

HIGH IMMOBILIZATION OF ANTIBACTERIAL MOIETIES ONTO MONODISPERSE MICROSPHERES BY DISPERSION POLYMERIZATION USING BICATIONIC VIOLOGEN SURFMER*

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Abstract In order to achieve monodisperse particles with high content of antibacterial groups covalently bonded on surface, a bicationic viologen, *N*-hexyl-*N'*-(4-vinylbenzyl)-4,4'-bipyridinium bromide chloride (HVV) was devised as a surfmer in dispersion polymerization of styrene (St) using a mixture of methanol (or ethylene glycol) and water as media. Effects of content of HVV, its addition profile and composition of reaction media on particles size and incorporation of HVV moieties were mainly investigated. The attachment of silver and gold nanoparticles on particle surface under UV irradiation ascertained the surface-bonded HVV segments. SEM, TEM observations and XPS, zeta potential measurements indicated that increase of initial HVV contents and addition of HVV (when polymerization had been performed for 3 h) led to grown particles and enhanced immobilization of HVV moieties. Using a mixture of ethylene glycol and water as reaction media, small particles (520–142 nm) with highly attached HVV moieties were prepared. Furthermore, antibacterial efficacy of the resultant particles against *S. aureus* was assayed, and particles with more HVV moieties anchored on surface demonstrated greater efficiency of antibacterial activity.

Keywords: Antibacterial microsphere; Monodispersity; Dispersion polymerization; Viologen; Surfmer.

INTRODUCTION

Insoluble polymeric materials with a variety of antimicrobial groups such as *N*-halamine, phenol derivatives and quaternary ammonium groups^[1] have great potentials in areas related to biomedicine, environmental protection and water treatment, thanks to their high antimicrobial activity, low residual toxicity, long durability and easy recovery. In recent decades, considerable efforts have been devoted to covalent immobilization of antimicrobial macromolecules on solid substrates^[2, 3], especially on polymeric particles^[4] because of their large specific surface area which would facilitate attachment of volumes of antibacterial groups and provide enhanced biocidal efficacies compared with bulk counterparts.

Generally, polymeric particles having permanent antibacterial surfaces can be produced by functionalization of preformed particles using molecules bearing antibacterial groups^[5, 6], or by direct copolymerization with monomers having antibacterial groups^[7]. The former way needs multiple steps to confer particles with biocidal properties, and functional groups on the preformed particles are limited to certain types of polymers, otherwise their introduction has some unfavorable binding characteristics. The latter one provides a simple technique to immediately fabricate antibacterial monodisperse spheres, particularly by employment of polymerizable quaternary ammonium cationic surfactants (surfmers) or others^[8, 9] in soap-free dispersion and

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emulsion polymerization^[10, 11].

Dispersion polymerization is a very attractive method for preparing functionalized, micron-size monodisperse polymeric microspheres by choosing an appropriate functional *co*-monomer or polymerizable agents^[12] to satisfy criteria in biological applications^[13]. Further, to avoid disturbance to uniformity of particle size caused by presence of *co*-monomers^[14] even in a small amount, methodology of “two-stage” dispersion polymerization (2-DisP) was developed, that is, adding functional *co*-monomers after a nucleation stage. However, high incorporation of functional moieties on particles surface to meet requirements in application, while keeping monodispersity of mature particles and long term stability, remains to be explored. Moreover, reactivity and hydrophilicity of comonomer or surfmer used in preparation of antibacterial particles received less attention previously, which would influence their incorporation degree on particles, as concluded by Guyot and Asua *et al.*^[15, 16].

Herein, we select a special viologen, *N*-hexyl-*N'*-vinylbenzyl-4,4'-bipyridinium bromide chloride (HVV), with moderate reactivity and hydrophilicity as a surfmer in dispersion polymerization of styrene (St) to confer antibacterial properties on particles. The bactericidal action and efficiency of viologen-containing vinyl groups and suitable alkyl chains have been extensively demonstrated in surface functionalized substrates^[17, 18], using the viologen as a monomer for grafting or further modification. But, little work to date involved application of viologen as a surfmer in dispersion polymerization. Moreover, the amount of incorporated HVV (enough to ensure antibacterial activity) and particles stability (coming from electrostatic repulsion and steric hindrance of HVV) need to be investigated.

