Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci.
DOI 10.1007/s40011-015-0517-x

RESEARCH ARTICLE

Bactericidal Activity of Nanopolymers Against Shrimp Pathogenic Bacterium *Vibrio harveyi*

K. K. Krishnani · V. Kathiravan · N. A. Shakil · Manish Kumar Singh · M. P. Brahmane · K. K. Meena · Biplab Sarkar · K. Choudhary · Mukesh Kumar Singh · Jitendra Kumar

Received: 28 August 2014 / Revised: 18 November 2014 / Accepted: 2 March 2015

Abstract The authors have reported the use of nanopolymers for the inhibition of shrimp pathogenic bacteria for aquaculture use. The antimicrobial activity of polyethylene glycols based amphiphilic polymers have different linker molecules such as suberic acid, adipic acid, glutaric acid and dimethyl-5-hydroxyisophthalate on *Vibrio harveyi*. It was examined by drawing growth curves using optical density measurements. Concentration dependent antimicrobial effects of nanopolymers were tested against *V. harveyi* which were further confirmed by agar well diffusion for determining minimum inhibitory concentrations. The increased concentrations of nanopolymer effectively reduced the bacterial growth and increased the diameter of inhibition zone. This study has important implications for disease management in coastal aquaculture.

K. K. Krishnani (✉) · V. Kathiravan
Central Institute of Brackishwater Aquaculture, RA Puram, Chennai 600028, Tamil Nadu, India
e-mail: krishnanik@hotmail.com

K. K. Krishnani · M. P. Brahmane · K. K. Meena · B. Sarkar
National Institute of Abiotic Stress Management, Malegaon, Baramati (Dist Pune) 413115, India

V. Kathiravan
Department of Botany, Madura College, Madurai 625011, Tamil Nadu, India

N. A. Shakil · M. K. Singh · M. K. Singh · J. Kumar
Division of Agricultural Chemicals, Indian Agricultural Research Institute, Pusa, New Delhi, India

K. Choudhary
Directorate of Weed Science Research, Maharajpur, Jabalpur 482004, India

Keywords Nanopolymers · Bactericidal activity · Shrimp pathogenic bacteria · *Vibrio harveyi*

Introduction

Aquaculture is one of the most important economy in many countries. Emerging major constraints in aquaculture production are the diseases caused by bacterial strains. Vibriosis has been identified as a common problem in shrimp aquaculture, all over the world predominantly in India creating economic loss [1]. Mass mortalities in hatcheries and grow-out ponds of shrimp attributed to outbreaks of vibriosis have been recorded from many regions [2–12]. The severity of infection depends on the species and strain of *Vibrio* involved, the stage of development, age of shrimp, and the ambient environmental conditions. Over a dozen species of *Vibrio* have been identified as having implication in the disease process [3–5]. Six species of *Vibrio* i.e. *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus*, and *V. splendidus* are associated with the diseased shrimp. *V. harveyi* has been implicated as the main bacterial pathogen of shrimps, which is a luminous gram negative bacteria causing mortalities among *Penaeus monodon* larvae, post larvae and cultured shrimp [13, 14]. Vibriosis is also having its impact in grow-out ponds and is frequently responsible for the morbidity and mortalities of shrimp [15–17]. Vibriosis is also rampant in the Indian region where brackishwater shrimp farming is the main aquaculture activity. The disease problem is particularly severe in hatcheries, and in the past few years many units were shut down due to the invasion by luminous *Vibrio* [18–20]. Because luminous *Vibrios* effect serious mortalities in shrimp larval rearing systems [21], it is imperative that measures for their control be developed. Chemical treatment of luminous vibriosis among shrimp larvae is quite limited because of the ineffectiveness of existing and

