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Reinforcement of antibiotic activity by nanoencapsulation of ampicillin against β-lactamase producing and non-producing strains of methicillin-resistant *Staphylococcus aureus*

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Ampicillin (ABPC) was encapsulated within n-butylcyanoacrylate by using dextran 70K, glucose, or the both mixtures as polymerization stabilizer, and many ABPC-nanocapsules with the various physicochemical properties were probed with the antibacterial activity against methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), β-lactamase producing MRSA (*blaZ* gene) and β-lactamase non-producing MRSA (no *blaZ* gene), and other germs. Morphological changes of MSSA and MRSA were assessed by scanning electron microscopy. The released ABPC was measured at various time points (1, 3, 6 or 24 h). Nanoencapsulation with ABPC resulted in an incremental increase in the antibacterial activity against MRSA penicillinase producing and non-producing strains. The nanocapsule was adhered on the cell wall of MRSA, and the morphological change was characteristically found on scanning electron microscope (SEM) image. The nanocapsulation of ABPC by n-butylcyanoacrylate was reinforced against β-lactamase producing and also non-producing strains of methicillin-resistant *Staphylococcus aureus,* and it will be a highly efficient treatment for infections caused by β-lactamase non-producing MRSA strains.

Key words: ABPC-nanocapsules; n-butylcyanoacrylate; β-lactamase non-producing MRSA.

INTRODUCTION

More than 50 years of widespread use of antibiotics has resulted in the gradual appearance of antibiotic-resistant bacteria (Leeb, 2004; Norrby et al., 2005). Methicillin-resistant *Staphylococcus aureus* (MRSA de-tection rate; ca 80%) have acquired antibiotic resistance due to the *mecA* gene that encodes alternative penicillin-binding protein (PBP 2'), resulting in the expression of an altered PBP with low affinity to methicillin (Ubukata et al., 1989).

The spread of infection by MRSA is now a serious problem. Indeed, the death toll from infection by MRSA was equal to the combined number of deaths caused by

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acquired immune deficiency syndrome (AIDS), lung cancer and road traffic accidents in the United States during 2005. Nowadays, MRSA is frequently isolated as multiple antibiotic-resistant pathogenic bacteria in clinical specimens, and infections of MRSA have spread from hospitals into the cities (Norrby et al., 2005). The emergence of antibiotic-resistant bacteria is worrying because the rate of discovery of novel antibacterial agents cannot keep pace. The development of new strategies to overcome the resistance mechanisms is now a global issue.

The antimicrobial-resistant mechanism of MRSA is classified into two principal types (Franciolli, 1991). One resistance mechanism is based on reduced binding affinity of β-lactam antibiotics to penicillin-binding protein (that is, from PBP to PBP2') encoded by the *mecA* gene. The second mechanism of resistance is hydrolysis of the β-lactam moiety of β-lactam antibiotics by β-lactamase, which MRSA secretes. The development of drug delivery systems (DDS) to combat the spread of antibiotic-resistant pathogens is currently attracting considerable interest (Garay-Jimenez et al., 2009; Litzinger et al., 1994; Liu et al., 2009). One such DDS comprises ampicillin enclosed by drug nano-carriers such as alkyl-cyanoacrylate. Covalent bonding of the ampicillin to *n*-butylcyanoacrylate (NBCA) occurs during production of the nanoparticles (NP). Intriguingly, this capsule was reported to protect the antibiotic from hydrolysis by β-lactamase (Fontana et al., 1998). However, β-lactamase non-producing MRSA accounts for ca 30% of clinical isolates in Japan (Yokoyama et al., 1996) and the development of a treatment for this type of MRSA remains largely unexplored.

The use of dextran70K or glucose as a polymerization stabilizer during synthesis of the nanoparticles gave the resulting preparation of a distinctive set of physico-chemical properties (Douglas et al., 1984, 1986). The present study focuses on the antimicrobial effect of various nanoparticles encapsulated with ampicillin (ABPC) on MRSA clinical isolates, which include β-lactamase producing and non-producing strains (Turos et al., 2007).

