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Antibacterial activity of immobilized silver nanoparticles on TEPA-Den-SiO_2 against shrimp pathogen, *Vibrio* sp. Persian1

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Abstract

In this study, we prepared silver nanoparticles immobilized onto silica sand beads as an antibacterial material against pathogenic luminous *Vibrio* sp. Persian1. Silica beads were modified with 3-aminopropyltriethoxysilane (APTES), cyanuric chlorid and tetraethylene pentamine, and silver nanoparticles were generated in various concentrations (0.5, 1, and 2 mM g^{-1} of silica beads) of $AgNO_3$ on the surface using chemical reduction. Ag/TEPA-Den-SiO_2 samples were characterized by TEM, FE-SEM/EDS, FT-IR and ICP OES and their antibacterial activity assayed by zone of inhibition and test tube tests against pathogenic *Vibrio* sp. The results of the zone inhibitory test revealed that all the Ag/TEPA-Den-SiO_2 samples had an antibacterial effect against luminous *Vibrio* sp. Persian1. In addition, the tube test results showed 100% killing of bacteria in 2 h contact period. Ag/TEPA-Den-SiO_2 samples maintained their antibacterial activity after 14-day immersion in seawater by slow release of silver ions. These results suggest that Ag/TEPA-Den-SiO_2 substrates could be effective antibacterial materials for disinfection of seawater used to culture Penaeid shrimp larvae.

Keywords: antibacterial activity, Silver nanoparticles, *Vibrio* sp., Shrimp

Introduction

Aquaculture is one of the main food providers in the world and shrimp culture is known as an important aquaculture activity in numerous areas. The shrimp aquaculture industry has been damaged especially by viral and bacterial diseases (Moriarty 1999). Vibriosis is the most predominant bacterial disease that causes mass mortalities of the cultured shrimp worldwide (Lavilla-Pitogo, Leano & Paner 1998). *Vibrio* species often cause mortality in both hatcheries and grow-out ponds (Mohney, Lightner & Bell 1994). Early mortality syndrome (EMS) or more descriptively called acute hepatopancreatic necrosis syndrome (AHPNS) has emerged as a new disease of shrimp since 2009. This disease has caused significant mortalities of Pacific White shrimp *Litopenaeus vannamei* and black tiger shrimp *Penaeus monodon* in Southeast Asian shrimp farms. EMS/AHPNS pathogen has been identified as a member of the *vibrio harveyi* clade, most closely related to *V. parahemolyticus* (Tran, Nunan, Redman, Mohney, Pantoja, Fitzsimmons & Lightner 2013). Chemical treatment of vibriosis among shrimp larvae is quite limited because of the ineffectiveness of existing drugs, resistance among *vibrio* species, high toxicity, human health hazards and high price of drugs (Krishnani, Kathiravan, Shakil, Singh, Brahmane, Meena, Sarkar, Choudhary, Singh & Kumar 2015). Therefore, there is a need to investigate novel control strategies for shrimp diseases.

Nanotechnology has provided novel opportunities to develop more efficient and cost effective materials for water purification. Nanomaterials have particular properties that make them attractive for water disinfection. They have much large surface areas than bulk particles, which allow them to interact closely with microbial membranes, and thus their bactericidal properties are increased (Bryaskova, Pencheva, Nikolov & Kantardjiev 2011). Silver has been used for disease prevention in many centuries. In recent years, silver nanoparticles have been extensively studied because of their possible application as bactericidal materials in the biomedical field (Bryaskova *et al.* 2011).

It is well known that colloidal silver nanoparticles have antimicrobial properties, but some problems may limit excessive use of them as an effective bactericidal agent (Gunawan, Guan, Song, Zhang, Leong, Tang, Chen, Chan-Park, Chang, Wang & Xu 2011; Agnihotri, Mukherji & Mukherji 2013). Colloidal AgNPs tend to aggregate in aqueous media that gradually reduces their efficiency in long-term use (Li, Lenhart & Walker 2012). In addition, the release of silver nanoparticles into the aquatic environment may lead to disastrous effects on aquatic organisms (De Gusseme, Hennebel, Christiaens, Saveyn, Verbeken, Fitts, Boon & Verstraete 2011; Asghari, Johari, Lee, Kim, Jeon, Choi, Moon & Yu 2012; Johari, Kalbassi, Soltani & Yu 2013a,b; Salarijoo, Kalbassi, Yu, Lee & Johari 2013; Johari, Kalbassi, Yu & Lee 2015; Johari, Sourinejad, Bärsch, Saed-Moocheshi, Kaseb & Nazdar 2014). For example, Bilberg, Hovgaard, Besenbacher and Baatrup (2012) found that 48-h median lethal concentration (LC_{50}) values to zebra fish (*Danio rerio*) were 84 μg L^{-1} for silver nanoparticles (81 nm) and 25 μg L^{-1} for silver ions ($AgNO_3$).

Silver nanoparticles can be coated on solid substances in order to deactivate microorganisms in water treatment (Nair & Pradeep 2007). Therefore, immobilization of AgNPs on the surfaces of materials provides an efficient disinfection activity with controlled silver release and decreases environmental threats (Agnihotri *et al.* 2013). Recently, immobilization of silver nanoparticles on silica substrate using coupling agents such as aminosilane and mercaptosilane has been widely considered in order to reduce the leaching of silver (Wu, Chang, Tsai, Huang & Yang 2010). One end of these molecules is connected to surface through a Si–O–Si bond, and the other end is connected to the silver atoms on the surface of the nanoparticles through an N–Ag coordination bond (Lv, Liu, Wang, Liu, Hao, Sang, Liu, Wang & Boughton 2009b).