In the present work, to achieve monodisperse particles with high degree of antibacterial viologen moieties covalently bonded on surface, increasing HVV contents, adding HVV when polymerization had been performed for 3 h and employing a mixture of ethylene glycol and water as media were attempted in dispersion polymerization of styrene. Furthermore, antibacterial efficacy of the resultant particles against *S. aureus* was assayed.

EXPERIMENTAL

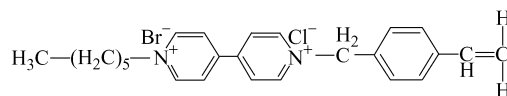
Materials

Vinyl benzyl chloride (VBC, 98%) and hexylbromide (C₆H₁₃Br, 98%) from Aldrich Chemical Co., and 4,4'-bipyridine (Bpy, 98%) from Beijing Chemicals Co. were used without further purification. Styrene (St) from Beijing Fine Chemical Industry Co. Ltd. was treated by passing through an inhibitor-removal column. Azobisisobutyronitrile (AIBN, 97%) from Beijing Chemicals Co. was recrystallized in anhydrous ethanol and stored in refrigerator before use. Solvents such as anhydrous methanol (MeOH), ethylene glycol (EG) *etc.* and other chemicals from Beijing Fine Chemical Industry Co. Ltd. were of AR grade and used as received.

Peptone, yeast extracts, agar and beef extract were purchased from Beijing Land Bridge Technology Co., Ltd, and *Staphylococcus aureus* (*S. aureus*) was obtained from Biological Resource Center, Institute of Microbiology, Chinese Academy of Sciences (IMCAS-BRC).

Synthesis of HVV

N-hexyl-*N'*-vinylbenzyl-4,4'-bipyridinium bromide chloride (HVV, Scheme 1) was synthesized according to procedure described in literature^[3]. Briefly, Bpy was mixed with C₆H₁₃Br at a molar ratio of 1.2/1 (Bpy/C₆H₁₃Br) and refluxed in acetone at 50°C for 24 h. The obtained mixture was separated by decantation, and the remaining precipitate was repeatedly washed with acetone and centrifuged for at least three cycles, then recrystallized with acetone and dried under vacuum. The produced intermediate Bpy-C₆H₁₃Br was subsequently reacted with VBC at a molar ratio of 1/1.2 (Bpy-C₆H₁₃Br/VBC) in acetonitrile at 70°C for 5 h. The precipitate was washed with acetonitrile and centrifuged for three cycles, and then recrystallized from ethanol and dried under vacuum. The desired end product was characterized by FTIR and ¹H-NMR analysis. FTIR (cm⁻¹, KBr): 1637 (—C=C), 1509 (Ar—), 719, 832, 921 (Ar—H), 1556, 1449 (BPY —C—H), 2920, 2851 (alkyl —C—H). ¹H-NMR (D₂O, δ): 1.3, 2.0 (—CH₂), 0.8 (—CH₃), 7.4, 8.6, 9.0 (Ar—H), 5.3, 5.8, 6.7 (—HC=CH₂).

Scheme 1 Chemical structure of HVV^[3]

Preparation of Poly(St-co-HVV) Microspheres

In a typical experiment, St (2.5 mL, 9.0 vol% in reaction solution), HVV (0.015 g, 0.6 mg/mL in reaction solution) and AIBN (0.045 g, 1.6 mg/mL in reaction solution) were introduced into a 150 mL four-neck round-bottom flask equipped with a PTFE stirrer, a reflux condenser and a thermometer, and containing a mixture of MeOH or EG (20 mL) and deionized water (5 mL). Polymerization was conducted with a stirring speed of 200 r/min at 70°C for 10 h under a nitrogen atmosphere. The formed microspheres were washed by extensive centrifugation/redispersion cycles with methanol and water, and then dried under vacuum at ambient temperature.

Characterizations

Fourier transform infrared (FTIR) spectra were collected on a Nicolet Nexus 670 FTIR spectrometer with KBr pellets at a scan resolution of 4 cm⁻¹. ¹H nuclear magnetic resonance (¹H-NMR) spectra were recorded on a Bruker AV600 NMR spectrometer with D₂O as a solvent.