MATERIALS AND METHODS

Normal-butyl 2-cyanoacrylate (NBCA: Histoacryl®) was generously provided by B/BRAUN Aesculap AG & Co. (Tuttlingen, Germany). Dextran70000 (Dex-70K), glucose and ampicillin (ABPC) were obtained from Sigma-Aldrich (St. Louis, MO). HCl and NaOH were obtained from Wako Chemical Co. (Tokyo, Japan). All other chemicals were of analytical reagent grade and were used without further purification. Ultrapure water was used for the preparation of all solutions.

ABPC-encapsulated nanoparticles

ABPC (80 mg) was dissolved in either 0.01 M or 0.001 M HCl (20 ml). Dex70K (200 mg), glucose (1 g), or a mixture of Dex70K and glucose (Douglas et al., 1984) was added to the ABPC-hydrochloric acid solution. NBCA (0.25 ml) was added in a dropwise fashion to the ABPC-Dex70K-glucose or -Dex70K+glucose hydrochloric acid solution under stirring at room temperature. The stirring rate (650 rpm) was carefully chosen to ensure that the monomer was fully dispersed. The pH of the resulting colloidal suspension was adjusted to 7.0 by addition of 0.1 N NaOH. The suspension was then filtered through a 5 μm filter. The weight of ABPC-encapsulated nanoparticles in suspension was determined by subjecting the sample to ultracentrifugation at 100,000 *g* for 60 min. The supernatant was then discarded and the pellet of ABPC-nanocapsule freeze dried and weighed prior to re-suspension in distilled water. Each preparation was carried out in duplicate to ensure the results were reproducible. In addition, ABPC concentration of the initial supernatant was obtained using the optical density method (λ_{max} 254 nm) and defined as the amount of released ABPC that was not encapsulated in ABPC-nanocapsules. The ABPC loading rate of ABPC-nanocapsules was calculated from the encapsulated amount of ABPC divided by the additive amount: (encapsulated amount = additive ABPC - initial supernatant ABPC).

Particle size and zeta potential

The size of NBCA-NPs was assessed using a dynamic light scattering spectrophotometer Zetasizer nano (Malvern Instruments Ltd., Malvern, UK). The colloidal suspension of the NPs was diluted with deionized distilled water, and the particle size analysis was carried out at a temperature of 25°C. The zeta potential was measured on a Zetasizer Nano system (Malvern Instruments Ltd.). The measurements were performed using disposable zeta cells in accordance with a general purpose protocol at 25°C.

Bacterial strains

The standard strains were methicillin-susceptible *Staphylococcus aureus* (MSSA); ATCC6538 and JCM2874, methicillin-resistant *Staphylococcus aureus* (MRSA); JCM8703 and N315 GTC01187, *Enterococcus faecium*; JCM5804, *Escherichia coli*; ATCC8739, *Pseudomonas aeruginosa*; ATCC9027, and *Klebsiella pneumoniae*; Tf399A. Clinical isolates of MRSA (30 isolates in total) were provided by Yokohama-City University Hospital (Yokohama, Japan). The *mecA* gene was detected in all the clinical isolates. Of the 30 isolates, 18 were β-lactamase producing MRSA (*blaZ* gene 14) and 12 were β-lactamase non-producing MRSA (no *blaZ* gene).

Determination of antibacterial activity

The minimum inhibitory concentrations (MICs) of ABPC-nanocapsules were determined by the microbroth dilution method (National Committee for Clinical Laboratory Standards Institute; CLSI).

Morphological analysis of MSSA and MRSA

MSSA an MRSA were incubated in Mueller Hinton Broth (M-H Broth) with or without ABPC-nanocapsules and/or antibiotics for 24 h. After incubation, the culture suspension was filtered using Nuclepore™ Track-Etch membrane of pore size 0.1 um (Whatman Inc, Clifton, NJ). Morphological changes of MSSA and MRSA were assessed by scanning electron microscopy (type: S-800; Hitachi Corp., Tokyo, Japan), as shown in Figure 1.

