the combined effect of time and CD treatment proved to be significant in the case of 6S-C12-BCD and QA-6S-C16-BCD. Unlike ACDs, there were no cases in which all three factors – the exposure time, the CD treatment, and their combination – proved significant.

Effect of β-cyclodextrins on the Cell Viability

In the case of the population growth assay (the optical density values), BCD, QABCD, QA-6S-C10-BCD, and QA-6S-C16-BCD exerted inhibition, which proportionally decreased with concentration, after 30 minutes of exposure, with a maximum of 34, 34, 32, and 33%; at 0.08, 0.08, 0.04, and 0.04 mM respectively. The 6S-C12-BCD derivative showed a high degree of inhibition, proportionally increasing with the concentration. The maximum inhibitory effect on cell proliferation was 72%, after 30 minutes, at 2 mM concentration.

Based on the enzyme activity assay, after 120 minutes of exposure, BCD and QABCD had a minor (maximum 15%) inhibitive effect on cell viability at the lowest concentrations (at 0.08 and 0.4 mM). The highest inhibition (64%) was achieved by 5 mM QA-6S-C10-BCDPS after 120 minutes; however, a vitality-reducing effect (35 and 41%) was observed for 5 mM QA-6S-C16-BCD and 2 mM 6S-C12-BCD as well. Similar to ACDs, these results indicate a potential cytotoxic effect of the β-CDs, which may interfere with the QQ effect of the examined cyclodextrins and requires further investigations.

Comparison of the Effectiveness of the Tested Cyclodextrins

Some earlier studies have demonstrated the QQ effects of different CD derivatives in QS model systems other than *A. fischeri* [20, 25, 26, 30]. However, the effect of cyclodextrins has been scarcely studied with *A. fischeri*. In this study, a series of CD derivatives were designed and synthesized to improve the QS inhibitory activity of native CDs. The AHL-mediated bioluminescence of *A. fischeri* was drastically decreased by adding 10 mM 6-mono-dodecanthiol substituted α- and β-CD (6S-C12-ACD and 6S-C12-BCD) by 79% and 70%, respectively. The *N,N,N*-trimethyl-aminopropyl-6-mono-decanethiol substituted α- and β-CD derivatives (1 mM QA-6S-C10-ACD and 5 mM QA-6S-C10-BCD) also showed outstanding QS inhibitory effect of 69% and 86%, respectively.

EC_{20} and EC_{50} values also revealed that 6S-C12-ACD and 6S-C12-BCD were the most effective among the tested QS inhibitors (Table 3).

Table 3. Effective Concentrations (EC_{20}, EC_{50}) of α- and β-CDs caused 20% and 50% inhibition of bioluminescence after 120 min exposure.

	Effective Concentrations [mM] – 120 min				
	ACD	6S-C12-ACD	QAACD	QA-6S-C10-ACD	
EC_{20}	0.08	0.06	3.14	0.20	
EC_{50}	nd	0.16	nd	0.24	
	BCD	6S-C12-BCD	QABCD	QA-6S-C10-BCD	QA-6S-C16-BCD
EC_{20}	nd	0.09	nd	2.19	0.17
EC_{50}	nd	0.76	nd	2.26	nd

*nd: not determined due to non-conventional concentration-response relationship.

An improvement in the QS inhibitory activity was also observed when applying both *N,N,N*-trimethyl-aminopropyl and mono-decanethiol groups on ACD and BCD parent molecules when administered in higher concentrations (1 and 5 mM, respectively).

At the same time, the longer alkyl chain of QA-C16-BCD did not result in further improvement of the QS inhibitory effect; on the contrary, 1 mM QA-C16-BCD resulted in a lower inhibitory effect of 48%.