Particles were observed with a Hitachi S-4700 scanning electron microscope (SEM) operated at an accelerating voltage of 20 kV, and a Hitachi H-800 transmission electron microscope (TEM) manipulated at an accelerating voltage of 200 kV. Samples for SEM and TEM observations were prepared by placing a drop of as-synthesized particles dispersion in methanol on a piece of clean cover glass or a carbon-coated copper grid, and then evaporating solvent at ambient temperature. The mean particle diameter ($\bar{d}_n$) and coefficient of variation (CV, %) of size distribution were evaluated statistically by measuring about 100 particles appeared in SEM images using an image processing program:

$$\bar{d}_n = \frac{\left(\sum_{i=1}^n d_i \right)}{n}, \quad CV(\%) = \frac{\left[\frac{\sum_{i=1}^n (d_i - \bar{d}_n)^2}{n-1} \right]^{1/2}}{\bar{d}_n} \times 100$$

where, n is the statistical number of particles. When CV (%) is less than 10%, particles are generally considered to be monodisperse^[19]. The less the value of CV (%) is, the more narrow the size distribution will be.

X-ray photoelectron spectroscopy (XPS) analyses of dried powder samples were conducted on an ESCALAB 250 apparatus with an AlK α radiation source at 1.2×10^{-6} Pa of analysis chamber pressure.

Zeta-potential measurements were carried out on a Brookhaven 90Plus/BI-MAS particle size analyzer with ultrasonically redispersed particles in 1.0 mmol/L NaCl aqueous solution at 25°C.

Assay of Antibacterial Effect

A colony of *Staphylococcus aureus* (*S. aureus*) was cultivated in 20 mL of a yeast-dextrose broth (containing 10 g/L peptone, 8 g/L beef extract, 5 g/L sodium chloride, 5 g/L glucose and 3 g/L yeast extract) at 37°C, shaking at 160 r/min for 18–24 h. The bacteria were diluted with 0.03 mol/L Phosphate Buffer (PBS: 7.13 g/L Na₂HPO₄·12H₂O, 1.36 g/L KH₂PO₄, pH 7.2) to the desired concentration^[3].

The conventionally prepared or as-prepared PSt particles (15 mg/mL) was immersed in 5 mL of a bacterial suspension and then shaken at 37°C for predetermined time. 1 mL of suspension was taken, diluted appropriately, and then 0.1 mL of diluted suspension was plated onto triplicate solid agar plates for overnight incubation. Each viable bacterium developed into a bacterial colony that was identified using an optical microscopy (Olympus BX60) and counted.

RESULTS AND DISCUSSION

Increase of Surfmer Fraction

In presence of a small amount of HVV (ranging from 0.6 mg/mL to 3.6 mg/mL), the reaction system of dispersion polymerization of St in a mixture of methanol and water using AIBN as an initiator exhibited high stabilization, due to the efficient electrostatic and steric effects given by bicationic viologen of HVV and its homo- or co-oligomers.

In order to improve incorporation degree of HVV moieties on particle surface, high fractions of HVV were employed, and monodisperse microspheres with large size ($\bar{d}_n$) were yielded (Table 1). XPS analysis and zeta-potential measurements demonstrated that content of element N (in moles) on particle surface and zeta-potential of particles in 0.1 mol/L NaCl aqueous solution were improved significantly with greater content of HVV charged into system (Table 1), suggesting that the increase of feed-in HVV fraction was conducive to attachment of HVV moieties on surface, and thus advantageous to stabilization and antibacterial application of particles.