Release of ABPC from the nanoparticles

One gram of dried nanoparticles encapsulated with ABPC was

Table 1. Physico-chemical property of ABPC-nanocapsules;A-D70 made by n-butyl cyanoacrylate(NBCA) and dextran 70K, A-Glucose by NBCA and glucose, A-DG by NBCA and mixture of Dex70K and glucose. Particle size: average of diameters of particles, PDI<0.131.

Polymerization pH in dil. HCl	Particle size (nm)		Zeta potential (mV)		Encapsulation Rate of ABPC (%)	
	pH2	pH3	pH2	pH3	pH2	pH3
A-D70	114	220	-20.5	-21.4	24.7	22.0
A-Glucose	99.4	190	-44.2	-49.2	25.5	22.8
A-DG	284	136	-20.4	-37.9	28.2	26.2

Table 2. MIC against ABPC sensitive Pathogensasogens; A-D70 made by n-butyl cyanoacrylate(NBCA) and dextran 70K, A-Glucose by NBCA and glucose, A-DG by NBCA and mixture of Dex70K and glucose. MIC:μg/ml upon CLSI.

Strain	*S. aureus*	*E. faecium*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
	ATCC6538	JCM5804	ATCC8739	ATCC9027	Kf399A
ABPC alone	0.06	2	2	256	64
A-D70	0.12	4	8	≥256	≥256
A-Glucose	0.12	4	4	≥256	≥256
A-DG	0.12	4	4	≥256	≥256

suspended in 100 ml of 0.9% saline. The suspension was sampled at various time points (1, 3, 6 or 24 h). The released ABPC was subsequently separated from the nanoparticles by centrifugation at 15,000 *g* for 15 min and then quantified by high performance liquid chromatography (HPLC) analysis. All experiments were performed in triplicate.

RESULTS

Physiological properties of nanocapsules with ABPC

The diameter of nanoparticles encapsulating ABPC was analyzed by the dynamic light scattering method using a Zetasizer Nano (Malvern Instruments) (Table 1). When dextran-70K or glucose was used as a polymerization stabilizer the diameter of the nanoparticles obtained in 0.01 N HCl solution (pH 2) was less than those in 0.001 N HCl (pH 3) solution. In contrast, a mixture of dextran-70K and glucose as stabilizer contributed to the production of larger nanoparticles in 0.01 N HCl solution by comparison to those generated in 0.001 N HCl solution (Table 1). Zeta potentials of nanoparticles were measured by electrophoresis using a Zetasizer Nano (Malvern Instruments). The zeta potential of nanoparticles encapsulated with ABPC using dextran-70K as stabilizer had a smaller negative charge than those prepared using glucose as stabilizer (Table I). The content of ABPC within nanoparticles in 0.01 N HCl solution was higher compared to those in 0.001 N HCl solution (Table 1).

Release of ABPC from nanocapsules

The elution profile of ABPC from the nanoparticles was biphasic with 30 to 40% of ABPC liberated after 1 to 3 h (Figure 2). The elution rate of ABPC from nanocapsules composed of dextran-70K was highest amongst the preparations analyzed in this study. The rate of release of ABPC from nanoparticles prepared in the presence of a mixture of dextran-70K and glucose was greater than those prepared in the presence of glucose only. The release profile of ABPC from nanocapsules made in the presence of glucose only was like monophasic that is, gradual release of ABPC from the capsule.