In our previous study [23], the concentration- and time-dependent bioluminescence inhibitory effect of nine CDs was monitored in the *A. fischeri* model system. 2-hydroxypropyl (HPACD, HPBCD), random-methylated (RAMEA, RAMEB, RAMEG), trimethyl-aminopropyl (QAACD), sulfobutyl-ether (SBEBCD) derivatives and their epichlorohydrin-crosslinked polymers (ACDPS and BCDPS) were tested besides the native α-, β- and γ-cyclodextrins (ACD, BCD, and GCD). It was found that the QQ efficiency of the tested CDs was influenced by the size of the interior cavity, their structure, the concentration, as well as the exposure time. In terms of the interior cavity size, only ACD, BCD, and their derivatives were able to efficiently inhibit the bioluminescence of *A. fischeri*, while GCD proved to be ineffective, which can be explained by the better fitting of the alkyl chain of the signaling molecule of *A. fischeri* (3-oxo-C6-HSL) into the narrow cavities of ACD and BCD than into that of GCD. It was also revealed that the 2-hydroxypropyl and trimethyl-aminopropyl substituted or the random-methylated α-CD derivatives did not improve the QQ ability of native ACD. On the contrary, in our recent study, a significant improvement of QQ ability was achieved by the 6-mono-dodecanthiol substitution of ACD (6S-C12-ACD). In our previous study [23], the random methylation and the 2-hydroxypropyl substitution of BCD did not improve the QQ ability of BCD, while SBEBCD exhibited a slightly higher quorum quenching effect than native BCD. On the contrary, carrying out random *N,N,N*-trimethyl-aminopropyl and 6-mono-decanethiol substitution on β-CD (QA-6S-C10-BCD) resulted in a tremendously high level of bioluminescence inhibition.

In the Gram-negative bacterial QS system of *Serratia marcescens*, Morohoshi and colleagues [21] observed the improvement of the QS inhibitory activity compared to native ACD, BCD, and GCD molecules in the case of 6-alkylamino-α- or γ-CDs and 2-alkylamino-CDs. In addition, 6,6′-dioctylamino-β-CD, which contains two octylamino groups, exhibited greater inhibitory activity than 6-mono-octylamino-β-CD. It was also revealed that the synthetic alkylamine-modified CD derivatives had higher equilibrium binding constants for binding with AHL than the native CDs. Strong QS inhibitory effects of the synthesized CD derivatives were also reported by Morohoshi *et al.* [21] in other Gram-negative bacterial QS systems, such as *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. However, by the *S. marcescens* prodigiosin production assay, the *C. violaceum* violacein production assay, and the *P. aeruginosa* elastase assay, the QQ effects of four β-CD derivatives were compared, but thorough investigation of the concentration- and time-dependence was not carried out. All tested CD derivatives were applied at 10 mg/mL concentration with a relatively long exposure (18–20 h) compared to the maximum 120 min exposure in our case. Another important difference in the experimental methodology is that in the study of Morohoshi *et al.* [21], the inoculated cell culture was grown together with the β-CD derivatives for 18–20 h, whereas in our case, a 16 h old (overnight) cell culture was applied for the tests. All these methodological alterations may result in different results compared to existing literature data.

Attaching a long alkyl chain to the CDs seems to be a good strategy to quench quorum sensing. Several fatty acids were found to mimic the signal molecules of various microbes and inhibit biofilm formation and virulence [36, 37]. Similar mimicking function might be postulated in case of the monoalkyl substituted CDs, too and this effect (occupying the receptors of the signals) combined with the trapping of the signals within

the CD cavity may result in effective quorum quenching agents against several microbes.

Conclusions

A possible strategy for inhibition of bacterial joint actions based on quorum sensing is trapping the signal molecules used by the bacteria for communicating the bacterial density.

Due to trapping these signal molecules cannot bind to the receptors and remain unperceivable for the microbial community. Special CD derivatives were synthesized which not only could serve as traps for the signal AHL molecules for the model organism *A. fischeri*, but also can mimic to some extent the structure of these signal molecules through their alkyl chains and bind to the AHL-receptors.