Published online: 31 March 2015

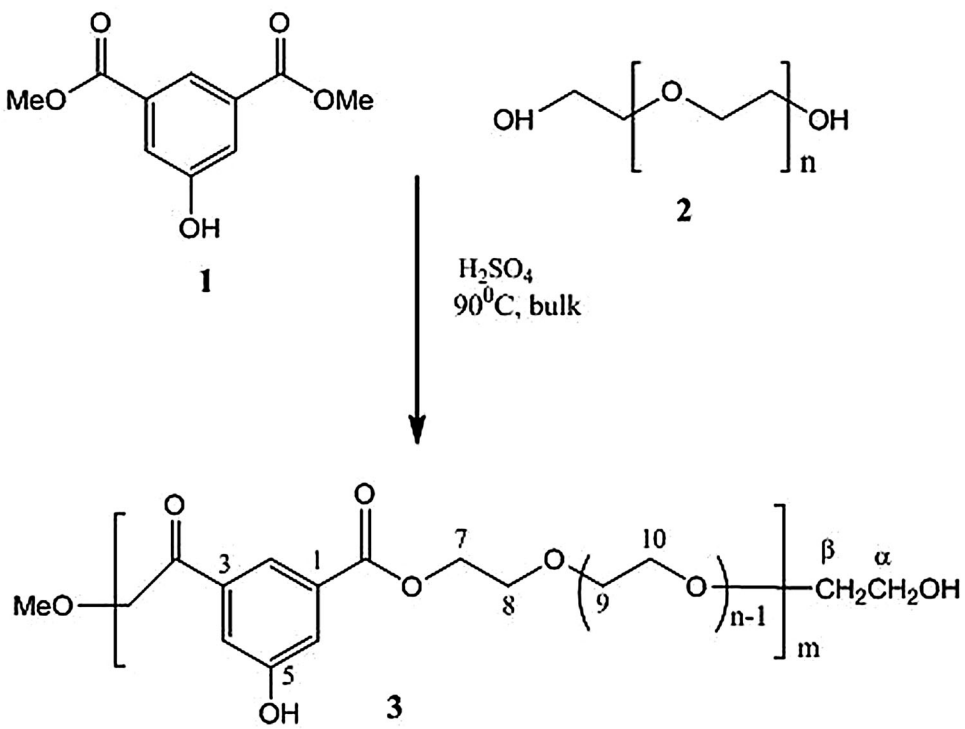
readily available drugs, possible development of resistance in bacteria, human health hazard, high toxicity and prohibitive cost of the drugs. The treatment of ponds with lime and chlorination also could not wipe out the *V. harveyi* [22]. Shrimp pathogenic bacteria *Vibrio* spp. are very difficult to be controlled in aquaculture and related aquatic environment when the stocking density is very high. There is always a need of augmentation method and developing novel bactericidal material to control the disease causing microbes. Nanomaterials have increasingly been used in water treatment because of economical and environmental viability and wider availability [23, 24]. Nanosilver and PEG conjugates are viable antibacterial agents [25, 26]. Krishnani et al. [27] have successfully demonstrated bactericidal activity of elastine like polypeptide biopolymer with polyhistidine domain and silver. However, reports on using PEG based nanopolymers with linker molecules for bactericidal activity against *Vibrio harveyi* for aquaculture are not available. In the present study, the authors report the use of PEG based nanostructured polymers with different linker molecules for the inhibition of pathogenic bacteria *V. harveyi* prevalent shrimp aquaculture.

Material and Methods

Synthesis of Nanopolymers

Amphiphilic co-polymers were synthesized and characterized according to the method reported in literature (Scheme 1) [28]. In a typical reaction, linker molecule such as suberic acid, glutaric acid, adipic acid and dimethyl 5-hydroxyisophthalate (1.0 mmol, 0.21 g) and PEG (1.0 mmol, MWt 600 (0.6 g)) were placed in a round-bottom flask (25 ml capacity). To this mixture was added the catalyst (10 % by weight w.r.t. monomers) and reaction flask was then placed in a constant temperature oil bath maintained at 90 °C under vacuum in solvent free conditions. The reaction was allowed to proceed for 48 h, after this time it was quenched by adding chloroform and filtering off the enzyme under vacuum. The organic solvent was then evaporated under vacuum and the residue was dialyzed using membrane (MWCO 10,000). After the completion of dialysis, the product polymers were freeze-dried. It was obtained as a viscous oil after freeze-drying in 90 % yield.

Scheme 1 General method of polymerization—Poly[poly(oxyethylene-600)-oxy-5-hydroxyisophthaloyl]



Characterization and Particle Size Analysis of Nanopolymers

Sample Preparation

The solutions of amphiphilic co-polymer were prepared by dispersing them in distilled water with gentle stirring for 30 min, followed by sonication for 15 min. The concentrations of the samples were varied from 0.1 mg/ml to 100 mg/ml, the sample solutions were purified by passing through a Millipore 0.2 μm filter.

Characterization by Laser Light Scattering Photometer

Static light scattering data was collected on a laser light scattering photometer (Wyatt Technology DAWN Model F)

equipped with a 632 nm He–NE laser as the light source.

Characterization of Nanopolymers by NMR

The synthesised amphiphilic polymers were characterised by ^{1}H and ^{13}C NMR. The amphiphilic co-polymers, when dissolved in water, aggregated to form nano-micelles. The size of nano-micelles, formed by the self assembly of co-polymers, varied with hydrophilic segments and was determined as reported earlier [29]. The structures of the polymers were characterized using NMR spectroscopy (Bruker 250 MHz).

