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Penicillin-bound polyacrylate nanoparticles: Restoring the activity of β-lactam antibiotics against MRSA

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Abstract—This report describes the preparation of antibacterially active emulsified polyacrylate nanoparticles in which a penicillin antibiotic is covalently conjugated onto the polymeric framework. These nanoparticles were prepared in water by emulsion polymerization of an acrylated penicillin analogue pre-dissolved in a 7:3 (w:w) mixture of butyl acrylate and styrene in the presence of sodium dodecyl sulfate (surfactant) and potassium persulfate (radical initiator). Dynamic light scattering analysis and atomic force microscopy images show that the emulsions contain nanoparticles of approximately 40 nm in diameter. The nanoparticles have equipotent in vitro antibacterial properties against methicillin-susceptible and methicillin-resistant forms of *Staphylococcus aureus* and indefinite stability toward β-lactamase.
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The continuing rise in microbial drug resistance has led to widespread problems in the treatment of bacterial infections.[1] Of particular concern are those illnesses caused by methicillin-resistant *Staphylococcus aureus* (MRSA), which are responsible for a majority of hospital-acquired infections, clinical complications, and nearly 100,000 deaths each year in the United States alone.[2,3] The loss of effectiveness of commonly used antibacterial antibiotics such as penicillin and other β-lactam drugs further adds to the dilemma, calling for the immediate need for improvements in drug design, discovery, and delivery. One of the major challenges in treating antibiotic-resistant bacterial infections is the need to develop agents that can stop the infection at the site of initiation, which frequently occurs in regions of the body where water-soluble drugs typically have poor access. However, the application of lipophilic agents to combat such infections likewise has limited effectiveness due to uptake and delivery issues resulting from low water solubility and biodistribution.[4] The ability to deliver antibacterial drugs to infections in fatty tissue or on the surface of implanted medical devices, for example, where microbial biofilms often develop, ultimately determines if the infection can be cleared without surgical intervention.[5] New drug delivery vehicles such as liposomes and nanoparticles offer a promising way to improve bioavailability, efficacy, and specificity of pharmaceutical compounds in general. Several groups have reported previously on the preparation and antibacterial testing of various penicillin- or ampicillin-entrapped polycyanoacrylates formed by anionic emulsion polymerization in water.[6–15] These emulsified suspensions consisted of drug-containing particles considerably larger than 100 nm in diameter, and reportedly provided enhancement of the drug's performance against some of the microbes tested. The use of antibiotic-laden nanoparticles such as those for drug-resistant bacteria such as MRSA has not been investigated, nor has there been an attempt to evaluate the effect of having the drug covalently bound to the nanoparticle versus non-covalently encapsulated within its matrix. In this report, we describe the results of our studies on the synthesis and evaluation of penicillin-bound polyacrylate nanoparticles against MRSA as a function of the

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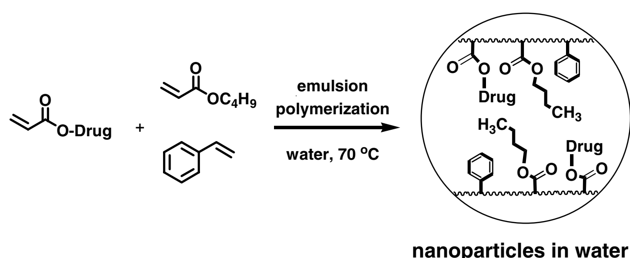


Scheme 1. Emulsion polymerization to make antibiotic-conjugated polyacrylate nanoparticles in water.

penicillin structure, linkage type, and location (for covalently bound systems), and covalently conjugated versus drug-encapsulated nanoparticle emulsions.

Recently we published[15] a procedure for preparing antibacterially active polyacrylate nanoparticles, which enhances the water-solubility and antibacterial properties of highly lipophilic N-thiolated β-lactam antibacterials.[16,17] In this method, the water-insoluble antibiotic was first converted to an acrylated form, then dissolved in a mixture of liquid monomers (butyl acrylate and styrene) before being emulsified with a surfactant and polymerized in the presence of a water-soluble radical initiator (Scheme 1). This procedure enhances the performance of water-insoluble antibiotics that act on targets within the bacterial cell.

