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Phospholipid Polymer, 2-Methacryloyloxyethyl Phosphorylcholine and Its Skin Barrier Function

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The effect of poly[2-methacryloyloxyethyl phosphorylcholine] (pMPC) on the skin permeation property was investigated by performing *in vitro* skin permeation study of a model drug, nicotinic acid (NA). Effect of pMPC polymer in donor solution on skin permeation rates was evaluated using side-by-side diffusion cells. Also, the structural alterations in the stratum corneum (SC), inter-lamellar bilayer (ILB) and dermis layers in pMPC-treated and -untreated skin sections were investigated with transmission electron microscopy (TEM). The permeation profile of NA without pMPC in donor solution showed biphasic mode: initial 1st phase and 2nd hydration phase. The sudden, more than 10-fold increase in flux from the initial steady state (43.5 μg/cm^2/hr) to the 2nd hydration phase (457.3 μg/cm^2/h) suggests the disruption of skin barrier function due to extensive hydration. The permeation profile of NA with 3% pMPC in the donor solution showed monophasic pattern: the steady state flux (10.9 μg/cm^2/h) without abrupt increase of the flux. The degree of NA permeation rate decreased in a concentration-dependent manner of pMPC. TEM of skin equilibrated with water or 2% pMPC for 12 h showed that corneocytes are still cohesive and epidermis is tightly bound to dermis in 2% pMPC-treated skin, while wider separation between corneocytes and focal dilations in inter-cellular spaces were observed in water-treated skin. This result suggests that pMPC could protect the barrier property of the stratum corneum by preventing the disruption of ILB structure caused by extensive skin hydration during skin permeation study.

Key words: Phospholipid polymer (pMPC), Stratum corneum, Skin permeation, Barrier function

INTRODUCTION

A polymeric phospholipid, poly[2-methacryloyloxyethyl phosphorylcholine] (pMPC) was synthesized as a new type of biomaterials (Ishihara *et al.*, 1990). Fig. 1 shows the molecular structure of pMPC, an analog of phospholipids, which bears the bio-mimic phosphorylcholine moiety as the side group and methacrylate unit. pMPC has been recognized for its unique water-holding capacity to give the excellent non-thrombogenic surfaces as the result of its ability of inhibiting the protein adsorption (Ishihara *et al.*, 1990). pMPC increases the biocompatibility with reduced protein adsorption and cell adhesion by enhancing free water content on the surface of polymer. The high level of free water content due to pMPC makes proteins contact the surface reversibly without significant conformational change (Ishihara, 1998). Since water is a native constituent of the stratum corneum affecting its plasticity and modulating its barrier function, a functional deficiency in water holding capacity of the stratum corneum was observed among the patients with various forms of dermatitis (Liron *et al.*, 1994; Tanaka *et al.*, 1998). Thus, pMPC which has a unique hygroscopic property has been studied for its dermatological application. In clinical study, pMPC could improve the dried skin condition in atopic dermatitis patients: the application of 1% pMPC lotion on human skin significantly increased the water content, reduced Trans Epidermal Water Loss (TEWL) and improved the skin surface condition (Kanegura, 2002; Murata, 2000).

Cutaneous permeability and hydration state are modulated by lipids in the SC which is composed of ceramides, cholesterol, free fatty acids, glycolipids and phospholipids (Kanegura, 2002). Within the stratum corneum, drug molecules meet restraining forces which impede their movement; these restraining forces are decreased as the tissue becomes hydrated (Batt *et al.*, 1987; Blank *et al.*, 1984). Since hydration could induce the weakening of the

