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The role of counterions in the membrane-disruptive properties of pH-sensitive lysine-based surfactants

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ABSTRACT

Surfactants are among the most versatile and widely used excipients in pharmaceuticals. This versatility, together with their pH-responsive membrane-disruptive activity and low toxicity, could also enable their potential application in drug delivery systems. Five anionic lysine-based surfactants which differ in the nature of their counterion were studied. Their capacity to disrupt the cell membrane was examined under a range of pH values, concentrations and incubation times, using a standard hemolysis assay as a model for endosomal membranes. The surfactants showed pH-sensitive hemolytic activity and improved kinetics at the endosomal pH range. Low concentrations resulted in negligible hemolysis at physiological pH and high membrane lytic activity at pH 5.4, which is in the range characteristic of late endosomes. With increasing concentration, the surfactants showed an enhanced capacity to lyse cell membranes, and also caused significant membrane disruption at physiological pH. This observation indicates that, at high concentrations, surfactant behavior is independent of pH. The mechanism of surfactant-mediated membrane destabilization was addressed, and scanning electron microscopy studies were also performed to evaluate the effects of the compounds on erythrocyte morphology as a function of pH. The *in vitro* cytotoxicity of the surfactants was assessed by MTT and NRU assays with the 3T3 cell line. The influence of different types of counterion on hemolytic activity and the potential applications of these surfactants in drug delivery are discussed. The possibility of using pH-sensitive surfactants for endosome disruption could hold great promise for intracellular drug delivery systems in future therapeutic applications.

1. Introduction

Many therapeutic agents, such as proteins, peptides, DNA and some drugs, act at intracellular sites, and thus their therapeutic efficacy depends on efficient intracellular trafficking pathways [1]. One of the challenges for the efficient intracellular delivery of therapeutic compounds is to manipulate or circumvent the non-productive trafficking from endosomes to lysosomes, where degradation may occur. This would allow delivery systems to escape endosomal compartments and consequently facilitate subsequent drug release into cytoplasm after internalization of the systems through endocytosis [2].

Carriers based on attenuated viruses have been studied extensively as pH-dependent membrane-disruptive components in gene delivery systems to enhance transport from endosomes to the cytoplasm; however, clinical use of these carriers is potentially limited by their antigenicity and toxicity, and the difficulties of large-scale production [3–5]. Safety issues have prompted the development of synthetic peptides structurally derived from viruses specifically to disrupt endosomal membranes [1]. However these peptides are also likely to be immunogenic *in vivo* [6,7]. To circumvent these problems, a variety of non-viral delivery vectors have been developed, such as synthetic polymers and surfactants. Polymerizable surfactants with tunable pH-sensitive amphiphilicity have recently been designed. These allow carriers to change their amphiphilic structure at endosomal–lysosomal pH, which results in the disruption of endosomal–lysosomal membranes [8,9]. The functionalization of one of these polymerizable pH-sensitive amphiphilic surfactants for the preparation of a peptide-directed siRNA delivery system has also been reported [10]. Moreover, cationic amino acid-based surfactants have been used to prepare novel biocompatible devices for the controlled encapsulation and release of DNA [11]. Cationic and anionic polymers with pH-sensitive activity, including non-biodegradable polymers and biodegradable poly(amino acids) and pseudo-peptides [2,7,12–18], have also been developed to promote endosomal escape. Cationic compounds have commonly been used to form stable cationic complexes; however, they show cytotoxicity and non-specifically adsorb serum proteins, thereby leading to rapid blood clearance as a result of the strong cationic surface charge [19–21]. In

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contrast, anionic pH-responsive polymers are considered of interest as drug carriers, because they mimic the structure and pH-dependent membrane–lytic behavior of endosomolytic viral peptides [1]. Moreover, recharging cationic complexes with anionic compounds has been reported as a promising method to overcome the adverse effects of cationic complexes [22].

Considerable research effort is devoted to delivery systems that specifically destabilize endosomal membranes in mildly acidic conditions following endocytic uptake [23]. In this context, amino acid-based surfactants with pH-sensitive activity and low toxicity deserve particular attention and could be a promising choice for application in non-viral drug delivery systems. Here, N^{α},N^{ε}-dioctanoyl lysine derivatives, a class of amino acid-based surfactants synthesized as lecithin analogs, were selected, since homologs with eight carbon atom chains are the least hemolytic and show the least irritant activity, thus proving the most suitable for practical applications [24]. Moreover, earlier studies by the authors' group demonstrated the biocompatibility and low *in vitro* toxicity of this series of anionic lysine-based surfactants [25–28].