Antibacterial activity of the ABPC-nanoparticles

Antibacterial activity as MIC was examined against several common pathogenic bacteria, *S. aureus*, *E. faecium*, *E. coli*, *P. aeruginosa* and *K. pneumonia,* as standard strains (Table 2). The antibacterial activity of the ABPC nanocapsules against *S. aureus* and *E. faecium* decreased to approximately 1/2 that of ABPC alone. Moreover, the antibacterial activity against *E. coli* decreased from 1/2 to 1/4 that of ABPC alone. *P. aeruginosa* and *K. pneumoniae* were resistant to both ABPC and ABPC-nanocapsules. By contrast, nanoencapsulation with ABPC resulted in an incremental increase in the antibacterial activity against MRSA. Moreover, the antibacterial activity of ABPC nanocapsules obtained in 0.01 HCl increased by 4 to 8 fold compared with ABPC alone (Table 3). The antimicrobial activity of ABPC nanocapsules against MRSA-*blaZ*(+) strains, which produce penicillinase, was compared with that against MRSA-*blaZ*(-) strains, which are penicillinase non-producers (Table 3). The MRSA-*blaZ*(+) strain was much more resistant to ABPC alone than the MRSA-*blaZ*(-) strain. However, the antimicrobial activity of ABPC nanocapsules against the MRSA-*blaZ*(+) and

Table 3. MIC against MRSA (producing penicillinase); A-D70 made by n-butyl cyanoacrylate(NBCA) and dextran 70K, A-Glucose by NBCA and glucose, A-DG by NBCA and mixture of Dex70K and glucose. MIC:μg/ml upon CLSI.

Polymerization pH in dil. HCl	MRSA: (N315 strain)		MRSA: (JCM8703 strain)	
	pH2	pH3	pH2	pH3
A-D70	8	16	16	16
A-Glucose	16	16	32	32
A-DG	8	16	8	8
ABPC alone	32		64	

MRSA-*blaZ*(-) strains was stronger by 8- and 4-fold, respectively, compared with ABPC alone.

Antibacterial activity of the ABPC nanoparticles to MRSA-clinical isolates

The antibacterial activity of ABPC nanocapsules was compared to ABPC, tetracycline (TC), clarithromycin (CAM), and vancomycin (VCM) alone (Table 4). Although many of the MRSA strains displayed multiple antibiotic drug resistance and were resistant to both TC and CAM, they were all sensitive to the ABPC-nanocapsules, as VCM. However, methicillin sensitive *S. aureus* were sensitive to ABPC, TC, CAM and VCM.

DISCUSSION

In this study, the antibacterial activity of the ABPC-nanocapsules against MRSA pathogens was evaluated based on the physiochemical properties of each of the nanocapsules *in vitro*. The antibacterial activity of the ABPC-nanocapsules against several common ABPC sensitive pathogens was assessed. Our findings show that the antibacterial activity of ABPC-nanocapsules was 1/2 that of ABPC alone against *S. aureus* and *E. faecium* (Table 2). Moreover, the antibacterial activity of ABPC-nanocapsules was 1/4 that of ABPC alone against *E. coli* (Table 2). The ABPC-nanocapsules had no antibacterial activity against *P. aeruginosa* and *K. pneumonia*, which were resistant to ABPC. The lower level of activity of the ABPC-nanocapsules towards Gram-negative bacteria by comparison to Gram-positive bacteria is thought to result from the structure of their outer cell wall. Specifically, the presence of lipopolysaccharide (LPS) in the outer cell wall in the Gram-negative bacteria is believed to act as an effective barrier to prevent uptake of the antibiotic into the cell (Snyder and McIntosh, 2000). LPS is absent in Gram-positive bacteria resulting in higher antimicrobial activity of ABPC-nanocapsules.