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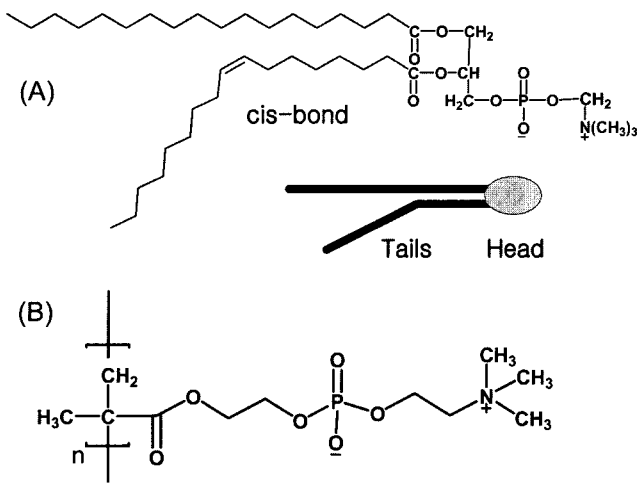

Fig. 1. Chemical structure of phospholipids (A) and its analog: PhospholipidPolymer, (2-methacryloyloxyethyl phosphorylcholine, pMPC). Due to a structural resemblance, pMPC shows high affinity with phospholipids. Quaternary ammonium salts in pMPC molecule would be positively charged, while phosphate ion is negatively charged. pMPC has about 3186 functional residues (Mw=940) per molecule and each residue is a zwitter ion which has both positive and negative ion in a molecule. pMPC can bind water molecules due to hydrogen bond between its oxygen atoms and water, that pMPC may serve as a humectant.

corneocytes cohesion of stratum corneum; this could cause a significant enhancement of skin permeation. Mak and coworkers also reported that water can loosen the inter-lamellar structure of stratum corneum and increase the skin flux (Mak *et al.*, 1991; Rawlings and Harding, 2004).

Considering the water holding property of pMPC (Ishihara *et al.*, 1998), we first investigated if pMPC could enhance a skin permeation of a drug or not. As a model drug, nicotinic acid was selected by considering its stability and its aqueous solubility. On the contrary to our expectation, a pronounced decrease of the skin flux of NA was observed with the presence of pMPC in donor solution during *in vitro* diffusion study. Thus we further investigated the effect of pMPC on the barrier property of the stratum corneum and the relationship between the stabilization of interlamellar structure and its water holding capacity.

MATERIALS AND METHODS

Materials

pMPC homopolymer (100% MPC; Mw=940k, Mn=252k) was provided as 5 wt% aqueous solution without any additives from Professor K. Ishihara, Tokyo University. pMPC solution was stored in cool and dark conditions. pMPC solution was used as 0.5-3% aqueous solution after dilution. Nicotinic acid (Sigma Chemical Co, USA) was used as received. All other chemicals were regent grade. The solvents used in HPLC were HPLC grade. Hairless mouse (Jackson Lab., 5-8 weeks age, 20±2 g weight) were purchased from Han-Lim-Won, Korea.

Skin permeation study

A full-thickness abdominal skin of a female hairless mouse (5-7weeks old, Jackson Lab. HRS/J strain) was excised freshly before the *in vitro* skin permeation experiment. The skin sample was then mounted between the donor and receptor half-cell of hydro-dynamically well calibrated side-by-side diffusion cells: the diffusion area 0.64 cm^2 (Tojo *et al.*, 1985). The donor and receptor cells were then charged in each cell compartment: cell volume 3.5 mL. Forty percent of PEG 400 aqueous solution was selected as a receptor solution since permeation profiles with different fractions of PEG 400 aqueous solution in receptor solution did not significantly influence the permeation rate and the lag time (Tojo *et al.*, 1987; Cho Lee, 1996). Chemical potential of NA in donor solution was controlled same by preparing the saturated solution of NA in each tested donor solution. Sink condition was maintained in receptor solution. At appropriate time intervals, 50 μL of receptor solutions were withdrawn and assayed for nicotinic acid. Twelve sets of the *in vitro* diffusion cells were employed in each permeation experiment. All experiments were carried out at a constant temperature (37 °C). The steady state skin permeation rates were obtained from slopes of the skin permeation profiles by employing the Least Linear Square method.