Table 1. Particle size and its distribution, *N* content determined by XPS analysis and zeta-potential of particles with respect to various initial content of HVV

No.	[HVV] (mg/mL)	$\bar{d}_n$ (nm)	CV (%)	N (%)	Zeta potential (mV)
S01	0.6	673	8.4	0.49	22.8
S02	1.2	809	6.9	0.70	45.1
S03	2.0	838	5.6	0.81	47.8
S04	3.6	902	10.6	0.96	55.4

[St] = 9.0%, [AIBN] = 1.6 mg/mL, $V_{\text{MeOH}}/V_{\text{H}_2\text{O}} = 4/1$

In view of reactivity and hydrophilicity of HVV, it was conceivable that (co-) polymers of HVV were preferentially located at the most top surface of particles. That means a low reactivity of HVV was displayed in nucleation stage of dispersion polymerization because of its low content relative to St, otherwise, efficient copolymerization of HVV with St would lead to its early consumption and burying within particles^[15, 16]. While, in particles growth stage, the reactivity of HVV was improved greatly due to large consumption of St, and elevated concentration of HVV relative to St in continuous phase, which facilitated it taking part in polymerization. On the other hand, the moderate hydrophilicity of HVV favored its distribution in interfacial layer, allowed copolymerization occur primarily on particle surface. Moreover, homo- or co-oligomer of HVV generated in continuous phase was prone to coagulate onto surface. Or else, excessive hydrophilicity of HVV would produce high level of dissolved homo- or co-polymer of HVV in continuous phase.

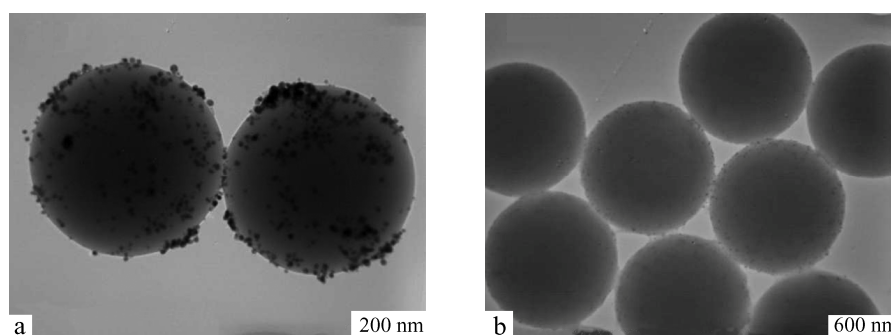


Fig. 1 TEM images of poly(St-co-HVV) microspheres with Ag (a) and Au (b) NPs attached on surface. Parameters for preparing particles: [St] = 10.0%, [HVV] = 0.6 mg/mL, [AIBN] = 1.8 mg/mL, $V_{\text{MeOH}}/V_{\text{H}_2\text{O}} = 4/1$, and for producing metal NPs: $c(\text{AgNO}_3) = 0.04$ mol/L, $c(\text{HAuCl}_4) = 0.006$ mol/L, $t(\text{irradiation time}) = 10$ min

The presence of HVV pendants on surface of particles was further confirmed by incorporation of silver and gold nanoparticles (NPs) (Fig. 1). With the aid of HVV pendants under UV irradiation, metal ions were reduced *in situ* from the corresponding salt solutions to their elemental state^[18], accompanying visually observed color of suspensions of particles changed from initial yellow to pale pink, red and violet.

When fraction of HVV was increased up to 3.6%, narrow distribution of particle size deteriorated. Further improved fraction of HVV brought about irregular aggregates of polymers instead of formation of microspheres (not showed here), and the end dispersion displayed somewhat pink-gray color, suggesting formation of polyelectrolyte which was commonly appeared in application of cationic surfmer at high contents^[8].

Supplementation of Surfmer

Apart from content of HVV fed in dispersion polymerization, the way in which to add HVV also had a marked effect on surface incorporation of HVV moieties. If additional HVV was supplemented to system when conversion of monomer was up to a high level, usually more than 80%, the reactivity of HVV could be enhanced greatly, giving rise to a completed conversion of HVV and high surface coverage of HVV moieties on particles, while not perturbing monodispersity of particles owing to the finish of nucleation.