The aim of the current study was to try to advance this nanoparticle methodology to penicillins that are used clinically in a water-soluble salt form and whose targets reside along the outer periphery of bacterial cell membranes. β-Lactam antibiotics such as the penicillins have been mainstays of clinical treatment for many types of bacterial infections, however, their effectiveness is significantly compromised in bacteria that produce β-lactamase enzymes which hydrolyze the β-lactam ring to an inactive ring-opened product. The hope was that penicillins could be incorporated into the nanoparticle framework during emulsion polymerization, despite their ready water-solubility, and be sheltered inside the matrix of the nanoparticle from bacterial penicillinase degradation. If this could be achieved, the β-lactam antibiotics could potentially be rendered highly effective against MRSA. As prototypes to explore this, five acrylated penicillins **1**–**5** were employed as monomeric substrates as a means to produce antibiotic-containing nanoparticles in aqueous media (Fig. 1). Our primary objective in selecting these particular penicillin derivatives was to evaluate the effect of the location, length, and polarity of the acrylate moiety linking the drug to the polymer framework (**NP1**–**NP5**), versus having the drug molecule non-covalently contained within the nanoparticle matrix (**NP6** and **NP7**).

Penicillin acrylimide **1** was synthesized from commercially available penicillin G potassium salt via its trimethylsilyl ester, which was reacted with acryloyl chloride followed by an aqueous work up (Scheme 2). It is our assumption that the acylation occurred selectively on the amido nitrogen rather than on the mesomeric oxygen center, to give the imide **1** as shown.

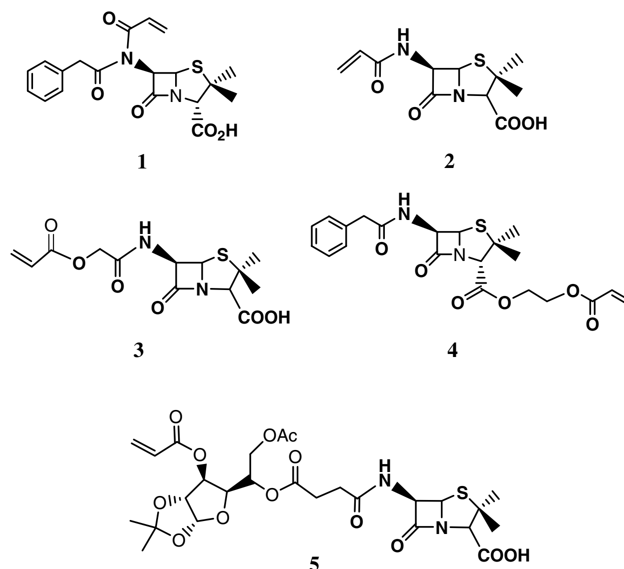
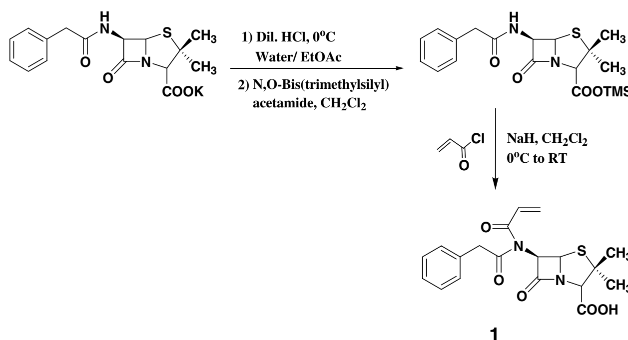


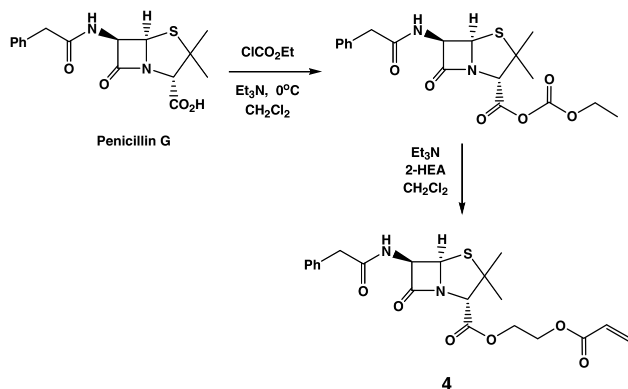
Figure 1. Acrylated penicillin monomers **1**–**5** used to prepare drug-conjugated nanoparticles **NP1**–**NP5**, respectively.