The autoinducer-dependent quorum sensing mechanism in *Aliivibrio fischeri* was markedly and significantly inhibited, the high quorum quenching effect of CDs was clearly demonstrated. The efficiency was influenced by several parameters; the size of the interior cavity, the structure and the concentration of CDs, as well as the contact time with the cells. It was found that the aggregation behavior is influenced by the alkyl chain length: at longer chain length both micelle-like aggregates and large nanoparticles are formed. In this work we studied the compounds with C10–C16 chain length but it seems to be worth to extend the investigations for the lower chain length region where both the solubility and aggregation could be more advantageous. An important conclusion of the present study is that although the presence of QA groups is important to improve the solubility of the molecules, it results in reduced QS inhibition potential.

The application of a cyclodextrin-trap for complexation of signal molecules may be a novel, promising method for influencing QS interfering strategies, for example, to enhance the efficiency of various biotechnologies, as well as to find alternative approaches against bacterial proliferation and infections. Furthermore, our results could also serve as a basis for further research with other bacterial or plant model systems, in which similar chemical signals may induce physiological responses.

Experimental

Synthesis of Cyclodextrins

The 6-mono-*O*-tosyl-BCD and DMF were weighed in a 3-neck round bottom flask under inert atmosphere and the proper alkylthiol and sodium methylate were added in sequence while stirring; the reaction mixture was then heated to 60 °C. The reaction was monitored by thin layer chromatography (TLC, eluent: dioxane:25% ammonia:*n*-propanol 10:7:3) and found to be completed within 2-4 h depending on the alkylthiol (the longer the alkyl chain the longer reaction time was needed). DMF was removed under reduced pressure at rotavapor and the yellow product was precipitated with methanol; the solid was filtered, extensively washed with methanol until a white solid was obtained and finally dried until constant weight in a vacuum drying box in the presence of KOH and P_2O_5 as drying agents. In case of 6S-C16-BCD further purification by chromatography was necessary (isocratic elution with acetonitrile:H_2O:25% ammonia 10:5:2). The yield varied between 50% and 90%.

The 6-monodeoxy-6-monobromo-ACD or -GCD was dissolved in DMF in a 3-neck round bottom flask under inert atmosphere, then the proper alkylthiol and sodium methylate were added in sequence and stirred while heated to 80 °C. The reaction was monitored by thin layer chromatography (TLC, eluent: dioxane:25% ammonia 10:7). According to the TLC the reaction was completed within 2 h. The solvent was removed under reduced pressure at rotavapor and the yellowish product was precipitated with methanol; the solid was filtered, extensively washed with methanol until a white solid was obtained and finally dried until constant weight in a vacuum drying box in the presence of KOH and P_2O_5 as drying agents. In case of 6S-C10-ACD further purification by chromatography was necessary (isocratic elution with acetonitrile:H_2O:25% ammonia 10:5:2).

A part of each of the 6-monodeoxy-6-monoalkylthio CD derivatives was solubilized in aqueous alkaline solution (NaOH). The reaction mixture was cooled down (T≈10-15 °C) and glycidyltrimethylammonium chloride was added in one portion and stirred overnight. The reaction was monitored by TLC (eluent: methanol:acetic acid: 0.1 N ammonium acetate 10:1:9). The reaction mixture was neutralized and the volatiles removed under reduced pressure at rotavapor. The obtained solids were washed with methanol and acetonitrile, then extensively dialyzed for 36 h. The dialysate was finally freeze-dried. The yield varied between 60% and 70%.

The characteristics: abbreviations (A), the molecular weight (MW), the solubility in water at 25 °C (WS), and the degree of substitution (DS) of the tested CDs are presented in Tables 4 and 5. The γ-CD series were not included in the biological tests as GCD itself was found ineffective in the preliminary experiments.

Table 4: Chemical properties of the tested α-cyclodextrins.

α-cyclodextrins (α-CDs)	A[1]	MW[2] [g/mol]	WS[3] [g/L]	DS[4]
Native α-CD	ACD	972	145	-
Trimethylaminopropyl-α-CD	QAACD	1430	500<	2.5–4
6-monodeoxy-6-monododecanthiol-α-CD	6S-C12-ACD		>10	~1
Trimethylaminopropyl-6-monodeoxy-6-monodecanethiol-α-CD	QA-6S-C10-ACD		~10	

[1]Abbreviation; [2]Molecular Weight; [3]Water Solubility at 25° C; [4]Degree of Substitution.