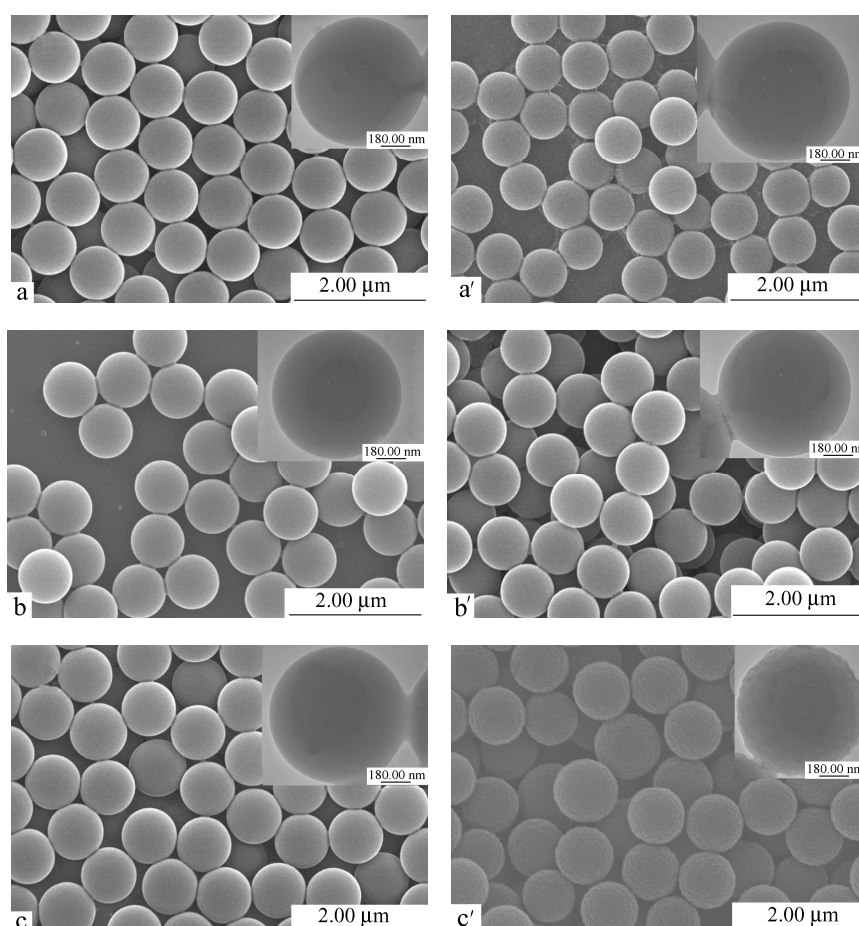


Fig. 2 SEM and TEM (the inserted) images of particles generated at reaction time of 360 (a, a'), 480 (b, b'), and 600 min (c, c'), without (a, b, c) or with (a', b', c') supplemented HVV (4.0 mg/mL). Initial reaction conditions: $[St] = 9.0\%$, $[HVV] = 2.0 \text{ mg/mL}$, $[AIBN] = 1.6 \text{ mg/mL}$, $V_{MeOH}/V_{H_2O} = 4/1$. Scale bar of inserted TEM images is 100 nm.

As shown in Fig. 2, when polymerization has been carried out for 3 h, certain amount of HVV was supplemented and then polymerization lasted for 7 h. The produced end particles grew somewhat bigger (particle diameters increased from 840 nm (Fig. 2c) to 996 nm (Fig. 2c')), and monodispersity in particle size was still maintained.

It was noted that after supplementation of HVV, the final particles presented rude surface (Fig. 2c'), implying a high coagglutination of HVV-containing oligomer on particle surface.

Further increasing the amount of supplemented HVV resulted in a slight increase in particle size, as summarized in Table 2. Moreover, both surface N element contents and zeta-potential values arose, which evidenced the enhancement of HVV moieties incorporated.

Table 2. Particle size and its distribution, N content determined by XPS analysis and zeta-potential of particles achieved with various contents of HVV supplemented in system

No.	[HVV]* (mg/mL)	$\bar{d}_n$ (nm)	CV (%)	N (%)	Zeta potential (mV)
S11	2.0	834	5.0	0.61	31.2
S12	3.2	863	2.6	0.73	37.3
S13	3.6	859	9.7	0.98	58.2
S14	4.0	896	4.8	1.20	61.1

Initial reaction parameters: [St] = 10.0%, [AIBN] = 1.8 mg/mL, [HVV] = 0.6 mg/mL, $V_{\text{MeOH}}/V_{\text{H}_2\text{O}} = 4/1$;

* HVV was supplemented when polymerization had undergone for 3 h.