The antibacterial activity of ABPC-nanocapsules against MRSA was found to be more potent than ABPC alone (Table 3). The mutation of PBP to PBP2' in MRSA decreases the affinity of this protein for β-lactam antibiotics (Hartman and Tomasz, 1981; Piddock et al., 1992). The binding properties of nanoparticles are strongly influenced by the zeta potential on their surface (Hu et al., 2002; McCarron et al., 1999). The integrated surface structure of the ABPC-nanocapsules is closely related to their enhanced affinity for PBP2' rather than PBP. Another antibiotic resistance mechanism found in MRSA is the production of β-lactamase. The covalent binding of ABPC to ethylcyanoacrylate nanoparticles has been reported (Fontana, 1998) to prevent the hydrolysis of β-lactam antibiotics by β-lactamase. The MIC50 and MIC90 of ABPC-nanocapsules against clinical isolates of MRSA were lower than those of ABPC alone, as shown in Table 4. For penicillinase producing clinical isolates, the ABPC-nanocapsules gave much greater antimicrobial activity over ABPC alone. The effect of encapsulating ABPC within nanoparticles to protect against hydrolysis by β-lactamase was first assessed in this study (Tables 3 and 4). Furthermore, the MIC50 and MIC90 of ABPC-nanocapsules against penicillinase non-producing clinical isolates were also lower than those of ABPC alone. These results show that the antibacterial activity of ABPC within nanoparticles is reinforced against MRSA penicillinase producing and non-producing strains.

Given that the antibacterial activity of ABPC is enhanced by nanoencapsulation against β-lactamase producing and non-producing strains, the improved antimicrobial activity does not solely arise from avoiding the effect of β-lactamase. Thus, the morphological changes in MRSA caused by ABPC-nanocapsules were different from those induced by ABPC alone (Figure 1). It is likely that binding of ABPC-nanocapsules to the cell wall will result in a release of ABPC at high concentration close to the adherence point. The release of ABPC from ABPC-nanocapsules was categorized as monophasic or biphasic depending on the polymerization stabilizer used to prepare the nanocapsules (Figure 2). For example, ABPC is released in a biphasic manner (i.e. ~40% ABPC after 4 h) from the ABPC encapsulation by ethylcyanoacrylate (Fontana, 1998).

In this study, 65% ABPC was released from ABPC-nanocapsules after 24 h. The surface property of

Table 4. MIC of ABPC, TC, CAM, VCM, and ABPC-nanocapsules against MSSA and MRSA (producing penicillinase strains upon *blaZ* gene, and non-producing penicillinase strains).

Strain	*blaZ*	ABPC	TC	CAM	VCM	ABPC-nanocapsules
MSSA 6538	-	≤0.06	0.125	≤0.125	1	0.125
2874	-	2	0.5	0.25	1	4
MRSA N315	+	32	0.125	≥128	1	16
8703	+	64	256	≥128	2	16
MRSA clinical isolates						
1423	-	16	0.5	0.5	1	2
1846	-	8	64	≥256	1	4
1801	-	16	64	≥256	1	8
1858	-	16	64	≥256	1	2
2022	-	16	64	256	1	8
2046	-	16	64	256	0.5	2
2137	-	16	0.5	256	0.5	4
2232	-	8	64	256	0.5	4
2790	-	8	64	256	1	2
3077	-	8	64	256	1	2
3223	-	8	64	256	1	2
3811	-	8	32	≥256	1	2
1447	+	32	32	≥256	0.5	4
1739	+	128	64	≥256	1	4
1847	+	64	0.5	≥256	1	4
1870	+	64	64	≥256	1	4
2005	+	64	8	128	0.5	16
2107	+	128	64	≥256	0.5	4
2370	+	128	2	128	0.5	8
2526	+	64	0.5	128	0.5	8
2836	+	16	64	256	1	4
2928	+	32	64	256	1	8
3137	+	16	16	256	0.5	4
3200	+	32	0.5	256	0.5	4
3334	+	16	64	≥256	0.5	4
3351	+	8	0.5	0.5	0.5	4
3428	+	32	64	≥256	1	4
3785	+	16	64	≥256	1	4
4147	+	64	64	≥256	1	4