Table 5: Chemical properties of the tested β-cyclodextrins.

β-cyclodextrins (β-CDs)	A[1]	MW[2] [g/mol]	WS[3] [g/L]	DS[4]
Native β-CD	BCD	1135	18	-
N,N,N-Trimethylaminopropyl-β-CD	QABCD	1665	500<	3–4
6-monodeoxy-6-monododecanthiol-β-CD	6S-C12-BCD		>10	~1
6-monodeoxy-6-monododecanthiol-β-CD	6S-C12-BCD		>10	~1
N,N,N-Trimethylaminopropyl-6-monodeoxy-6-monodecanethiol-β-CD	QA-6S-C10-BCD		~10	
N,N,N-Trimethylaminopropyl-6-monodeoxy-6-monohexadecanethiol-β-CD	QA-6S-C16-BCD		~10	

[1] Abbreviation; [2] Molecular Weight; [3] Water Solubility at 25 °C; [4] Degree of Substitution.

NMR spectra

^{1}H and HSQC NMR spectra were recorded on Varian VXR-600 (Agilent Technologies, Palo Alto, CA, USA) at 298 K, equipped with a 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences and processing routines available in Vnmr J 3.2C/Chempack 5.1 were used, using residual solvent signals (HDO at 4.79 ppm) as internal reference. 32 transients were coadded. Before integration automatic baseline correction was performed.

MALDI-TOF mass spectra

The spectra were recorded on a Bruker Microflex LRF system The microflex LRF operated in positive ion mode using the linear detector.

Ion generation was achieved using a 60 Hz N2-Cartridge-Laser including variable power attenuator and UV optics. The laser operated at 337 nm and 2,5-dihydroxybenzoic acid (DHB) was used as matrix. For sample preparation 2,5-dihydroxybenzoic acid was used as matrix.

Aggregation studies

The aggregation behavior (nanoparticle formation) was studied by photon correlation spectroscopy (dynamic light scattering) using Malvern Zetasizer Nano ZS (Malvern Instruments, UK) equipment at 25 °C using 1% aqueous solution. The size distribution curves according to the volume of particles were recorded and the average of 5 parallel measurements was calculated.

Examination of the effect on bacterial communication

The time- and concentration-dependent effect of native α- and β-CDs (ACD and BCD), their *N*,*N*,*N*-trimethyl-aminopropyl derivatives, and novel alkyl thioether derivatives were tested in a series of short-term (30–120 min) experiments at 0.008–10 mM concentration range.

To differentiate between the quorum-quenching effect and the cytotoxic effect of the CDs, not just bioluminescence but also cell viability (the population growth and the enzyme activity) was determined.

Bacterial Strains and Culture Conditions

The bacterial strain *Aliivibrio fischeri* (NRRL B-111 77) was cultured and maintained in the laboratory under axenic circumstances. The 16 h old (overnight) cell culture applied for the tests was prepared by inoculating 30 mL of Photobacterium medium with an ampoule of lyophilized bacteria. The culture was shaken in the dark, at 160 rpm, 24°C, to an optical density of 0.6 at 600 nm (OD_{600}).

The ingredients of the Photobacterium growth medium were (pH=7.2): 30 g NaCl, 6.1 g $NaH_2PO_4 \cdot H_2O$, 2.75 g K_2HPO_4, 0.204 g $MgSO_4 \cdot 7 H_2O$, 0.5 g $(NH_4)_2HPO_4$, 5 g peptone, 0.5 g yeast extract, 3 cm^3 glycerol per 1 L distilled water as it is described by Fekete-Kertész *et al.* [38]. The measurements were performed following the description of Leitgib *et al.* [39] by the modification of the ISO 11348-3 protocol.

Preparation of CD stock solutions

The tested CD molecules were suspended in sterile distilled water and sterilized in an autoclave. A dilution series was prepared from the stock solutions (suspensions), covering a 0.04–50 mM concentration range.