Production of Small Particles

The incorporation of HVV moieties onto particle surface depended severely on particle size. Suppressing particle volume growth in polymerization was favorable to surface incorporation of HVV moieties, due to large specific surface area of small particles. In this work, selecting a solvent of ethylene glycol (EG) rather than methanol or commonly used acetone and ethanol as a component of reaction media in dispersion polymerization, particle size could be confined.

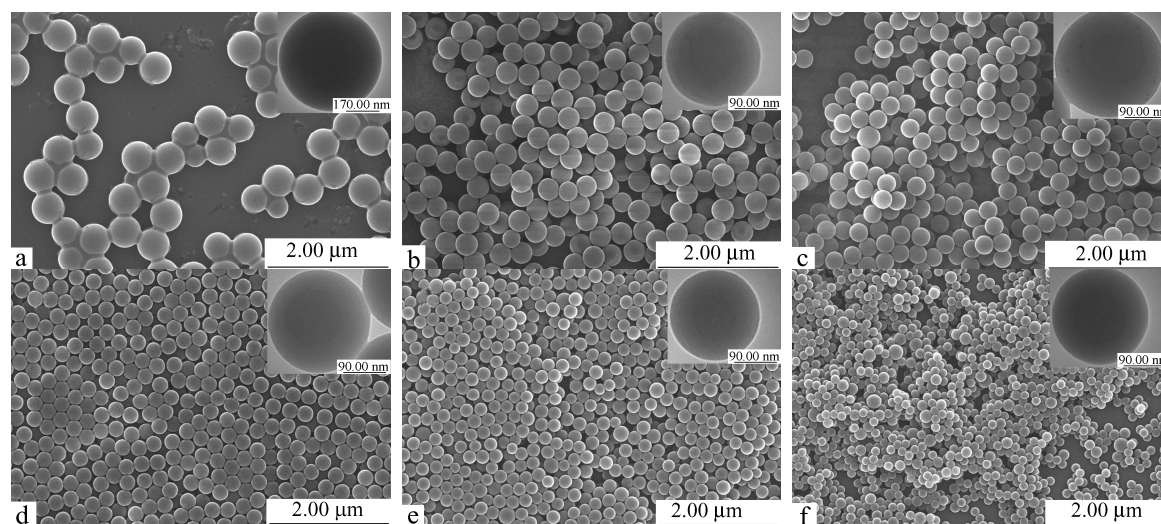


Fig. 3 SEM and TEM (the inserted) images of particles obtained with various ratios of EG/[EG + H₂O] (vol%): (a) 100, (b) 90, (c) 80, (d) 70, (e) 60, (f) 50
[St] = 9.0%, [HVV] = 0.6 mg/mL, AIBN = 1.6 mg/mL. Scale bar of inserted TEM images (a) is 170 nm, and scale bar of other TEM images (b–f) is 90 nm.

As revealed in Fig. 3 and Table 3, monodisperse microspheres with diameter of 520–140 nm were prepared in media composed of various contents of EG, substantially smaller than that synthesized in mixture of methanol and water (usually particle size is around 1 μm). With the reduction of EG content in media, particles size

declined, and at EG content of 50%, monodispersed particles of about 140 nm were achieved. When methanol, acetone or ethanol used as a component in media, and its content reduced to a value less than 85%, it is not feasible to preserve a narrow size distribution.

Furthermore, N element contents on particle surface and zeta-potential values of particles prepared in media containing EG (Table 3) were extremely greater than those obtained when mixture of methanol and water was used (Tables 1, 2), suggesting the achievement of higher incorporation degree of HVV moieties. Less EG charged into system was negative to high incorporation of HVV moieties, as indicated by the decrease in N element contents and zeta-potential values (Table 3).