Resistance upon CLSI: ABPC ≥ 0.5 μg/ml, TC ≥ 16 μg/ml, CAM ≥ 8 μg/ml, VCM ≥ 32 μg/ml. MIC50 against MRSA *blaZ*(+) strains: 4 μg/ml, MIC90: 8 μg/ml. MIC50 against MRSA *blaZ*(-) strains: 2 μg/ml, MIC90: 8 μg/ml.

the nanoparticles differ depending on the type of cyanoacrylate derivative and polymerization initiator (Table 1) used in their preparation. In addition, the surface property affects the release rate of ABPC (Figure 2). The antibacterial activity of ABPC-nanocapsules can be deduced from the following equation:

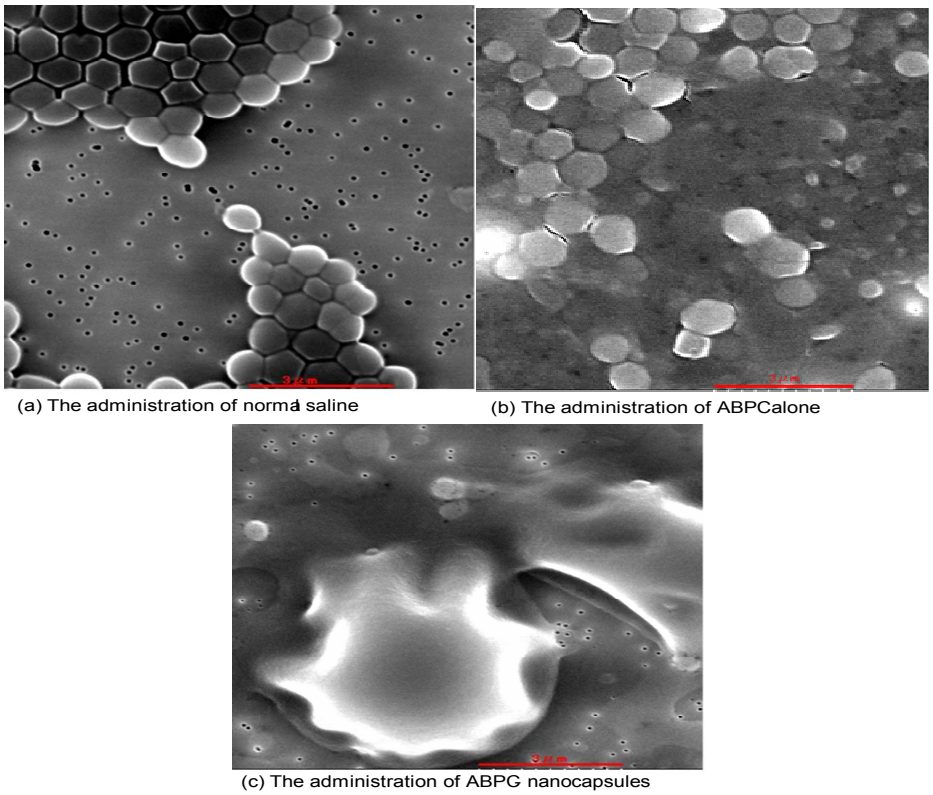
(Antimicrobial activity of ABPC-nanocapsules) = (activity of released ABPC) + (activity(X) on binding nanocapsules)

The MIC value of ABPC alone was put into the (Antimicrobial activity) part of the equation. The concentration of released ABPC was put into (activity of released ABPC), and the (activity(X) on binding nanocapsules) was calculated from the conjugation index.

Table 5. The antimicrobial activity (X) by binding ABPC-nanocapsules against MSSA and MRSA (producing penicillinase strains upon *bla*Z gene, and non-producing penicillinase strains).