Bioluminescence Inhibition Assay

The experiments were carried out with overnight *A. fischeri* cell culture in 96-well microtiter plates with a total volume of 250 µL. 50 µL of the members of the fivefold dilution series made from stock solutions were added to the wells of a sterile, transparent, 96-well, round-bottomed Sarstedt microtiter plate in four replicates. The concentrations of the members of the dilution series are presented in Table 6. Distilled water was used as a negative control in the same volume.

To ensure the validity of data and verify the sensitivity of the bacteria, a five-member CuSO$_4$ dilution series (1.6 ppm, 8 ppm, 40 ppm, 200 ppm, and 400 ppm) was used as a positive control. After that, 200 µL of the diluted overnight culture was added to the wells, except for the blank samples for which 200 µL of Photobacterium medium was added instead. The bioluminescence intensity was measured with Fluostar Optima BMG Labtech microplate reader (fluorescence detection limit >30 amol/well ATP) after 30, 60, and 120 minutes of exposure. The evaluation of the results was carried out as described by Ujaczki et al. [40].

Table 6. The concentration of the members of the dilution series.

Cyclodextrin	Concentration [mM]			
6S-C12-BCD	0.016	0.08	0.4	2
QA-6S-C10-ACD	0.008	0.04	0.02	1
QA-6S-C10-BCD, QA-6S-C16-BCD	0.04	0.2	1	5
ACD, QAACD, 6S-C12-ACD, BCD, QABCD	0.08	0.4	2	10

Cell viability – The Population Growth Assay

To investigate whether the CDs had a cytotoxic effect, the growth of the bacterial population was determined through the measurement of the optical density (OD). The measurements were carried out as described by Molnár et al. (2021). The OD was measured after the incubation period, following the bioluminescence inhibition assay with DIALAB ELx800 ELISA Microplate Reader (Dialab GmbH, Austria) at the wavelength of 630 nm.

Cell Viability – The Enzyme Activity Assay

To examine the potential influence of the CDs on the vitality of the bacteria, an enzyme activity assay was applied 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The measurements were carried out as described by Molnár *et al.* (2021) with a few modifications. Metabolically active cells reduce the green-colored MTT into a black formazan product, whose concentration is directly proportional to the activity of the viable cells. Following the population growth assay, the assay was performed in the same microplates by adding 30 µL of 4 mg/mL sterile MTT solution to each well. The microtiter plate was then incubated for 30 min until color development. The absorbance was measured spectrophotometrically with DIALAB ELx800 ELISA Microplate Reader (Dialab GmbH, Austria) at the wavelength of 630 nm and with Fluostar Optima BMG Labtech microplate reader at the wavelength of 544 nm.

Statistical Analysis

Repeated measures analysis of variance (RM ANOVA) was performed with TIBCO Statistica™ 13.5 (TIBCO Software, Inc., Palo Alto, CA, USA) software to investigate whether the CD concentrations, the exposure time (incubation time), and their interactions affected the bioluminescence intensity of *A. fischeri*. CD concentration was considered a grouping factor, and the within-subject factor was the exposure time, which varied within the grouping factor. The Mauchley sphericity test was applied to confirm the criteria. Statistical analyses were performed at the $p < 0.05$ significance level. Tukey's honestly significant difference test was used for comparison of the

effects of the treatments. The significant effects are marked by an asterisk (*) in all figures ($p < 0.05$).

Pearson Product Moment Correlation Analysis was also performed by TIBCO Statistica™ 13.5 (TIBCO Software, Inc., Palo Alto, CA, USA) to examine the relationship between the measured endpoints and the cyclodextrin concentrations. The level of significance was $p < 0.05$. The correlation was considered strong when the correlation coefficient (r) was higher than 0.60 and very strong at r > 0.85

Effective Concentration (EC20, EC50) values causing 20, and 50% inhibition of bioluminescence intensity were determined using OriginPro 2018 software following the concentration-response analysis with Logistic function fitting ($y = A2 + (A1-A2)/(1 + (x/x0)^p)$).

Supporting Information

Table S1 is included in SI file.

Funding

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