In the present study, the membrane lytic properties were studied as a function of pH of five anionic lysine-based surfactants differing in the nature of their counterion. To evaluate the potential applications in cytoplasmic delivery carriers, the pH-sensitive cell membrane disruptive activity of these compounds was examined using a standard hemolysis assay of rat erythrocytes at a range of pH values as a model for endosomal membranes. The mechanism involved in cell membrane disruption, the kinetic properties of each surfactant in the endosomal pH range, and their effects on erythrocyte morphology as a function of pH are also presented, together with *in vitro* cytotoxicity assays in the 3T3 fibroblast cell line. Furthermore, to gain insight into the structure-dependent interaction of these compounds with membrane bilayers, the influence of the surfactant structure and counterions on hemolytic activity is also discussed.

2. Materials and methods

2.1. Materials

L-Lysine monohydrochloride, L-lysine, Tris, the bases NaOH, LiOH and KOH, sodium dodecyl sulfate (SDS), glutaraldehyde, NaCl, Na_2HPO_4 and KH_2PO_4 were purchased from Merck (Darmstadt, Germany). PEG-10,000, D-glucose, dimethyl sulfoxide (DMSO), 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT) and neutral red dye (NR) were from Sigma–Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin–EDTA solution (170,000 U l^{-1} trypsin and 0.2 g l^{-1} EDTA) and penicillin–streptomycin solution (10,000 U ml^{-1} penicillin and 10 mg ml^{-1} streptomycin) were obtained from Lonza (Verviers, Belgium). The 75 cm^2 flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland).

2.2. Surfactants tested

Five anionic amino acid-based surfactants derived from N^{α},N^{ε}-dioctanoyl lysine and with counterions of distinct chemical nature were evaluated: two salts with organic counterions—lysine salt (77KK) and tris(hydroxymethyl) aminomethane salt (77KT); and three salts with inorganic counterions—sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP) (Fig. 1). These surfactants were synthesized in the authors' laboratory as previously described [25,29]. The commercial anionic surfactant SDS was used as reference compound.

2.3. Preparation of red blood cell suspensions

Rat blood was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. The procedure was approved by the institutional ethics committee on animal experimentation. Red blood cells were isolated by centrifugation at 3000 rpm at 4 °C for 10 min, and washed three times in an isotonic PBS containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.6 mM KH_2PO_4 in distilled water (pH 7.4; 300 mOsmol l^{-1}). The cell pellets were then suspended in PBS solution at a cell density of 8×10^9 cell ml^{-1}.

2.4. Hemolysis assay

The membrane–lytic activity of the surfactants was examined by hemolysis assay. PBS buffers in the pH range 5.4–8.0 were prepared to be isosmotic to the inside of the erythrocyte and cause negligible hemolysis. The 25-μl aliquots of erythrocyte suspension were exposed to various concentrations (from 100 to 800 μg ml^{-1}) of the surfactants dissolved in PBS solution in a total volume of 1 ml. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). The samples were incubated at room temperature under constant shaking for various periods up to 90 min,

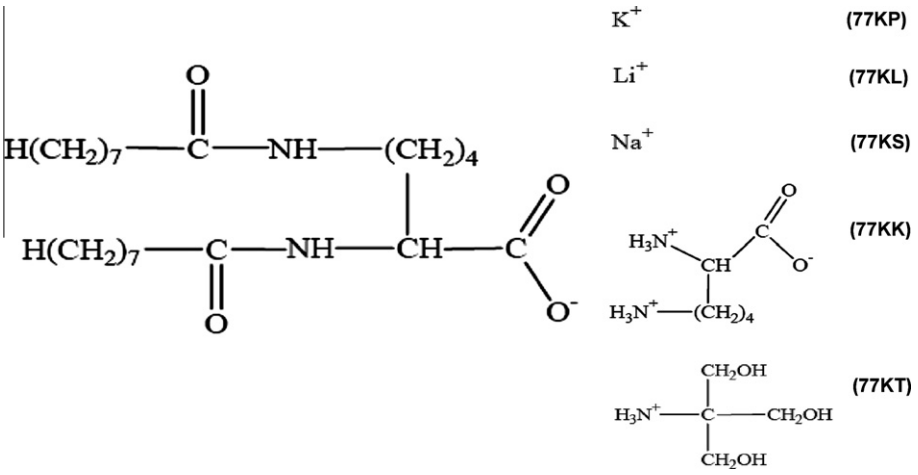

Fig. 1. Molecular structure of lysine-based anionic surfactants with different counterions. Codes P, L, S, K, and T represent potassium, lithium, sodium, lysine and Tris, respectively.

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