Test Bacteria

The antibacterial activity of biopolymers was assessed against pathogenic bacterial strain *V. harveyi* B5 (GenBank accession no 16S rRNA: JF264473) strain isolated from *P. monodon* hatchery in the locality of Chennai in the state of Tamil Nadu. The virulent nature of this train has been confirmed by the presence of *vhh* gene (GenBank accession no *vhh* gene: KJ000877). Following primer set was used for amplification of *vhh* gene 5′-CTTCACGCTTGATGGCTACTG-3′ and 5′-GTCACCCAATGCTACGACCT-3′. The polymerase chain reaction was performed on the samples along with negative control (water) with a 20 µl reaction mixture using Eppendorf thermal cycler (Master cycler gradient). The following composition was used for a single reaction: IX 20 µl: Water 13.6 µl; buffer (× 10 Tris with 15 mM $MgCl_2$) 2 µl, 10 mM dNTP (2.5 mM) 1 µl, forward primer (30 pM) 1 µl, reverse primer (30 pM) 1 µl, *Taq* (3 U/µl) 0.2 µl, BSA (20 mg/ml) 0.2 µl, DNA template 1 µl. The amplification programs were as follows: one cycle consisting of 94 °C for 2 min, followed by 34 cycles consisting of denaturation (94 °C for 45 s), annealing (57 °C for 45 s), elongation (72 °C for 45 s) and a final extension step consisting of 72 °C for 6 min. Aliquots (10 µl) of the PCR products were electrophoresed and visualized in 1 % agarose gel by using standard electrophoresis procedures. Voltage and run time of the electrophoresis were 100 V for 20 min initially and then 150 V for 40 min.

The isolates were maintained in trypticase soy broth (TSB) at—20 °C; 300 µL of each stock-culture was added to 1.7 ml of TSB. Overnight cultures were kept for 24 h at 36 ± 1 °C and the purity of cultures was checked after 8 h of incubation. After 24 h of incubation, bacterial suspension (inoculum) was diluted with sterile physiological solution, for the diffusion to 10^{8} CFU/ml.

Test of Inhibitory Effect of Nanopolymers on Growth and Viability of Bacterial Cells

The antibacterial activity of nanopolymers at different concentrations (0–2.84 µg) has been performed by agar well diffusion method against *V. harveyi*. The bacterial inoculum was uniformly spread using sterile cotton swab on a triptic soya agar. Varying concentration of nanopolymers were added to each of the wells (4 mm diameter). The plates were stored for 30 min to allow for pre-diffusion of the sample into the agar medium. Subsequently, the plates were incubated at 37 °C and zone of inhibition was read after 24 h. Inhibition of the bacterial growth was measured in mm. For statistical studies, triplicates were used for each concentration. PEG 600 (0.058–2.84 µg) and sterile water were used in the control well.

Culture Growth Suppression Spectrophotometric Method

For bacteriological tests of nanopolymers at different concentrations, the bacterial strain was grown in broth for 18 h and then grown on solid medium. 100 µl of the proper dilution was transferred directly using special *V. harveyi* medium [30]. From agar plates, fresh colonies were inoculated into 5 ml of broth medium. Growth was monitored under UV–visible spectrophotometer, till the optical density reached 0.2 at 600 nm. Subsequently, 0.1 ml from the above was further added to freshly prepared broth medium supplemented with varying concentrations of nanopolymers. Broth solution without nanopolymers was served as control. All the tubes were incubated in a rotary shaker at 200 rpm at 37 °C. The growth was monitored at an interval of 12 h for 48 h by measuring absorbance at 600 nm, for which 0.1 ml of culture from the control and treated sample were taken out and diluted in 10fold increments. Each concentration was tested in triplicate. The lowest concentration of antibacterial solution needed to prevent visible growth of test microorganism was defined as the minimal inhibitory concentrations against the microorganism.

Statistical Analysis

There were three replica in all the experiments. Duncan's multiple range test was applied to identify significant differences among treatments. The SPSS 16.0 statistical software was used to perform statistical analysis.

Results and Discussion

Chemical Composition and Characterization of Polymers

The nanopolymers used were adipic acid (AA), suberic acid (SA), glutaric acid(GA), dimethyl-5-hydroxyisophthalate (DMHP) with PEG-600. Nanopolymers were

