Diblock copolymers composed of poly(ε-caprolactone) (PCL) and poly(N,N-dimethylamino-2-ethyl methacrylate) (PDMAEMA), or methoxy polyethylene glycol(PEG), were synthesized via a combination of ring-opening polymerization and atom-transfer radical polymerization in order to prepare polymeric nanoparticles as an antifungal drug carrier. Amphotericin B (AmB), a natural antibiotic, was incorporated into the polymeric nanoparticles. The physical properties of AmB-incorporated polymeric nanoparticles with PCL-b-PDMAEMA and PCL-b-PEG were studied in relation to morphology and particle size. In the aggregation state study, AmB-incorporated PCL-b-PDMAEMA nanoparticles exhibited a monomeric state pattern of free AmB, whereas AmB-incorporated PCL-b-PEG nanoparticles displayed an aggregated pattern. In vitro hemolysis tests with human red blood cells, AmB-incorporated PCL-b-PDMAEMA nanoparticles were seen to be 10 times less cytotoxic than free AmB (5 µg/ml). In addition, an improved antifungal activity of AmB-incorporated polymeric nanoparticles was observed through antifungal activity tests using Candida albicans, whereas polymeric nanoparticles themselves were seen not to affect activity. Finally, in vitro AmB release studies were conducted, proving the potential of AmB-incorporated PCL-b-PDMAEMA nanoparticles as a new formulation candidate for AmB.

Keywords: Amphotericin B, infectious disease, nanoparticle, hemolysis, Candida albicans

Amphotericin B (AmB), an amphoteric macrocyclic natural antibiotic, is known to bind strongly to sterol components, such as ergosterol, in susceptible fungal cell membranes and to induce changes in permeability that can substantially induce lethal cell injury [10, 12, 24]. Since AmB has a broad antifungal spectrum activity, it is one of the first considerations for standard antibiotic therapy when faced with life-threatening fungal infections, such as visceral leishmaniasis and mucocutaneous leishmaniasis, amongst others [3]. Although there are well-known side effects of AmB, such as nephrotoxicity, which limit its clinical usage, AmB is also a useful antibiotic for the treatment of systemic fungal infections [7]. Other major drawbacks of AmB include poor aqueous solubility and its amphiphilic properties [2, 6]. Owing to its amphiphilic nature, AmB can easily be aggregated in an aqueous solution in a micellar form, and it is known that the toxic side effects of AmB are closely related to its aggregated form [2]. For this reason, AmB should be completely solubilized in an aqueous solution in a monomeric state to make it therapeutically active and less cytotoxic to the human body. Commercially available Fungizone®, a colloidal dispersion of AmB in sodium deoxycholate, has acute
toxic side effects and has proved less effective on immunocompromised patients [9]. Liposome preparations of AmB (Amphotec™ and Abelcet®), also commercialized, are known to be less toxic than Fungizone® [21, 25]. Another candidate for AmB formulation is AmBisome®, a liposomal preparation. AmBisome® is known to be less toxic than other kinds of AmB liposomal formulations, and it is possible to increase the doses of AmB without correspondingly producing more pronounced toxic side effects. Larabi et al [18] reported that a lipid formulation of AmB exhibited reduced intrinsic toxicity in vitro and in vivo. Although many formulations of AmB are commercially available, it still remains a challenge to find a new formulation that reduces its toxicity.