HPLC assay

High pressure liquid chromatography system (Waters, USA) was employed to quantify the amount of the drug permeated through the skin. Mobile phase was prepared by acetonitrile and water (containing 10 mM of 1-sodium hexane sulfonic acid) with the ratio of 95 to 5 and pH was adjusted to 2.0 with phosphoric acid. The flow rate was 1 mL/min. μ-Bondapak C-18 reverse phase (4×15 mm) column (Waters, USA) was used. Samples were detected using UV-detector at the wavelength of 260 nm.

Transmission electron microscopy (TEM)

Fixation of the skin specimen was processed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution for 3 h. As a post-fixation process, fixed skin specimen was exposed to 0.2% OsO4 in cacodylate buffer solution for 1hr. Thin sections were stained and examined using electron microscopy (JEM-1200EX-II. JEOL Co., Japan).

Statistical analysis

At least, triplicate experimental values were averaged for the calculation of the flux of NA permeation across skin, and data were expressed as mean±standard deviation.

Error bars in figures represent the standard deviation. Student's *t*-test was applied for the calculation of p-values to verify the difference between experimental data. The cases of *p*-value less than 0.05 were considered statistically different.

RESULTS

Understanding skin barrier function

Fig. 2-A shows the histology of normal hairless mouse skin stained with Hematoxylin and Eosin (×800). The upper most layers, stained in red, are the stratum corneum; below the viable epidermis , stained in blue for nuclei are shown. Fig. 2-B shows the schematic diagram of the epidermis in mammalian skin. The epidermis is a multi-layered epithelium composed largely of dead keratinized layer of squames, granular cell layer, prickle cell layer and basal cell layer. While some basal cells are dividing, others are slipping out of the basal cell layer into the prickle cell layer, taking the first step on their outward journey (Alberts *et al.*, 1994). As they reach granular layer, the cells start to lose their nuclei and cytoplasmic organelles and are transformed into the keratinized squames of the keratinized layer, in which corneocytes are embedded in inter-lamellar bilayer (ILB) composed of ceramides, cholesterol and fatty acids. These 10-20 layers of dead keratinized brick-like structure functions as a major diffusion barrier for skin penetration of an exogenous compound and controls water holding capacity of the skin (Mak *et al.*, 1991; Gay *et al.*, 1994).

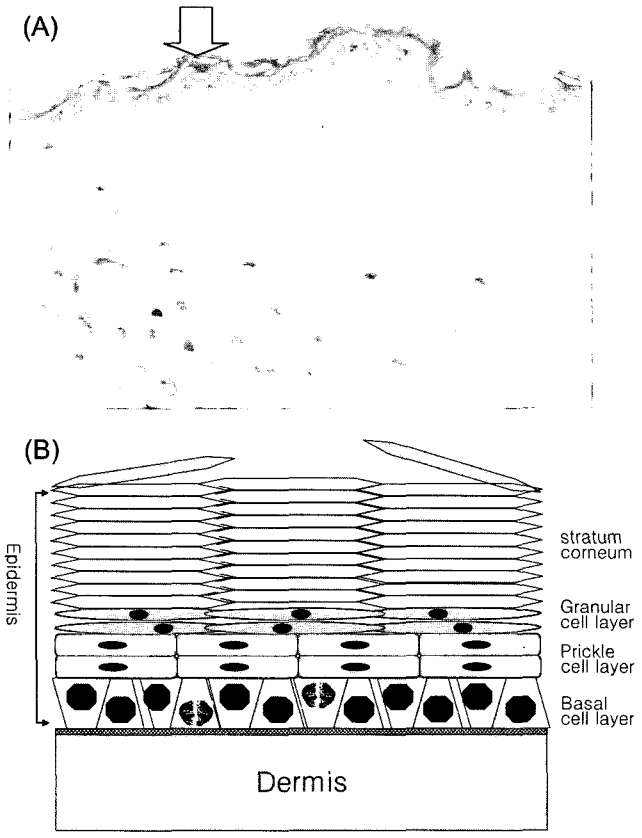

Fig. 2. (A): Histology of normal hairless mouse skin stained with Hematoxylin and Eosin (×800). The upper most layers, stained in red, are the stratum corneum; below the viable epidermis with nuclei are shown, stained in blue. (B): Schematic diagram of the skin structure focusing on the epidermis consisting of stratum corneum (keratinized squames), granular cell, prickle cell and basal cell layers. Squames are about to flake off from the surface. Stratum corneum lipids such as ceramides, cholesterol and fatty acids serve as a bound-water modulator (Alberts *et al.*, 1994).