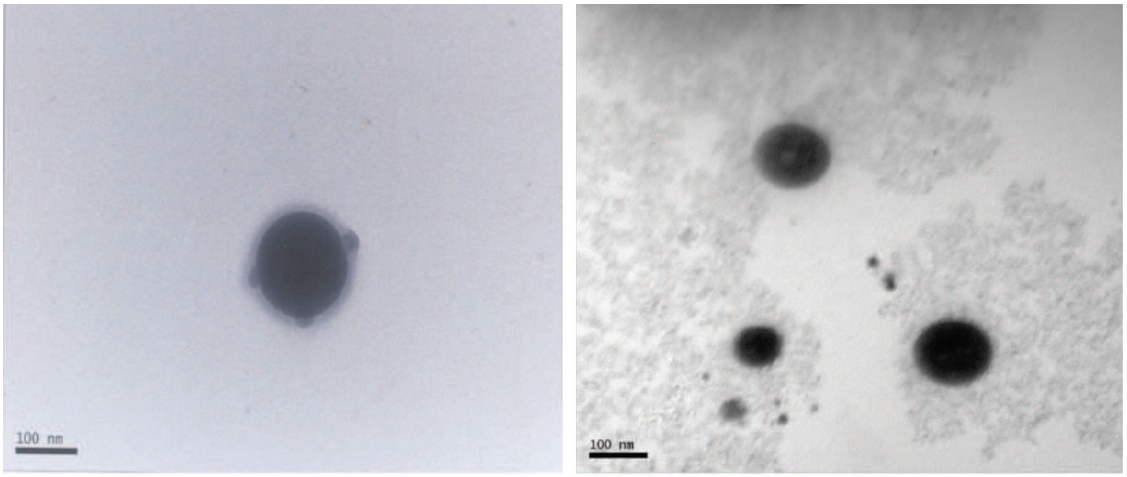


Fig. 1 TEM images of suberic acid nanopolymer

obtained by heating linker molecule (1 mmol) with PEG 600 (1 mmol, 0.6 g) in the presence of catalyst at 90 °C in solvent free conditions for 48 h under vacuum. It was obtained as a viscous oil after freeze-drying in 90 % yield. The molecules used for the synthesis of polymers are used for drug delivery and are FDA approved. Figure 1 shows the transmission electron microscopy images of suberic acid. Figure 2 represents the 1H-NMR spectrum for synthesized polymer with glutaric acid. The spectral data of one representative example of polymer is reproduced below.

1H-NMR Data ($CDCl_3$): δ 3.64–3.68 (brs, methylene PEG protons on C-9 and C-10 carbons of the repeating units and on C-α and C-β), 3.82 (t, 2H, C-8H), 3.93 (s, 3H, -COOCH3), 4.48 (t, 2H, C-7H), 7.71 (m, 2H, C-4H and C-6H) and 8.21 (s, 1H, C-2H).

13C-NMR Data ($CDCl_3$): δ 52.74 (-OCH3 end group), 62.07 (C-α), 64.74 (C-β), 69.44 (C-8), 70.93 (repeating PEG units' carbons), 72.90 (C-7), 121.43 (C-4 and C-6), 122.53 (C-2), 131.18 (C-1 and C-3), 157.57 (C-5) and 166.11 (–COOMe).

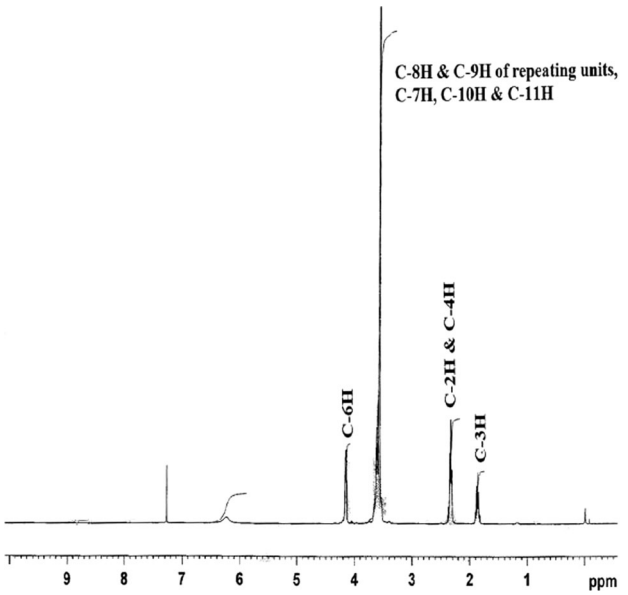


Fig. 2 ^{1}H-NMR spectrum for synthesized polymer with glutaric acid as linker

Particle Size Determination

The particle size of the aggregated polymers was determined as reported earlier [29]. The amphiphilic co-polymers when dissolved in water above their critical micelle concentration (CMC) aggregate to form nano-micelles. The CMC values were determined by static light scattering and found independent on the size of hydrophilic segment PEG as no significant change in CMC values were observed in going from PEG 300 to PEG 1000. However, the size of nano-micelles formed by the self assembly of co-polymers varied with hydrophilic segments. The radius of gyration (Rg) increased with the increase in the size of hydrophilic segment, (PEG size) and was found to be in the range of 29.21–67 [31]. Sizes of nanopolymers are as follows: glutaric acid polymer = 354 nm, adipic acid polymer = 145.7 nm, suberic acid polymer = 134.3 nm, DMHP polymer = 17.85 nm.