In this study, silver nanoparticles were immobilized on the surfaces of silica sand beads by using 3-aminopropyltriethoxysilane (APTES) as a cross-linker molecule. Then, the antibacterial activity of silica sand beads that contained different concentrations of silver nanoparticles (Ag/TEPA-Den-SiO_2) was evaluated against luminous *Vibrio* sp. Persian1 isolated from the cultured shrimp. Release of silver ions from these synthesized substrates into seawater was also measured in the *in vitro* condition.

Experimental

3-Aminopropyltriethoxysilane and *N*, *N*-diisopropylethylamine (DIEA) were purchased from Aldrich. Cyanuric chloride (CC), tetraethylenepentamine (TEPA), silver nitrate, tetrahydrofuran (THF) and Toluene were purchased from Merck.

Synthesis of amine functionalized silica beads (NH_2-SiO_2)

First, we activated the silica beads (0.4–0.8 mm) by adding silica beads (25 g) and HCl (50 mL, 6 M) to a 100 mL round bottom balloon. The resultant mixture was refluxed for 24 h. After cooling, the mixture was decanted and washed several times with distilled water and acetone, then dried overnight at 80°C. Various amounts of mM g^{-1} of APTES silica beads were used to prepare miscellaneous amine functionalized silica beads. For example, in a 50 mL round bottom balloon, 0.23 mL of APTES (1 mmol) was added to silica beads (10 g) in toluene (30 mL) and the mixture was stirred for 24 h at 100°C. Then, the reaction was cooled, filtered, washed several times with acetone, followed by water, then dried overnight at 80°C.

Synthesis of aliphatic amines Dendron on functionalized silica beads (TEPA-Den-SiO_2)

A solution of CC (1 mmol, 0.18 g) in dry THF (25 mL) was added dropwise at 0°C to a mixture of the NH_2-SiO_2 (10 g) and DIEA (1 mmol, 0.085 mL) in dry THF (30 mL), which were

introduced in a 100 mL round bottom flask. The mixture was stirred for 1 h to synthesize triazine anchored silicas (CC-NH-SiO_2). The solution was decanted and washed with dry THF (2 9 15 mL), after which fresh THF (25 mL) was poured onto the remaining hybrid silicas. Next, a solution of TEPA (2 mmol, 0.34 mL) in THF (15 mL) was added dropwise for 5 min, then stirred for 1 h at 0°C, 2 h at 25°C and 24 h at reflux condition. After completion of reaction, the mixture was cooled, decanted and washed several times with THF and acetone. The desired product (TEPA-CC-NH-SiO_2) was dried overnight at 50°C. The attachment of various amounts of amine Dendron to the surface of silicas was obtained (Table 2).

Synthesis of Ag nanoparticles on TEPA-Den-SiO_2

In a 250 mL round bottom balloon, 25 mL of water was added to 10 g of TEPA-CC-NH-SiO_2. An aqueous solution of $AgNO_3$ in various concentrations (for example: 2 mmol/50 mL) was added slowly to it at room temperature. The mixture was warmed to 80°C for 4 h. The solution gradually became dark. After completion of reaction, the mixture was cooled, decanted and washed several times with water to remove any Ag nanoparticles that had not loaded on the surface. The residue was dried to obtain various loaded amounts of Ag on the aminated surface (Ag/TEPA-Den-SiO_2).

Characterization

Imaging and surface morphology of the silver nanoparticles coated on silica beads were investigated using field emission scanning electron microscopy (FE-SEM; Mira 3 XMU, TESCAN ORSAY HOLDING, Brno, Czech Republic) coupled with X-ray energy dispersive spectroscopy (EDS). Transmission electron microscopy (TEM) image was taken by a Philips CM30 300KV instrument that operated at an accelerating voltage of 150 KV. Infrared spectra were prepared on a spectrometer (Bruker Alpha, Ettlingen, Germany) over range of 400–4000 cm^{-1}. The total Ag content of Ag/TEPA-Den-SiO_2 samples was determined by acid digestion of 1 g sample in 4 mL 65% HNO_3 (Suprapur grade, Merck, Germany) and the amount of dissolved silver analyzed with ICP-OES[1] (Optima 8000 model, Waltham, MA, USA) .

[1]Inductively coupled plasma optical emission spectroscopy.

Antibacterial activity test

The target bacterium was luminous *Vibrio* sp. Persian1 (KC505639.2). This bacterium has a pathogenic effect on *Litopenaeus vannamei* and isolated from diseased postlarvae and seawater (Mirbakhsh, Akhavan sepahy, Afsharnasab, Khanafari & Razavi 2014). This *Vibrio* species was cultured in tryptic soy broth (TSB) + 2.5% NaCl (Merck, Darmstadt, Germany) and incubated overnight at 30°C in a shaking incubator at 200 rpm (JSSI-200CL; JSR, Gongju-City, Korea).

Zone of inhibition test

First, we poured Mueller–Hinton agar + 2.5% NaCl onto sterilized petri dishes and allowed them to solidify. Then, the surface of MHA was completely inoculated with bacteria (1/5 9 10^8 CFU mL^{-1}) by using a sterile swab. Silica beads coated with different concentrations of silver nanoparticles (Ag/TEPA-Den-SiO_2 samples, Table 2) and uncoated (control) weighting 30 mg were placed over solidified agar gel in round shape with diameter in the range of 7–8.5 mm. The plates were incubated at 30°C for 24 h. Finally, the diameter of the growth inhibition zones was measured by digital caliper (Guilin Guanglu Measuring Instrument, Zone Guilin, China). All tests were conducted in triplicate.