Table 3. Particle size and its distribution, N content determined by XPS analysis and zeta-potential of particles with respect to various EG contents in media

No.	EG (%)	$\bar{d}_n$ (nm)	CV (%)	N (%)	Zeta potential (mV)
S21	100	520	7.9	1.72	73.1
S22	90	341	3.7	1.63	71.3
S23	80	314	1.7	1.53	66.8
S24	70	252	1.6	0.96	54.1
S25	60	218	4.3	0.78	46.8
S26	50	142	5.5	1.43	65.4

Reaction parameters: [St] = 9.0%, [HVV] = 0.6 mg/mL, [AIBN] = 1.6 mg/mL

The above results explicitly manifested the great effects of polarity of reaction media exerted on particle size and incorporation of HVV moieties in dispersion polymerization. The introduction of EG having greater polarity than methanol, or increase of water content in media reduced solvency of (*co*-)polymer of St in media, producing smaller, more contracted and compact nuclei and formation of smaller mature particles due to unfavorable diffusion of hydrophobic monomers for growth.

On the other hand, using EG instead of methanol in media, polymer portions containing rich HVV moieties could stretch fully to continuous phase and provide more static stability. In addition, high viscosity of the media containing EG could effectively prevent or mitigate aggregation of primary nuclei, permitting the formation of small particles and maintenance of narrow size distribution. However, more water content in media allowed (*co*-) polymer of HVV preferably to stay in continuous phase, which was adverse to the incorporation of HVV segments, and gave rise to slight reduction in N element contents and zeta-potential values.

When EG content was further reduced to 50%, poor solubility of St in reaction medium would make polymerization somewhat analogous to emulsifier-free emulsion polymerization, or a combination of dispersion polymerization and emulsifier-free emulsion polymerization. The latter polymerization appeared to contribute greatly to the formation of very small particles and relative high incorporation of HVV segments.

Antibacterial Effect of Microspheres

Typical sample of poly(St-*co*-HVV) microspheres with various amounts of HVV moieties immobilized on surface was applied to assess antibacterial activity through contacting the microspheres with viable cells in suspension. As shown in Fig. 4, the number of viable cells in suspension decreased by only about 2% after 3 h in contact with pristine PSt particles (control sample, containing no HVV moieties on surface), while for particles anchored high amount of HVV moieties on surface (indicated by N element content and zeta-potential value), the viable cell number decreased greatly, and after 3 h, only less than 40% of the cells was survival (S23).

CONCLUSIONS

In this paper, the possibility to employ a viologen of HVV as bicationic surfmer in dispersion polymerization of St was explored deliberately to prepare monodisperse particles with antibacterial activity. The unique properties of HVV pertaining to its moderate reactivity and hydrophilicity allowed its copolymerization occur predominantly at interfacial layer and coagglutination of its oligomers onto surface of particles, ensuring high

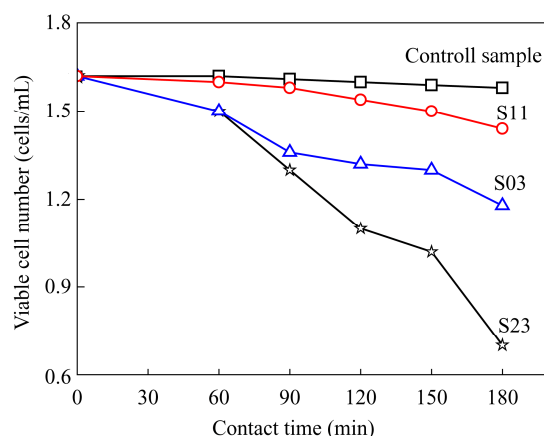


Fig. 4 Number of viable *S. aureus* cells in suspension as a function of time in contact with particles immobilized various amounts of HVV moieties on surface, indicated by N element content and zeta-potential value shown in Table 2 (S11), Table 1 (S03) and Table 3 (S23). Initial concentration of *S. aureus* in suspension: 1.62×10^{-6} cells/mL

incorporation of HVV moieties on surface and efficient stabilization to particles. To pursue antibacterial activity in a high efficiency, great amounts of HVV moieties covalently immobilized on particle surface were demanded. The obtained results indicated that increase of initial content of HVV and supplemented addition of HVV after nucleation were useful measures to enhance the incorporation degree of HVV moieties. Moreover, small particles produced in media containing ethylene glycol, were favorable to achieve high incorporation of HVV moieties.

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