PCR Amplification of *vhh* Gene in Test Organism *V. harveyi*

235 bp fragment of hemolysin *vhh* gene (Fig. 3) was PCR amplified from *V. harveyi* strains B5 (GenBank accession no. JF264473) to confirm the pathogenicity nature.

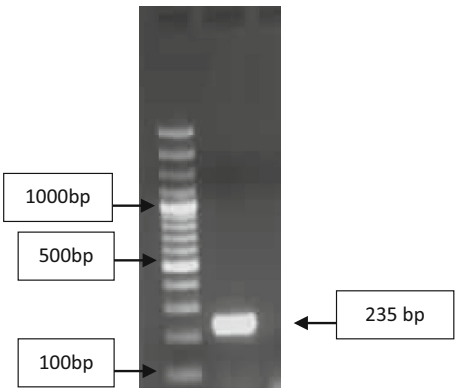


Fig. 3 PCR amplification of *Vhh* gene in *V. harveyi*

Inhibitory Effect of Polymers on Growth and Viability of Bacterial Cells

Agar Well Diffusion Method

The diameter of the inhibition zones of test bacteria using nanopolymers is given in Fig. 4. The diameter of the inhibition zones increased from 1 to 18 mm with increased concentration of the nanopolymers. A strong inhibition zone (18 mm) was obtained by AA polymer due to its higher concentration and nanosize. In spite of less concentrations used in the assay, the DMHP and SA showed 15 and 10 mm inhibition zones respecively due to their nanosizes. GA was not much active as it showed less zone of inhibition due to its higher particle size. PEG 600 at the selected concentration range of 0.058–2.84 µg and sterile water didn't show any inhibition.

Culture Growth Suppression Spectrophotometric Method

The growth curves obtained from the bacteriological tests to assess the inhibitory effect of nanopolymers are shown in Fig. 5, which revealed that nanopolymers caused substantial decrease in the cell growth and viability of *V. harveyi*. The antibacterial activity of the nanopolymers determined by growth curve method showed no effect on the bacterial growth of blank and control samples. Data are shown with mean values and standard errors of bacterial counts. All the experimental data were statistically analysed and the difference was considered statistically significant when $P \leq 0.05$. Nanopolymer of DMHP were active inhibitors of *V. harveyi,* as compared to other nanopolymers used in the present study. DMHP almost completely inhibited the growth of pathogenic bacteria at minimum concentration (0.294 µg/ml). There is no bacterial growth at 0.4 µg/ml level for SA polymer. AA polymer inhibited bacterial growth at 0.625 µg/ml. However, there

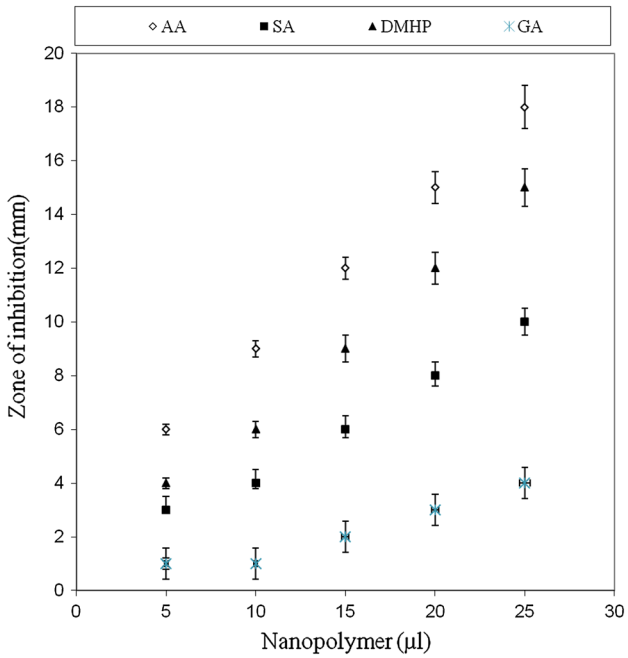


Fig. 4 Inhibition zones of samples as a function of different concentrations of nanopolymers against *V. harveyi*. Each point represents an average of triplicate measurements (5, 10, 15, 20 and 25 µl dilutions contain 0.2, 0.4, 0.625, 0.833 and 1 µg of AA nanopolymer, 0.1, 0.2, 0.3, 0.4 and 0.5 µg of SA nanopolymer, 0.058, 0.117, 0.176, 0.235 and 0.294 µg of DMHP nanopolymer and 0.568, 1.136, 1.704, 2.272 and 2.84 µg of GA nanopolymer respectively)

is no complete reduction for GA polymer even at higher concentration. The results showed that minimum inhibitory concentrations of polymers of DMHP, SA and AA are 0.294, 0.4 and 0.625 µg/ml respectively. The present result revealed that the particle size plays a major role on the inhibition of bacterial growth. Lower size nanoploymer (DMHP-17.85 nm) reduced the growth at lower concentration (0.294 µg/ml). This is due to the fact that the nano size of molecules can easily enter in the bacterial cells and damage their viability.