Test tube test

In this experiment, the test tubes that contained 5 mL of sterilized seawater were used. The properties of natural seawater used as the water source are shown in Table 1. Silica beads that contained different concentrations of silver nanoparticles (Ag/TEPA-Den-SiO_2 samples, Table 2), bare silica beads and amine functionalized silica beads (TEPA-Den-SiO_2) as control (1.5 mg or 0.5 mg per mL) were placed in each tube test. Then, 100 µl of bacteria (approximately 1.5 9 10^5 CFU mL^{-1}) harvested by centrifugation (2600 *g* at 15°C, 10 min) were added. All tubes were incubated in a shaker incubator at 30°C. The suspension (100 µL) fractions were withdrawn after 0, 2, 6 and 24 h intervals and poured onto TCBS + 1/5% NaCl (Merck) plates according to a serial dilution method. All the plates were incubated at 30°C for 24 h to obtain viable counts. All the experiments were performed in triplicate.

Table 1 The properties of natural seawater source used for antibacterial activity experiments

Parameters	Units	Concentration
Hardness ($CaCO_3$)	g L^{-1}	5.88
Salinity	g L^{-1}	37
pH	–	8.2
Chloride (Cl^-)	g L^{-1}	20.47
PO_4^{3-}	mg L^{-1}	0.040
Nitrite (NO_2^-)	mg L^{-1}	0.019
Nitrate (NO_3^-)	mg L^{-1}	0.083

Table 2 Different concentrations of 3-aminopropyltriethoxysilane (APTES) and $AgNO_3$ used for immobilization of silver nanoparticles on the surfaces of silica beads

Ag/TEPA-Den-SiO_2 samples number	APTES (mmol g^{-1} SiO_2)	$AgNO_3$ (mmol g^{-1} SiO_2)	Ag content (mg Ag g^{-1} SiO_2)
1	0.5	0.5	0.111 ± 0.011^{b}
2	0.5	1	0.133 ± 0.018^{c}
3	0.5	2	0.096 ± 0.005^{ab}
4	1	0.5	0.079 ± 0.002^{a}
5	1	1	0.139 ± 0.003^{c}
6	1	2	0.143 ± 0.012^{c}

The data with different letters are significantly different ($P < 0.05$).

Antibacterial activity of Ag/TEPA-Den-SiO_2 substrates in contact with seawater

For this purpose, we put 1 g of Ag/TEPA-Den-SiO_2 samples in 100 mL of sterilized seawater (37 g L^{-1}) and agitated on a shaker for 14 days at 150 rpm. After this period, the antibacterial activity was examined as described in the Section Test tube test.

Antibacterial activity of seawater in contact with Ag/TEPA-Den-SiO_2 substrates

We investigated antibacterial activity of seawater medium in contact with Ag/TEPA-Den-SiO_2 samples by immersion of 1 g of these samples in 100 mL of sterilized seawater (37 g L^{-1}) and holding them on a shaker for 14 days at 150 rpm. After this period, 5 mL of seawater was sampled and poured into a sterilized tube test. The antibacterial activity of seawater samples was investigated as described in the Section Test tube test.

Release of silver into seawater medium

We studied the release of silver from Ag/TEPA-Den-SiO_2 samples into seawater medium by adding 1 g of silica beads that contained different silver concentrations to 100 mL sterilized seawater (37 g L^{-1}) in a glass bottle in triplicate. The bottles were rotated for 14 days at a rate of 150 rpm. Then, a volume of sample was taken and digested by 65% HNO_3 (Suprapur grade; Merck). The concentration of silver ions was analyzed by ICP-OES (Optima 8000 model).

Statistical analysis

Data were presented as means ± SD. Differences between the means were analyzed using one-way analysis of variance (ANOVA). Duncan's new multiple range test was conducted to determine significant differences between the means using SPSS software (version 19.0) (IBM SPSS, Armonk, NY, USA). Statistical significance was accepted at the level of $P < 0.05$.