Scheme 2. Synthesis of penicillin acrylamide monomer **1**.

Penicillin acrylamide monomers **2** and **3** were synthesized from 6-aminopenicillanic acid using either acryloyl chloride or acryloyloxyacetyl chloride in the presence of triethylamine in methylene chloride. Similarly, glycosylated penicillin monomer **5** was synthesized from 6-aminopenicillanic acid and the relevant carbohydrate acyl chloride. Acrylated penicillin G monomer **4** was prepared by stirring penicillin G free acid with triethylamine and an equivalent of ethyl chloroformate at 0 °C, then further with 2-hydroxyethyl acrylate (2-HEA) as a means to produce the esterified penicillin G (Scheme 3).

Two penicillin G analogues (**6** and **7**) were also prepared that lack an acrylated side chain in order to construct nanoparticles (**NP6** and **NP7**) where the drug is incorporated non-covalently (Fig. 2). These two analogues were chosen because while penicillin G (**6**) possesses the free carboxyl acid needed for binding to the transpeptidase target, analogue **7** does not and would presumably



Scheme 3. Synthesis of penicillin monomer **4**.

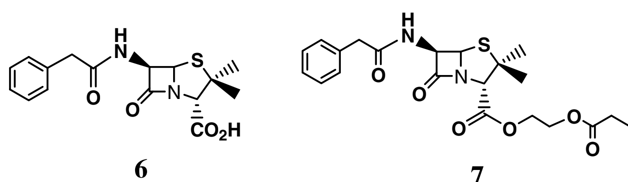
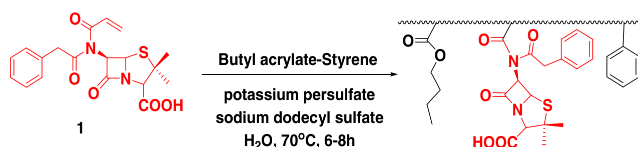


Figure 2. Penicillin G (**6**) and penicillin ester **7** used for preparing drug-encapsulated nanoparticles **NP6** and **NP7**, respectively.

require cleavage of the ester moiety to be effective as an antibiotic.

Commercially available penicillin G potassium salt was first converted to the more highly organic-soluble free acid **6** using dilute hydrochloric acid, which was then subjected to EDCI coupling with 2-hydroxyethyl propionate to produce ester derivative **7**. Penicillin analogues **1**–**7** were rigorously purified by column chromatography prior to use in the emulsion polymerization.

Penicillin nanoparticles were prepared by dissolving one of the seven antibiotics (1% w/w) in a 7:3 (w/w) mixture of butyl acrylate and styrene (Scheme 4).[8] This mixture was then pre-emulsified by adding sodium dodecyl sulfate (3% w/w) as a surfactant with stirring in nano-purified water. The resulting micelles were then heated in a 70 °C oil bath under an atmosphere of nitrogen and treated with potassium persulfate (0.5% w/w), a water-soluble radical initiator, to induce free radical polymerization. The reactions were allowed to run at 70 °C for 6 h with rapid stirring. After cooling to room temperature, the samples were characterized to determine their antibacterial capabilities, average size, shape, and stability in solution.



Scheme 4. Representative example of an emulsion polymerization using acrylated penicillin monomer **1**.

In vitro experiments were conducted to determine if the penicillin-containing nanoparticles displayed antibacterial activity against a methicillin-susceptible form of *S. aureus* (MSSA), and if so, whether this activity could be retained against a methicillin-resistant form (MRSA). Minimum inhibitory concentration values (determined by broth dilution assays) for each of the samples are provided in Table 1. These assays showed that penicillin-bound nanoparticles (**NP1**–**NP4**) and the penicillin-encapsulated nanoparticles (**NP6** and **NP7**) displayed significant antibacterial activity against both MSSA and MRSA, while the antibiotic-free nanoparticles (**NP0**) were completely inactive. Penicillin G (free drug form) is about 1500 times less active against MRSA compared to MSSA, indicative of the presence of penicillinase in MRSA.