Current study depicted the synthesis and function of PEG derived nano polymers in the inhibition of bacterial shrimp pathogen, *V. harveyi*. PEG variety like PEG-400 is a neutral odourless, water soluble molecule having roles in cell adhesion, clumping and pharmaceutical applications. As mentioned by earlier researchers [32], concentrated PEG 400 can induce the lowering of water activity, aggregation and morphological variability of bacterial cells. Nanopolymer synthesized from different PEG types can have higher inhibitory role as nano- size enhances proficiency. This phenomenon has also been reflected in current experiments where nanopolymer exhibited efficacious inhibition in bacterial growth and colony formation. The results suggest the role of PEG nanopolymer as a potential

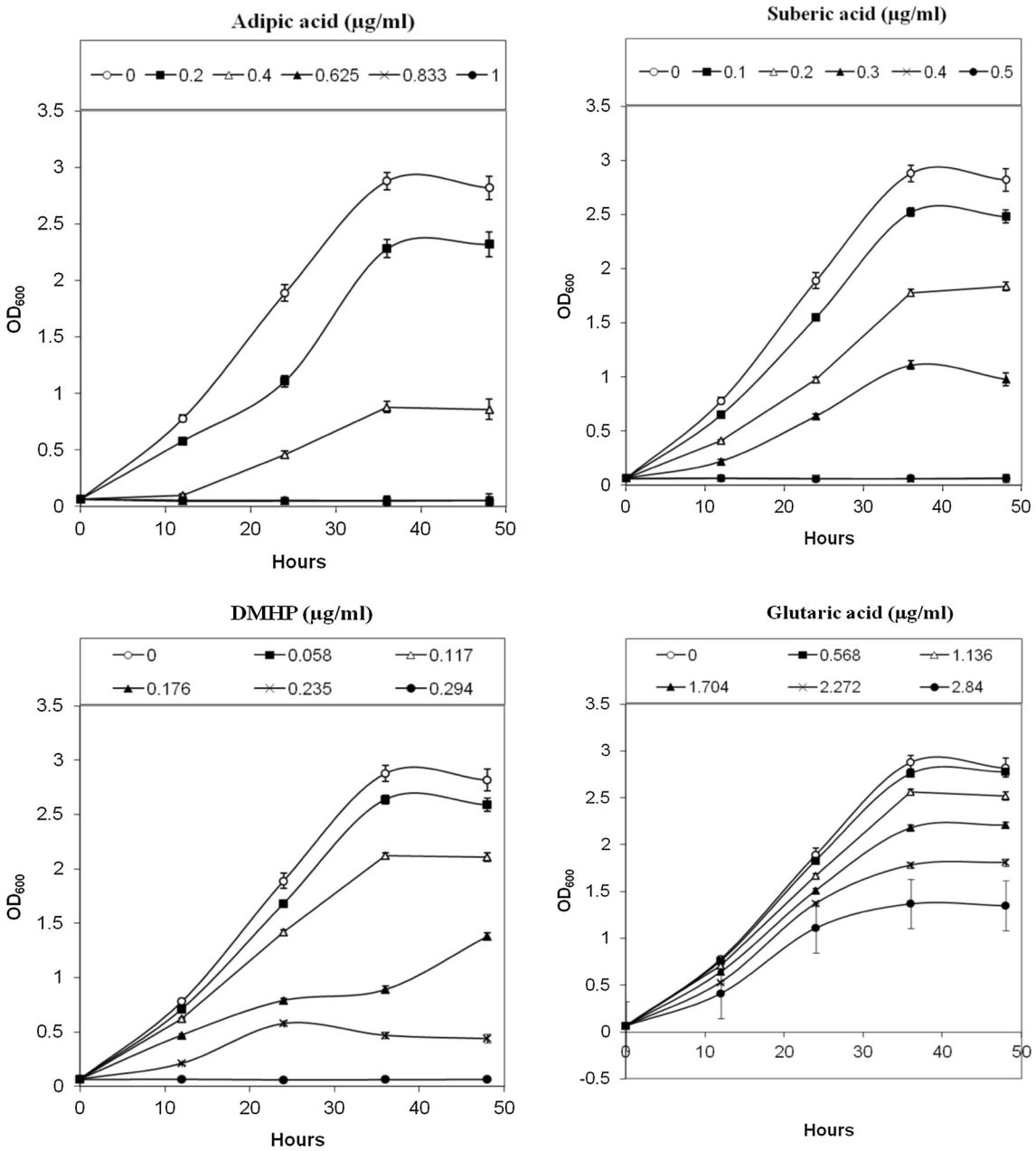


Fig. 5 Bacterial growth curve with increasing concentration of nanopolymers. Each point represents an average of triplicate measurements