Results and discussion

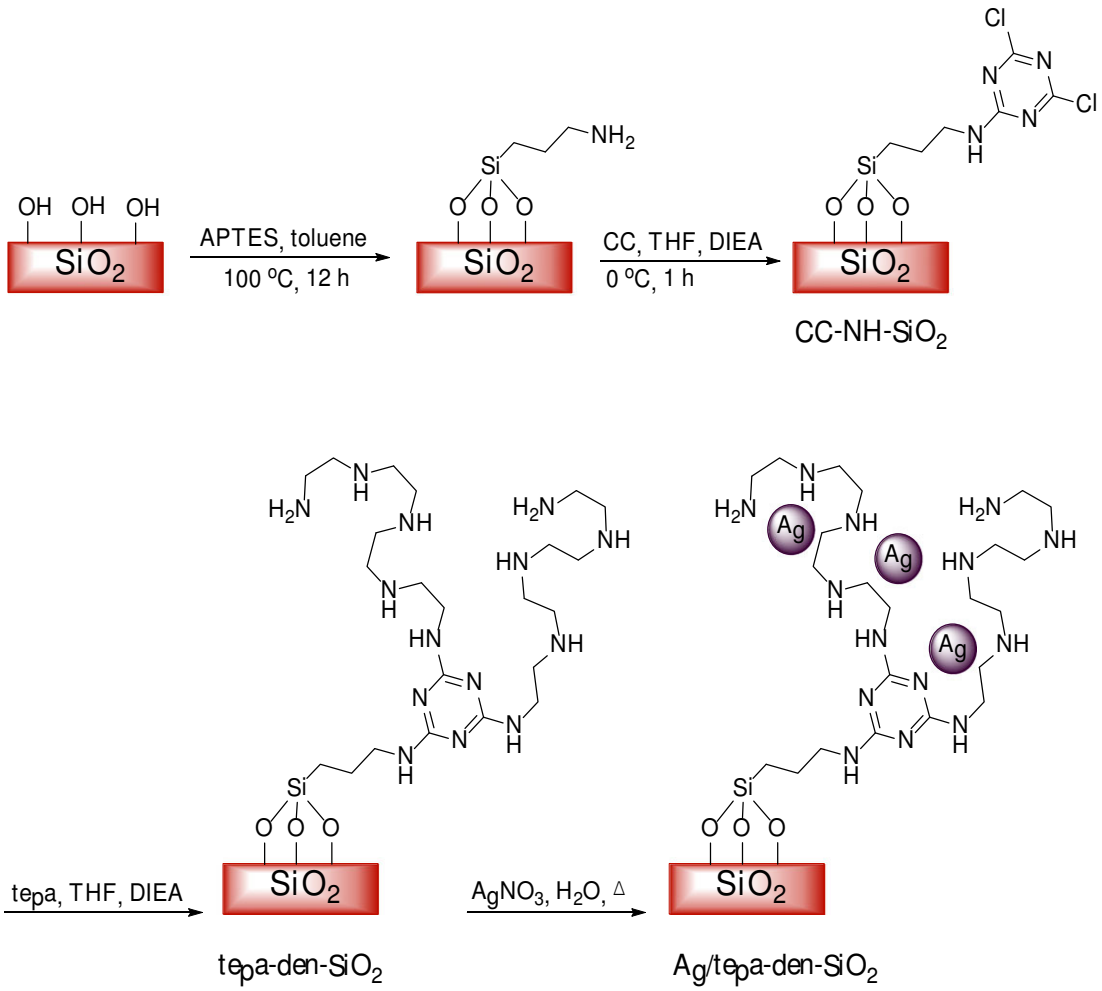
Characterization of Ag/TEPA-Den-SiO_2

The schematic pathways for the synthesis of Ag/TEPA-Den-SiO_2 are depicted in Scheme 1. First, the silica beads were activated with refluxing of SiO_2 in HCl (aq) and Ag/TEPA-Den-SiO_2 was prepared according to the similarly reported method (Veisi, Kordestani, Hemmati, Faraji & Veisi 2014). Then, silver ion source was added to the hybrid surface in water and the mixture was stirred and refluxed to obtain final Ag loaded product. Silanol groups (Si-OH) on the surface of active silica beads play very important roles in the functionalization of silica beads (Quang, Sarawade, Hilonga, Kim, Chai, Kim, Ryu & Kim 2011a). Silica substrate can be functionalized with amino functional groups (Park, Park & Shin 2007). In this study, silica beads were modified with APTES, CC and then TEPA, respectively, to link multi-amino functional groups on their surfaces. Silver ions can be complexed on the surface-exposed amino groups through covalent bond and/or electrostatic interactions (Quang, Sarawade, Hilonga, Kim, Chai, Kim, Ryu & Kim 2011b). Various amounts of APTES and $AgNO_3$ were used to prepare the several hybrid surfaces (Table 2) in order to receive maximum antibacterial ability as our favourite goal. The amounts of Ag contents of Ag/TEPA-Den-SiO_2 samples measured with ICP-OES have been shown in Table 2. It seems when the amount of APTES as a linker

increases, the silver absorption may also increase, although this increase does not follow a certain order while addition of $AgNO_3$ usually increases Ag contents of the surface. After antimicrobial study of all samples, it was clear that the all the samples had the same performance in the relevant field. Therefore, it was attempted to evaluate as much as possible of the sample no.1 because of the use of low amounts of APTES and $AgNO_3$ to synthesize this sample.

Digital photos of uncoated silica beads and Ag/TEPA-Den-SiO_2 are shown in Figure 1. The colour of silica beads changed from white (A) to brownish yellow (B) when silver cations immobilized onto silica beads reduced to silver nanoparticles using the methods described above. Silver nanoparticles usually have yellow colour and this colour is remained when these nanoparticles are incorporated into silica structure (Quang *et al.* 2011a). FE-SEM micrographs of the sample are shown in Figure 1c. This image showed the distribution of white, small spherical nanoparticles on the surfaces of the silica beads. We also studied the hybrid material with EDS analysis. The EDS analysis results, shown in Figure 1d, have confirmed the elemental composition of Ag/TEPA-Den-SiO_2 and the existence of Ag in the surface as an elemental form attached to the surface of the silica structure.

The presence of tetraethylene pentamine linked to triazine cycles as an organic layer on silica maintained silver nanoparticles on the surface can be inferred from FT-IR technique. Figure 2a shows spectrum of bare SiO_2. A signal at 1100 cm^{-1}



Scheme 1 Schematic pathway for preparation of Ag/TEPA-Den-SiO_2.

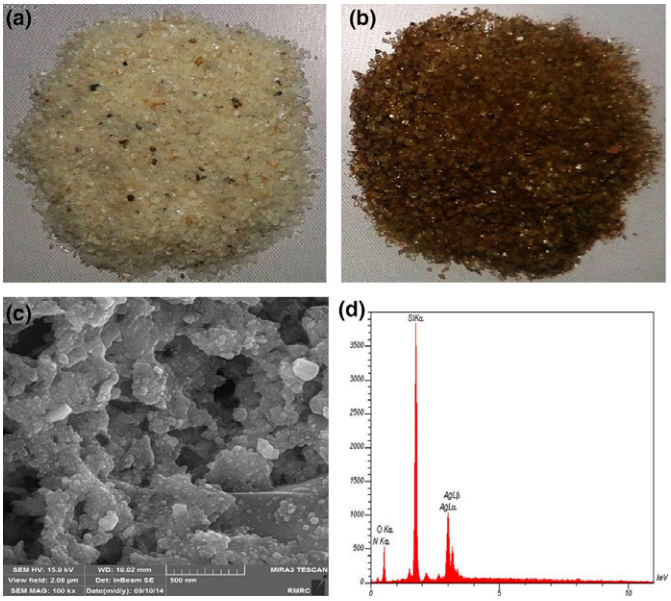


Figure 1 Digital photos of bare silica beads (a) and Ag/TEPA-Den-SiO_2 (b); FE-SEM image of Ag/TEPA-Den-SiO_2 (sample 1) (c) and its energy dispersive X-ray spectroscopy pattern (d).

was attributed to Si-O-Si anti symmetric stretching vibration. The intense bands located at 1100 and 800 cm^{-1} are attributed to Si–O–Si stretching and Si–O–Si bending vibration respectively (Yanagisawa, Fujimoto, Nakashima, Kurata & Sanada 1997).