While none of the penicillin nanoparticles had the antibacterial activity of penicillin G alone against *S. aureus*, the nanoparticles **NP1** did display much stronger activity than penicillin G against MRSA. In fact, the penicillin-containing nanoparticles displayed equal antibacterial activities for *S. aureus* and MRSA, indicating that the β-lactamase enzyme exuded by the bacterium does not at all affect the activity of the drug-bound nanoparticles. This was further demonstrated with a control experiment on agar plates in which the nanoparticle emulsion **NP1** was screened against *S. aureus* (MSSA) in the absence versus the presence of added β-lactamase enzyme (Fig. 3). The image on the left shows three different amounts of **NP1** (20, 50, and 100 μg of antibiotic) as well as penicillin G (20 μg) as a positive control. The penicillin G free drug produces a large clearing zone due to inhibition of MSSA growth. However, when 100 μg of commercial penicillinase (Type I from *Bacillus cereus*) is added to the media (right image), penicillin G loses all of its antibiotic activity, while the nanoparticles **NP1** retain their original inhibition capabilities. This confirms the ability of the nanoparticle to protect the antibiotic from enzymatic degradation by penicillinase (even at unusually high concentrations of protein) without curtailing its antibacterial properties. While this agar diffusion experiment is illustrative, the broth MIC

Table 1. Antibacterial activity of penicillin-containing nanoparticles **NP1**–**NP7** against *Staphylococcus aureus* (ATCC 25923) and MRSA (ATCC 43300), as determined by broth dilution MIC assays[a]

Sample	Microbe	
	Staphylococcus aureus (μg/mL)	MRSA (μg/mL)
NP0 (control)	>256	>256
NP1	2	2
NP2	64	64
NP3	16	16
NP4	16	16
NP5	>256	>256
NP6	64	64
NP7	64	64
Penicillin G	0.012	16

[a] MIC values refer to the lowest concentration of the bound drug (μg/mL) required to completely inhibit bacterial growth for 24 h in culture. MIC determinations were run in triplicate and the values obtained for each compound were found to be invariant.

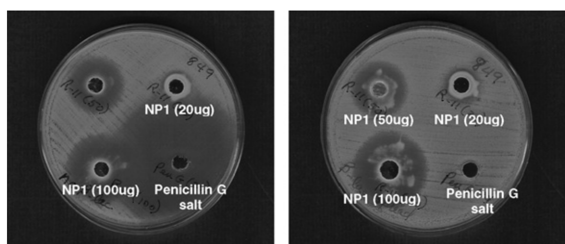


Figure 3. Kirby–Bauer studies of **NP1** using *Staphylococcus aureus* (ATCC 25923). Assays were performed first in the absence of added penicillinase protein (left image) and then in the presence of 100 μg of penicillinase added to the agar (right image). The control, penicillin G, lost all of its activity in the presence of the enzyme, while **NP1** retained its original activity at all three drug amounts, as noted. The cloudy white spots appearing in the inhibition zones in the penicillinase-treated plate (right) appear to be due to uneven diffusion of the polymer through the agar in the presence of the added protein, which we have observed occasionally in Kirby–Bauer experiments (and seen around the edges of the wells), and not from surviving bacterial colonies. These spots were sampled and cultured on agar to confirm that no surviving bacteria were present.

measurements in Table 1 are more meaningful for quantitatively assessing in vitro activity of these nanoparticle-bound antibiotics. **NP1** also retained its full antibacterial activity against MRSA after being exposed to 10% fetal bovine serum (FBS) for 24 h, indicating that the nanoparticles are stable to the various enzymes and other degradative components of serum. MIC assays performed by substituting the commonly used Mueller–Hinton broth with 10% FBS solution confirmed that the MIC values of **NP1** remained at 2 μg/mL, respectively, against both the MSSA and MRSA strains.