therapeutic to control tropical bacterial infection like that of *V. harveyi*. But dose of application is also an important parameter as PEG in higher dose can be lethal to normal cellular behavior in shrimp. Nanopolymer can be more suitable as it can bring more output at lower administered dose. Moreover, antibacterial efficacy of nanopolymer will also vary with size, shape and attached molecular derivatives. In this experiment, dimethyl-5-hydroxyisophthalate attachment with nanopolymer exhibited higher bactericidal effect due to its nanosize. Impact of nanopolymer also depends on the nature of medium, mode of delivery and conjugation approach.

Conclusion

Vibrosis is a fatal disease inhibiting the mass production of shrimp. There are many routes like application of antibiotics, probiotics and prebiotics etc. to control these infections. Among all, antibiotics are widely applied, commercial method. But, with the invoke of global restrictions on antibiotic application in food commodity including shrimp industry, nanotechnology based therapeutic tools have appeared as a novel candidate and are getting popularity where nanopolymer can be a suitable alternative. Nanopolymer application can inhibit the infectivity in

a mechanism avoiding all conventional, well described routes. Antibacterial activity of nanopolymers was investigated, which has shown to have promising bactericidal activity against *V. harveyi* for aquaculture use. Current research on the synthesis of PEG based nanopolymers with linker molecules to enhance the antibacterial efficacy is a meaningful value addition in this direction. But, challenge trials of nanopolymer delivery at infected shrimp will be conducted to conclude upon its field application dose and biosafety issues. Successful further studies on the use of nanopolymers could be beneficial for disease management in aquaculture systems. However, they are required to undergo toxicity test prior to their use in coastal aquaculture.

Acknowledgments The authors are grateful to Director, Central Institute of Brackishwater Aquaculture, Chennai and Head of Division of Agricultural Chemicals, Indian Agricultural Research Institute, Pusa, New Delhi for their encouragements to carry out this work.