The broad band at 3200–3600 cm^{-1} at spectrum A referred to O–H stretching frequency of Si–O–H groups and/or water in atmosphere (Quang *et al.* 2011a). The bands appeared at 1478 and 1625 cm^{-1} in spectrum B were attributed to C=N and C-N stretching of triazine ring and amines. We believed that the signal at 3424 cm^{-1} referred to the N-H stretching of amine groups on Ag/TEPA-Den-SiO_2. In addition, the signal at 2926 cm^{-1} was related to C-H stretching of tetraethylene pentamine groups.

The morphology of the modified surface (sample 1) was studied with transmission electron microscopy (TEM). The TEM observation indicates that silver nanoparticles deposited onto substrate had spherical shape with the mean diameter of 8.48 ± 4.29 nm and a size distribution that ranged from 2.35 to 31.28 nm (Fig. 3).

Antibacterial activity of silver nanoparticles immobilized on silica beads

Inhibitory zone formed by silica beads coated with different concentrations of silver nanoparticles (Ag/TEPA-Den-SiO_2 samples) and bare silica beads (control) against *Vibrio* sp. Persian1 in plating test is shown in Figure 4. An inhibition zone diameter of 13.1–15.07 (mm) was observed around Ag/TEPA-Den-SiO_2 samples, whereas no inhibition zone was seen around uncoated silica beads. Results of statistical analysis showed that the mean diameter of inhibitory zone formed around Ag/TEPA-Den-SiO_2

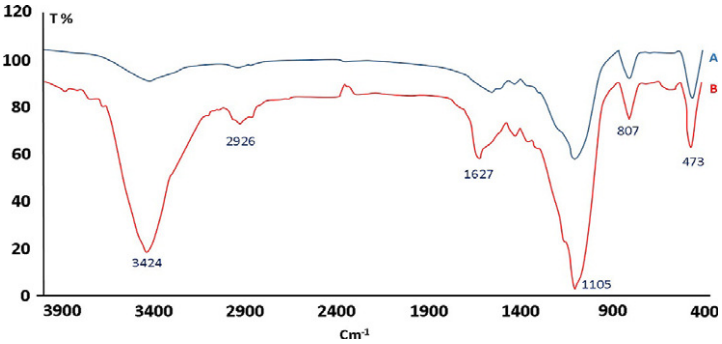


Figure 2 FT-IR spectra of SiO_2 (A) and Ag/TEPA-Den-SiO_2 (B).

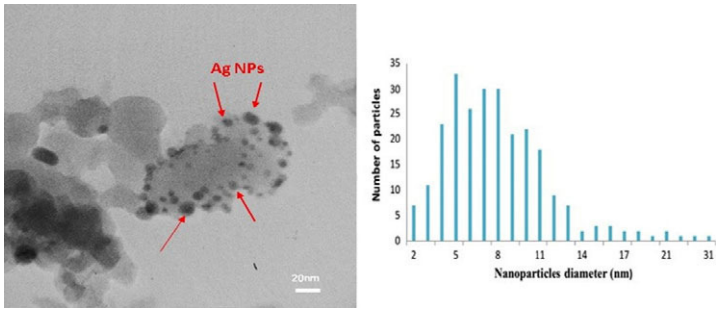


Figure 3 TEM images of silver nanoparticles immobilized on the surface of Ag/TEPA-Den-SiO_2 and histogram for particles size distribution of silver nanoparticles.

samples was not significantly different ($P < 0.0.5$, Fig. 5). It was revealed that all the Ag/TEPA-Den-SiO_2 samples with the total Ag content that ranged from 0.079 ± 0.002 to 0.143 ± 0.012 (mg g^{-1} silica beads) had antibacterial effect against *Vibrio* sp. Persian1. According to Quang *et al.* (2011a), zone inhibitory tests proved that silver nanoparticle-supported silica micro beads (Ag-NPBs) were effective against both Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* bacteria. An inhibition zone diameter of about 10 mm for *E. coli* was observed around silver nanoparticle-decorated porous ceramic composite, whereas bacterial growth was seen in case of the undecorated porous ceramic (Lv *et al.* 2009b).

The tube test results for *Vibrio* sp. Persian1 is shown in Figure 6. The bacteria count in seawater after 0, 2, 6 and 24 h contact with Ag/TEPA-Den-SiO_2 samples, amine functionalized silica beads (TEPA-Den-SiO_2) and bare silica beads as positive control indicated that 100% of bacteria were inactivated within 2 h contact with all of the Ag/TEPA-Den-SiO_2 samples, whereas substantial growth was counted in the control samples (Table 3). Silica substrates functionalized with amine group had no inhibitory effect against *Vibrio* sp. Persian1. This result emphasizes that disinfection potential of Ag/TEPA-Den-SiO_2 samples against this pathogenic *Vibrio* species could be attributed to silver nanoparticles immobilized on the silica bead surfaces. Karnib, Holail, Olama, Kabbani and Hines (2013) reported 100% reduction in *E. coli* count after 1 h of contact with silver nanoparticles impregnated activated carbon at different concentrations in the shaker flask technique. Tube test results of silver nanoparticles decorated-porous ceramic composite against *E. coli* showed no detectable bacteria after a 24 h contact period (Lv *et al.* 2009b).