In order to address this issue, we used a diblock copolymer for the preparation of AmB-incorporated core-shell type nanoparticles. Core-shell type nanoparticles have been reported as promising solubilizing agents of hydrophobic drugs, and favor drug delivery via a passive targeting mechanism, minimizing irritation at the injection site and toxic side effects. They have also been reported to have advantages when compared with conventional drug formulations such as emulsion preparations, liposomes and plain nanoparticles [16, 19]. Recently, we reported original synthesis of diblock copolymers (abbreviated as CD) composed of poly(ε-caprolactone) (PCL) and poly(N,N-dimethylamino-2-ethyl methacrylate) (PDMAEMA) via atom transfer radical polymerization (ATRP), which is one of the most robust controlled/living radical polymerization methods, using an optimized catalyst/ligand system [5]. PDMAEMA is a weak polybase polymer (pKₐ ~7.0), which is soluble both at a neutral pH and in acidic medium, owing to protonation of the tertiary amine groups, and displays a well-marked polyelectrolyte behavior in water [22]. PDMAEMA (co)polymers have been extensively used as DNA binding agents either as pure compounds or as mixtures in nonviral systems, forming so-called polyplexes related to gene delivery systems, and are of particular interest in the field of gene therapy [8]. We hypothesized that the PDMAEMA component in the CD diblock copolymer would form the hydrophilic outer-shell of the core-shell type nanoparticles owing to its aqueous solubility, whereas PCL would form the inner core of the polymeric micelle because of its hydrophobic properties. To the best of our knowledge, this represents the first such formulation of AmB-incorporated CD nanoparticles prepared by ATRP.

In the present work, we studied the physical properties of AmB-incorporated nanoparticles with CD by way of comparison with PCL-b-methoxy poly(ethylene glycol) (abbreviated as CE) in relation to morphology, particle size, and aggregation states. Furthermore, examinations of the hemolysis and antimicrobial activities in vitro, in addition to drug release studies, were also conducted.

**Materials and Methods**

**Materials**

AmB and methoxy poly(ethylene glycol) (PEG, Mₘ=5,000 g/mol) were acquired from Sigma (St. Louis, USA). Dialysis membranes with a molecular weight cutoff (MWCO) of 8K and 12K g/mol were purchased from Spectra/Pro Membranes. Methanol (MeOH) and dimethyl sulfoxide (DMSO) were of HPLC grade and used without further purification. ε-Caprolactone (Acros), toluene (Labscan), and THF (Labscan) were dried over calcium hydride for 48 h at room temperature and distilled under reduced pressure prior to use. Aluminum triisopropoxide [Al(OPr)₃; Acros] was distilled under vacuum, quenched in liquid nitrogen, rapidly dissolved in dry toluene, and stored under a nitrogen atmosphere. The Al(OPr)₃ concentration was determined by back complexometric titration of Al using the ethylenediaminetetraacetic acid (EDTA) disodium salt and ZnSO₄ at pH 4.8. 2-Bromo-2-isobutyl bromide (BrBuBr; Aldrich), 1,1,4,7,10,10-hexamethylenetetramine (HMTETA; Aldrich) and copper(I) bromide (CuBr; Fluka) were used without further purification. N,N-(Dimethylamino-2-ethyl) methacrylate (DMAEA; Aldrich) was passed through a column of basic alumina to remove the stabilizing agents and then stored under a nitrogen atmosphere at −20°C.

**Synthesis of PCL-b-PEG (CE) Diblock Copolymers**

The CE diblock copolymer was prepared as previously described [15]. Briefly, PEG and ε-caprolactone were mixed in a round-bottomed flask under vacuum. The mixture was cooled and degassed with a pump. The round-bottomed flask was then sealed off and placed in an oil bath at 180°C. After the polymerization was completed, the resulting product was cooled at room temperature and then dissolved in methylene dichloride. The solution was precipitated into an excess amount of cold ethanol and filtered to remove unreacted PEG homopolymer and ε-caprolactone monomers. The precipitates were washed with diethyl ether three times and then dried in a vacuum oven for 3 days.

**Synthesis of Poly(ε-caprolactone)-b-PDMAEMA (CD) Diblock Copolymers**

CD diblock copolymers were obtained by way of a three-step technique protocol as has previously been described [5, 22]. In a typical experimental run, all equipment was initially flame-dried, resulting in a clean airtight system. Particular attention was given to the bottom flask equipped with a three-way stopcock and a rubber septum. The flask was cleaned, dried, and then purged with nitrogen.