References

1. Raju GA (1994) Shrimp aquaculture in India. In : Turnbull J et al. (eds) Proceedings. of the SEAADEP, Prawn health management work-shop, Aquatic Animal Health Research Institute, DOF Bangkok, Thailand, pp 39–44
2. Couch JA (1978) Diseases, parasites and toxic responses of commercial penaeid shrimps of the Gulf of Mexico and south Atlantic coasts of North America. Fish Bull (Wash DC) 76:1–4
3. Overstreet RM (1978) Marine maladies? worms, germs, and other 353 symbionts from the Northern Gulf of Mexico. Mississippi-Alabama Sea Grant Consortium MASGP-78-021, Ocean Springs, p 140
4. Lightner DV (1988) *Vibrio* disease of penaeid shrimp. Elsevier, New York
5. Sindermann CJ (1990) Principal diseases of marine fish and shellfish, vol 2, 2nd edn. Academic Press, New York
6. Ruangpan L, Kitao T (1991) *Vibrio* bacteria isolated from black tiger shrimp, Penaeus monodon Fabricius. J Fish Dis 14:383–388
7. Chen SN, Huang SL, Kou GH (1992) Studies on the epizootics and pathogenicity of bacterial infections.in cultured giant tiger prawns, Penaeus monodon in Taiwan. In: Fulks W, Main KL (eds) Diseases of cultured penaeid shrimp in Asia and the United States. Honolulu, The Oceanic Institute, pp 20–195
8. Yang J, Wu Y, Zhu X (1992) Pathogenic biology studies on the black gill and brown spot of shell disease syndrome of penaeid shrimp infected by bacteria. Donghai Mar Sci Donghai Haiyang 10:27–33
9. De La Pena LD, Tamaki T, Momoyama K, Muroga K (1993) Characteristics of the causative bacterium for vibriosis in the kuruma prawn, Penaeus japonocus. Aquaculture 115:1–12
10. Jiravanichpaisal P, Miyazaki T, Limsuwan C (1994) Histopathology, biochemistry and pathogenicity of *Vibrio harveyi* infecting black tiger prawn, Penaeus monodon. J Aquat Anim Health 6:27–35
11. Mohney L, Bell TA, Lightner DV (1994) An epizootic of vibriosis in Equadorian pond-reared Penaeus vannamei Boone (Crustacea:Decapoda). J World Aqua Soc 25:116–125
12. Lavilla-Pitogo CR, Leano EM, Paner MG (1998) Mortalities of pond-cultured juvenile shrimp, Penaeus monodon, associated with dominance of luminescent *vibrios* in the rearing environment. Aquaculture 164:337–349
13. Moriarty DJW (1999) Disease control in shrimp aquaculture with probiotic bacteria. In: Proceedings of the 8th international symposium on microbial ecology
14. Le Groumellec M, Goarant C, Haffner P, Berthe F, Costa R, Mermoud I (1996) Syndrome 93 in New Caledonia: investigation of the bacterial hypothesis by experimental infections, with reference to stress-induced mortality. In: SICCPPS book of abstracts. SEAFDEC, Iloilo City, p 46
15. Abraham TJ, Manley R (1995) Luminous and non-luminous *Vibrio harveyi* associated with shell disease in cultured Penaeus indicus. J Aqua Trop 10:273–327
16. Hameed ASS (1994) Experimental transmission and histopathology of brown spot disease in shrimp (Penaeus indicus) and lobster (Panulirus homarus). J Aqua Trop 9:311–332
17. Jayasree L, Janakiram P, Madhavi R (2000) Characteristics, pathogenicity and antibiotic sensitivity of bacterial isolates from white spot diseased shrimps. Asian Fish Sci 13:327–333
18. Karunasagar I, Pai R, Malathi GR, Karunasagar I (1994) Mass mortalities of Penaeus monodon larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture 128:203–220
19. Hameed ASS, Farmer JJ, Hickmann Brenner FW, Farming GR (1996) Characteristics and pathogenicity of a *Vibrio campbelli* like bacterium affecting hatchery reared P. indicus (Milne Edwards 1837) larvae. Aquac Res 27:853–858
20. Shome R, Shome BR, Soundararajan R (1999) Studies on luminous *Vibrio harveyi* isolated from Penaeus monodon larvae reared in hatcheries in Andamans. Indian J Fish 46:141–147
21. Lavilla-Pitogo CR, Baticados CL, Cruz-Lacierda ER, de la Pena L (1990) Occurrence of luminous bacteria disease of Penaeus monodon larvae in the Philippines. Aquaculture 91:1–13
22. Karunasagar I, Otta SK, Karunasagar I (1996) Effect of chlorination on shrimp pathogenic *Vibrio harveyi*. World aquaculture′96, book of abstracts. The World Aquaculture Society, Baton Rouge
23. Zhang LC, Shi ZW, Wang Y, Yang R, Shi DX, Zhang GY (2011) Catalyst-free growth of nanographene films on various substrates. Nano Res 4(3):315–321
24. Ali J, Ali M, Baboota S, Sahani JK, Ramassamy C, Dao L, Bhavna (2010) Potential of nanoparticulate drug delivery systems by intranasal administration. Curr Pharm Des 16(14):1644–1653
25. Shameli K, Ahmad MB, Jazayeri SD, Sedaghat S, Shabanzadeh P, Jahangirian H, Mahdavi M, Abdollahi Y (2012) Synthesis and characterization of polyethylene glycol mediated silver nanoparticles by the green method. Int J Mol Sci 13(6):6639–6650
26. Bhattacharya D, Samanta S, Mukherjee A, Santra CR, Ghosh AN, Niyogi SK, Karmakar P (2012) Antibacterial activities of polyethylene glycol, tween 80 and sodium dodecyl sulphate coated silver nanoparticles in normal and multi-drug resistant bacteria. J Nanosci Nanotechnol 12(3):2513–2521
27. Krishnani KK, Hao Jumin, Meng X, Mulchandani A (2014) Bactericidal activity of elastin-like polypeptide biopolymer with polyhistidine domain and silver. Colloids Surf B 119:66–70
28. Kumar R, Tyagi R, Shakil NA, Parmar VS, Kumr J, Watterson AC (2005) Self-assembly of PEG and diester copolymers: effect of PEG length, linker, concentration and temperature. J. Macromol. Sci. Part A 42:1523–1528
29. Shakil NA, Singh MK, Pandey A, Kumar J, Parmar VS, Pankaj, Singh MK, Pandey RP, Arthur C, Watterson AC (2010) Development of poly(ethylene glycol) based amphiphilic copolymers for controlled release delivery of carbofuran. J Macromol Sci-Pure App Chem 47:241–247
30. Lachlan H, Leigh O, Sandra S (1996) A selective and differential medium for *Vibrio harveyi*. Appl Environ Microbiol 62(9):3548–3550

31. Kaushik P, Shakil NA, Kumar J, Watterson AC (2012) Synthesis and characterization of novel poly(ethylene glycol) based amphiphilic polymers. J Macromol Sci Pure Appl Chem Part A 49(2):111–115

32. Chirife JF, Herszage L, Joseph A, Bozzini JP, Leardini N, Kohn ES (1983) In vitro antibacterial activity of concentrated polyethylene glycol 400 solutions. Antimicrob Agents Chemother 24(3):409–412