The effectiveness of bactericidal property of AgNPs-ETS-10 samples against *E. coli* improved after 3 h and samples with different contents of silver nanoparticles had a similar bactericidal profile (Lv, Luo, Ng & Zhao 2009a). Different mechanisms by which silver nanoparticles inactivate bacteria are debate in the literature (Jin, Li, Wang, Marambio-Jones, Peng, Hung, Damoiseaux & Hoek 2010) and include: (1) attachment to cell membrane and disruption of the permeability and respiration functions of the cell, and ultimately cell death (Morones, Elechiguerra, Camacho & Ramirez 2005), (2) generation of reactive oxygen species (ROS) on the surface of silver nanoparticles, and thus damage of DNA by exerting oxidative stress (Feng, Wu, Chen, Cui, Kim & Kim 2000) and (3) cellular uptake of silver ions released from AgNPs, disruption of ATP production and DNA replication (Marambio-Jones & Hoek 2010). In general,

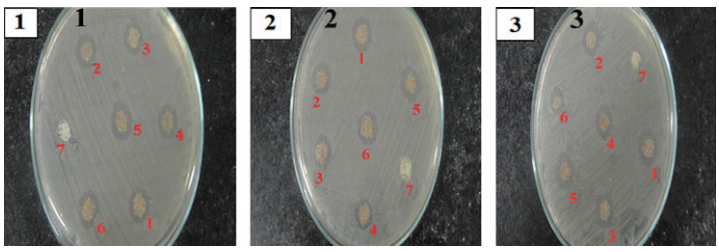


Figure 4 Zone inhibitory test results: comparision of the inhibitory zone formed by Ag/TEPA-Den-SiO_2 samples (1–6) and bare silica beads sample (control, 7) for *Vibrio* sp. Persian1 in plate test. Test was done in triplicate.

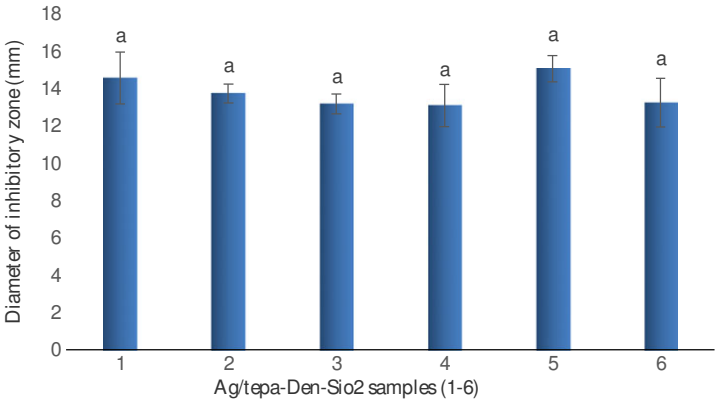


Figure 5 Diameter of inhibitory zone formed by Ag/TEPA-Den-SiO_2 samples (1–6, Table 2) against *Vibrio* sp. Persian1 in the plate test. Bars represent mean ± SD. $P < 0.05$.

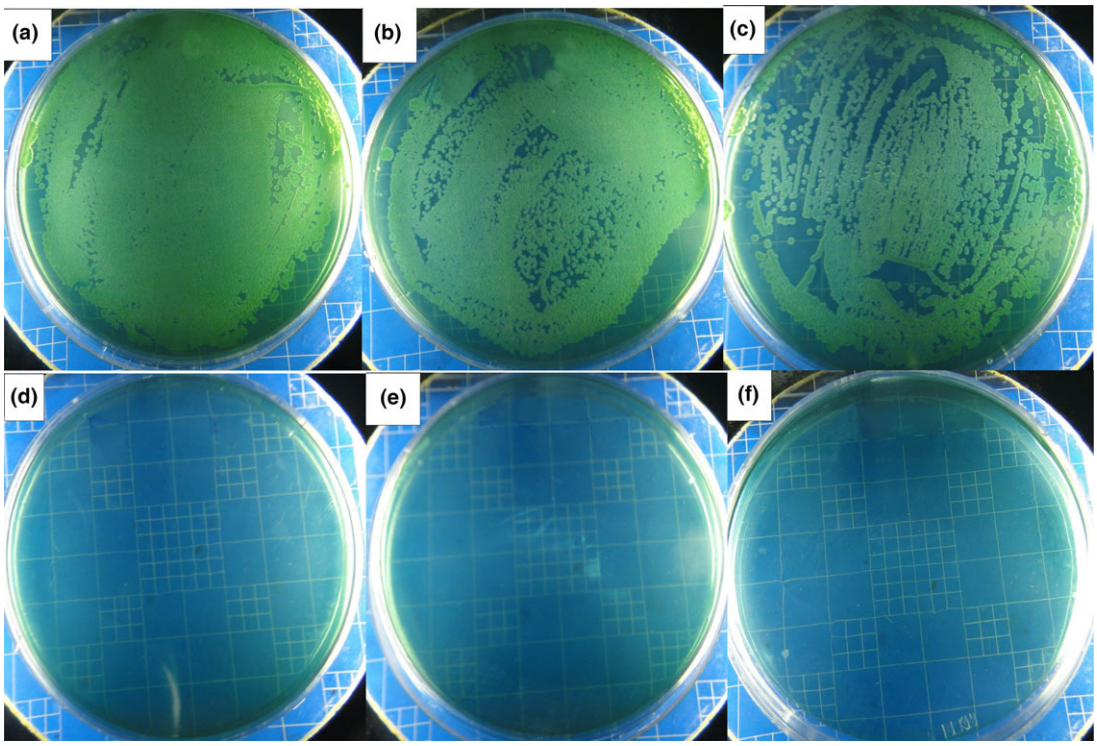


Figure 6 Test tube test results for *Vibrio* sp. Persian1 (10^5 CFU mL^{-1}). After 2 h (a), 6 h (b), 24 h (c) contact with bare silica beads (control) and after 2 h (d), 6 h (e), 24 h (f) contact with Ag/TEPA-Den-SiO_2 samples.