Staphylococcus aureus	**Activity(X) by binding ABPC-nanocapsules**					
	Nanocapsules from Dex-70K		**Nanocapsules from glucose**		**Nanocapsules from Dex-70K and glucose**	
MSSA	-0.43	<0	-0.01	<0	-0.05	<0
ATCC6538	-4.22	<0	-0.04	<0	-1.38	<0
JCM2874						
MRSA (*bla* Z+strain)	+28.77	>0	+23.86	>0	+28.66	>0
N315	+57.54	>0	+48.07	>0	+60.66	>0
JCM8703						
Clinically isolated MRSA (*bla* Z-strain)	+9.54	>0	+7.86	>0	+9.36	>0
1801	+9.54	>0	+7.86	>0	+9.36	>0
2022						
Clinically isolated MRSA (*bla* Z+strain)	+57.54	>0	+48.07	>0	+60.66	>0
2005	+57.54	>0	+48.07	>0	+57.36	>0
2526						

X>0: the decrement of antibacterial activity. X<0: the increment of antibacterial activity.



(a) The administration of normal saline

(b) The administration of ABPCalone

(c) The administration of ABPC nanocapsules

Figure 1. The morphological changes of MRSA(N315) caused by ABPC-nanocapsules after 12 h (on SEM image× 10,000). Black spots are filter holes (approx. 100nm). Nanocapsules were binding on surface of MRSA.

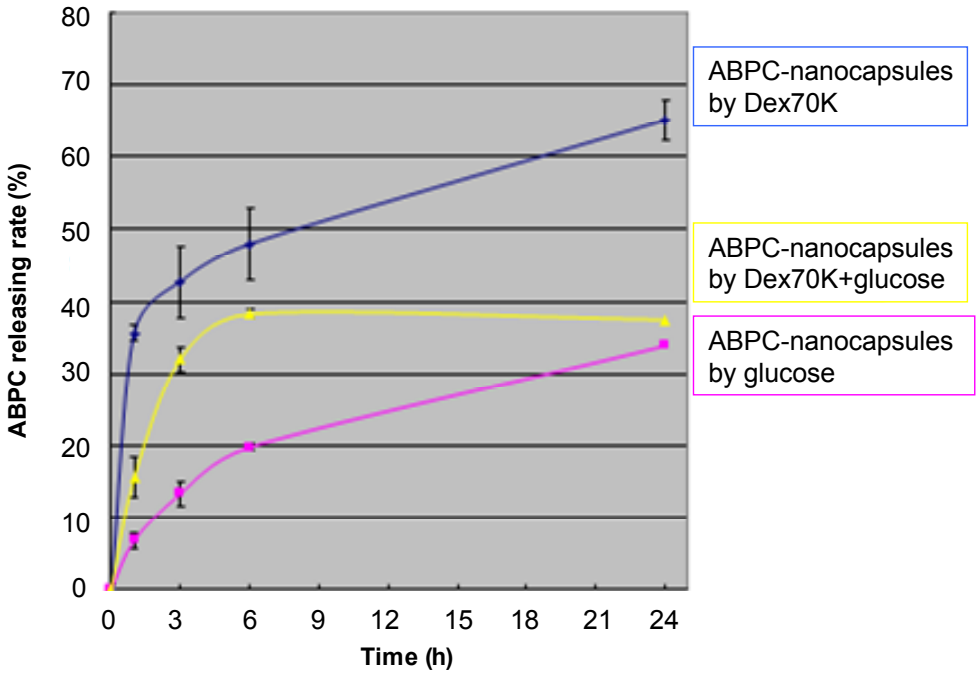


Figure 2. ABPC releasing profiles from ABPC-nanocapsules made by Dextran 70K, by Dextran 70K+glucose, or by glucose.

When the "activity of a nanoparticle" was set to X from this formula, X ≥ 0 shows antimicrobial activation reinforcement, whereas X ≤ 0 shows an antimicrobial activity attenuation effect (Table 5).

We conclude that the nanocapsulation of ABPC by n-butylcyanoacrylate was reinforced against β-lactamase producing and also non-producing strains of methicillin-resistant *S. aureus,* and that it will be a highly efficient treatment for caused by β-lactamase non-producing MRSA strains.

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Conflict of interest

Authors declare that there are no conflicts of interest.

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