The microbiological data in Table 1 suggest that the penicillin G-conjugated nanoparticles (**NP1** and **NP4**) are significantly more active than the 6-aminopenicillanic acid-containing nanoparticles (**NP2** and **NP3**), and that the type of linkage used to covalently attach the drug moiety to the polymer backbone significantly alters the activity of the drug-conjugated nanoparticle. Both the type and the location of the linkage were varied in order to optimize the antibacterial activity of the penicillin nanoparticle system. The data suggest that the bioactivity follows the relative order of hydrolytic sensitivity of the linkage, with the imide > ester > amide (**NP1** > **NP3** = **NP4** > **NP2** ≫ **NP5**). The fact that the carboxylic ester-linked system, **NP4**, is active against both MSSA and MRSA is notable, in that it implies that the active drug is cleaved from the nanoparticle framework, perhaps by esterases in the bacterial cell or along the bacterial membrane, since the free carboxylic acid form of penicillin is required for interaction with the target transpeptidases of *S. aureus*. These data also indicate that antibacterial activity is somewhat greater when the drug is covalently bound to the polymeric nanoparticle, as opposed to being non-covalently encapsulated within the nanoparticle, as in **NP6** and **NP7**. This suggests that the nanoparticle may enhance delivery of the bound antibiotic to the bacterial cell. Additional studies are underway in our laboratory to understand this interaction and to obtain detailed insight as to how the drug is being released into the cell. The MIC data obtained for all of the penicillin nanoparticles against MSSA and MRSA infer that drug release must occur *after* interaction of the particle with the cell, since release of the β-lactam drug outside the cell would likely result in rapid hydrolytic cleavage of the antibiotic by extracellular penicillinases exuded by the MRSA, and thus loss of bioactivity. This expected loss of activity is clearly not what we observe for the nanoparticles. Our attempts to measure the rate of cleavage of the drug molecules from the nanoparticle matrix using various commercial esterases failed to provide any useful information thus far. We suspect that the cleavage event may require not only the presence of the esterase but also the interaction between the nanoparticle and the cellular membrane.

Particle size analysis of the emulsions was performed using transmission electron microscopy (TEM), atomic force microscopy (AFM), and dynamic light scattering (DLS) analysis, which all indicated that the nanoparticles possess uniformly spherical morphology with diameters of 25–40 nm (Fig. 4). Zeta potential analysis showed that the particles' surface charge in the emulsions ranged from −40 to −80 mV, suggesting high particle stability. The thermal stability of the nanoparticle emulsions was also examined over a range of storage conditions. Experiments indicated that the original emulsions (containing 20% solid polymer content) are stable from pH 1 through pH 10, and to temperatures from 5 to 40 °C without precipitation or changes in particle size or morphology, as determined by DLS. Moreover, nanoparticle samples **NP0** (no drug), **NP1**, and **NP4** were analyzed for cytotoxicity against human

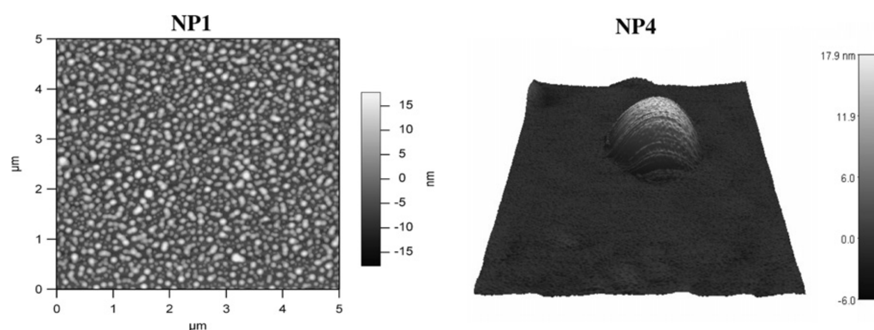


Figure 4. AFM images show the particles from the **NP1** and **NP4** emulsions are uniformly in the range of 25–40 nm in diameter.

dermal fibroblast cells using a CyQUANT DNA binding assay. These experiments showed that all three nanoparticle formulations are non-toxic to the fibroblast cells at 1 mg/mL drug concentrations, which are more than 10 times the bacterial MIC levels.

In summary, these studies have established that penicillin-containing polyacrylate nanoparticles can be easily prepared by free radical emulsion polymerization in water and are effective at helping to rejuvenate the in vitro antibacterial activity of penicillin drugs against beta-lactamase-producing MRSA. The favorable features of this methodology include simple, one-step preparation of penicillin-containing nanoparticles in aqueous media, rigid control of nanoparticle size in the 25–40 nm range, absence of cytotoxic effects in healthy human cell culture, and ability to incorporate drugs covalently onto the polymer framework or non-covalently (encapsulated) without the need for post-synthetic modification of the nanoparticle. Ongoing studies in our laboratory are attempting to elucidate the mechanism of bioactivity and drug release of the nanoparticles, and examining their in vivo properties in animal infection models to determine their efficacy and potential for therapeutic applications.

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