antibacterial mechanism of substrate that contained AgNPs is attributed to the release of silver ions but some researchers have hypothesized that contact killing mechanism enhances their antibacterial efficiency (Sotiriou & Pratsinis 2010; Agnihotri *et al.* 2013). Immobilized AgNPs with a size distribution that ranged from 4 to 10 nm may act through both the release of Ag ions and direct contact behaviour (Sotiriou & Pratsinis 2010). Small silver nanoparticles (<10 nm) may pass directly through cell membranes and produce ROS, and subsequently cause oxidative stress (Carlson, Hussain, Schrand, Braydich-Stolle, Hess, Jones & Schlager 2008). The bactericidal activity of AgNPs (8.6 nm) immobilized on amine functionalized silica surfaces showed that contact killing was the predominant bactericidal mechanism against *E. coli* and *B. subtillis* (Agnihotri *et al.* 2013). A number of studies have reported that the bactericidal effect of silver nanoparticles

Table 3 *Vibrio* sp. Persian1 count in seawater in the presence of Ag/TEPA-Den-SiO_2 samples after various contact times (mean ± SD)

Sample		Contact time (h)/Vibrio sp. Persian1 count (10^5 CFU mL^{-1})			
		0	2	6	24
Seawater (negative control)		0	0	0	0
Seawater + bacteria (positive control)		1.3 ± 0.32	1.6 ± 0.47	9.3 ± 1.1	1.67 ± 0.14
Seawater + bacteria + silica beads (positive control)		1.18 ± 0.12	1.4 ± 0.42	9.6 ± 1.13	1.75 ± 0.15
Seawater + bacteria + Tepa-Den-SiO_2 (positive control)		1.14 ± 0.21	1.32 ± 0.18	8.6 ± 0.87	1.42 ± 0.13
Seawater + bacteria +	Sample 1	1.22 ± 0.10	0	0	0
	Sample 2	1.0 ± 0.11	0	0	0
	Sample 3	0.96 ± 0.10	0	0	0
	Sample 4	1.08 ± 0.12	0	0	0
	Sample 5	1.21 ± 0.14	0	0	0
	Sample 6	1.28 ± 0.13	0	0	0

against Gram-negative bacteria increases in the presence of divalent cations (hardness). Divalent cations can form ion bridges between the negatively charged silver nanoparticles and lipopolysaccharide molecules on the bacteria surface, and facilitate the transfer of silver nanoparticles across the outer membrane (Jin *et al.* 2010). In this study, the amount of silver nanoparticles with mean diameter of 8.48 ± 4.29 that were immobilized onto the Ag/TEPA-Den-SiO_2 samples was significantly different (Table 2), but they had a similar antibacterial activity. Therefore, it can be inferred that direct contact may be involved in killing the bacteria cells. In addition, seawater medium with hardness of 5.8 g L^{-1} $CaCO_3$ may be involved in the attachment of silver nanoparticles to cell membranes of Gram-negative *Vibrio* sp. Persian1.

Silver release into seawater medium

The release of silver is an important parameter in evaluating the stability and durability of the bactericidal agents. The release of silver may have ecotoxicological effects and restrict the practical usability of these agents (Lv *et al.* 2009a; Agnihotri *et al.* 2013). Figure 7 shows the amount of silver release from silica beads that contained different silver nanoparticle concentrations after immersion in seawater (37 g L^{-1}) for 14 days. The silver concentration in seawater that contained Ag/TEPA-Den-SiO_2 samples increased significantly compared with the seawater as control ($P < 0.05$). The amount of silver released from Ag/TEPA-Den-SiO_2 samples was consistent with the total Ag content of these samples (Table 2). These results show that the increase in silver content increases the release of silver into seawater. Agnihotri *et al.* (2013) demonstrated that

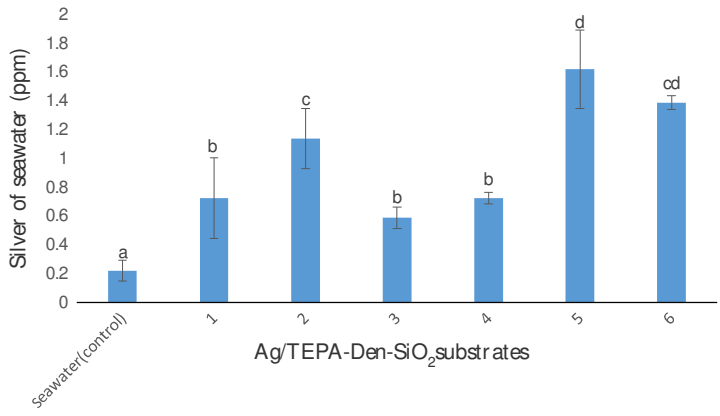


Figure 7 The silver concentration in seawater after contact with Ag/TEPA-Den-SiO_2 samples (1–6, Table 2) for 14 days (mean ± SD, $P < 0.05$).

the maximum amount of silver released from AgNP-glass substrate after 60 min immersion in 100 mL fresh DI water was 25 ppb, which constituted 1.15% of the total amount of silver immobilized on the AgNP–glass substrate. The amount of silver that migrated from chitosan/silver nanoparticles films (0.25 g) with silver concentration of 0.2% into the diluted Muller–Hinton broth after 360 h (15 days) was 0.35 mg L^{-1} (Lopez-Carballo, Higueras, Gavara & Hernandez-Munoz 2012). In this study, the maximum and minimum levels of silver released from Ag/TEPA-Den-SiO_2 samples after 14 days immersion in seawater were 1.62 ± 0.27 and 0.58 ± 0.07 mg L^{-1} for samples 5 and 3, respectively, which constituted 1.16% and 0.59% of the total silver immobilized onto the these samples. Taglietti, Arciola, D'Agostino, Dacarro, Montanaro, Campoccia, Cucca, Vercellino, Poggi, Pallavicini and Visai (2014) showed that the maximum amount of silver ions released from AgNP-modified glass slide with APTES after 3 days was about 16% of the total silver bound on the surface.

Antibacterial activity of Ag/TEPA-Den-SiO_2 samples in seawater medium over time

Table 4 shows the antibacterial capacity of Ag/TEPA-Den-SiO_2 samples immersed in seawater medium for 14 days against *Vibrio* sp. Persian1. In addition, antibacterial activity of seawater in contact with Ag/TEPA-Den-SiO_2 and control samples for 14 days against this bacterium species after incubating at 30°C for 24 h is shown in Table 5. In both experiments, initial bacterial density in seawater after 2 h contact decreased to 0, but no reduction in bacterial density was seen in control samples. The results showed that the antibacterial efficiency of Ag/TEPA-Den-SiO_2 samples did not change over time. All the seawater mediums that contained different Ag/TEPA-Den-SiO_2 samples killed all the bacterial cells after 2 h contact period. This result could be explained by the fact that the total silver (silver ions and silver nanoparticles) concentration released from different Ag/TEPA-Den-SiO_2 samples into seawater medium ranged from 0.58 to 1.62 mg L^{-1}.

Table 4 Antibacterial capacity of Ag/TEPA-Den-SiO_2 samples after immersion in seawater for 14 days against *Vibrio* sp. Persian1 (mean ± SD)

		Contact time (h)/Vibrio sp. Persian1 count (10^5 CFU mL^{-1})			
Sample		0	2	6	24
Sea water (negative control)		0	0	0	0
Sea water + bacteria (positive control)		3.3 ± 0.81	2.72 ± 0.23	4.07 ± 0.28	1.1 ± 0.31
Sea water + bacteria + silica beads (positive control)		3.9 ± 0.89	3.5 ± 0.18	7 ± 0.27	8.8 ± 0.42
Sea water + bacteria +	Sample 1	3.5 ± 0.84	0	0	0
	Sample 2	3.3 ± 0.81	0	0	0
	Sample 3	2.8 ± 0.75	0	0	0
	Sample 4	2.9 ± 0.76	0	0	0
	Sample 5	3.4 ± 0.85	0	0	0
	Sample 6	3.2 ± 0.82	0	0	0

Table 5 Antibacterial activity of the seawater in contact with Ag/TEPA-Den-SiO_2 samples for 14 days against *Vibrio* sp. Persian1 (mean ± SD)

		Contact time (h)/Vibrio sp. Persian1 count (10^5 CFU mL^{-1})			
Sample		0	2	6	24
Sea water (negative control)		0	0	0	0
Sea water + bacteria (positive control)		9.3 ± 1.1	1.6 ± 0.47	4.5 ± 0.40	10.67 ± 1.4
Bacteria + sea water in contact with	Sample 1	3.8 ± 0.6	0	0	0
	Sample 2	2.3 ± 0.5	0	0	0
	Sample 3	5.2 ± 0.72	0	0	0
	Sample 4	4.7 ± 0.69	0	0	0
	Sample 5	4.5 ± 0.67	0	0	0
	Sample 6	5.1 ± 0.72	0	0	0

Silver exerts its antimicrobial activity by several mechanisms. Silver ions bind to sulphur or phosphorus groups in membrane proteins, create holes by which cytoplasmic content flow out the cell and then cause bacterial cell death (Taglietti *et al.* 2014). When silver ions are inside the bacterial cell, bind to DNA molecule and damage its ability to replicate (Feng *et al.* 2000) and lead to the formation of ROS, which are toxic to bacterial cells (Taglietti *et al.* 2014). Antibacterial mechanisms of silver nanoparticles may originate from their binding to the cell membrane, altering its permeability and attacking the respiratory chain (Kvitek, Panacek, Soukupova, Kolar, Vecerova, Prucek, Holecova & Zboril 2008). In addition, they can penetrate inside bacterial cell and release silver ions (Lopez-Carballo *et al.* 2012). In fact, it could be concluded that Ag/TEPA-Den-SiO_2 samples maintain their antibacterial activity for a long time by reserving of silver in the form of silver nanoparticles and slow release of silver ions in a liquid medium.

Conclusion

Different amounts of silver nanoparticles were immobilized onto silica beads by using APTES as a cross-linker molecule. Ag/TEPA-Den-SiO_2 samples showed effective antibacterial activity and inactivated 100% of *Vibrio* sp. Persian1 within a 2 h contact period. Ag/TEPA-Den-SiO_2 maintained their antibacterial effect after immersion in seawater medium for 14 days. In addition, immobilized silver on the silica beads surfaces minimized the silver released into seawater medium, so that the maximum silver released was 1.16% of the total silver immobilized on silica substrates. These results indicate that Ag/TEPA-Den-SiO_2 samples are potential antibacterial agents that can be used for disinfection of water used in shrimp larval rearing system. However, antibacterial activity of the filters that contained Ag/TEPA-Den-SiO_2 substrates and the toxicity of silver released into water for target species should be examined.

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Conflict of interest

The authors have no conflict of interest to declare.

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