Effect of pMPC polymer on the skin permeation

Fig. 3 shows the effect of pMPC in the donor solution on the skin permeation of NA. 40% PEG 400 was used as a receptor solution. Significant decrease of skin permeation rate in the presence of pMPC (0.5 and 3.0%) suggests the possible protective mechanism against skin hydration.

Effect of skin hydration on the skin permeation

Fig. 4 shows the cumulative amount of NA permeated through the skin during *in vitro* skin permeation study without pMPC in donor solution. The permeation profile of NA without pMPC in donor solution shows biphasic mode: initial 1st phase and 2nd hydration phase. The sudden, more than 10-fold increase in flux from the initial steady state (43.5 μg/cm^2/h) to the 2nd hydration phase (457.3 μg/cm^2/h) suggests the disruption of skin barrier function due to extensive hydration. Exposing the skin to extrinsic water is usually considered to be innocuous. Thus few studies have been focused on the effect of water on the skin permeation property of a drug. However, Warner and

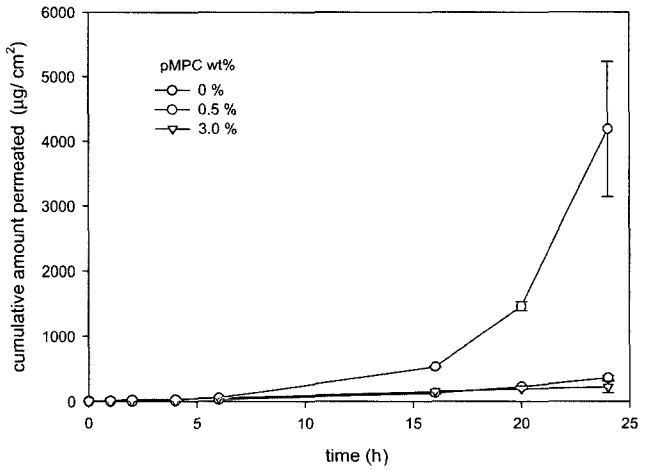

Fig. 3. Plot of cumulative amount-time profiles of nicotinic acid permeated across hairless mouse skin into receptor solution over a period of 24 h without/with pMPC (0, 0.5, 3%) in the donor solution.

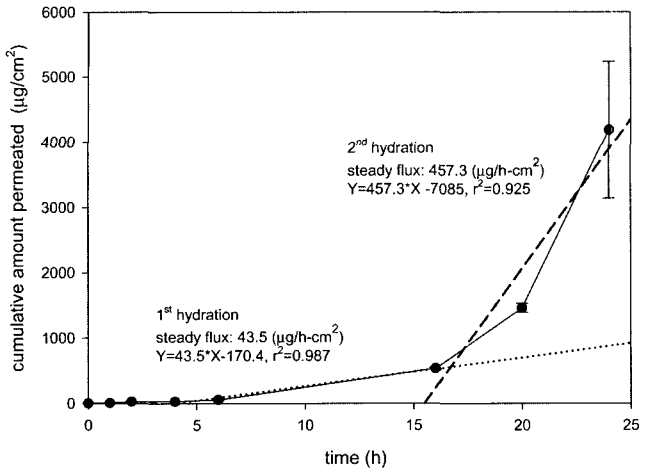

Fig. 4. Skin permeation profile of NA from saturated solution of NA (Cs: 13 mg/mL) in water. Without pMPC in donor solution, skin flux sharply increased after 16h in vitro skin permeation study as arrow denote on the profile. As skin hydrates further, a significant increase in skin flux is observed. The straight line represents the best linear fit to the data. 1st and 2nd phase permeation rates were determined by least square method.

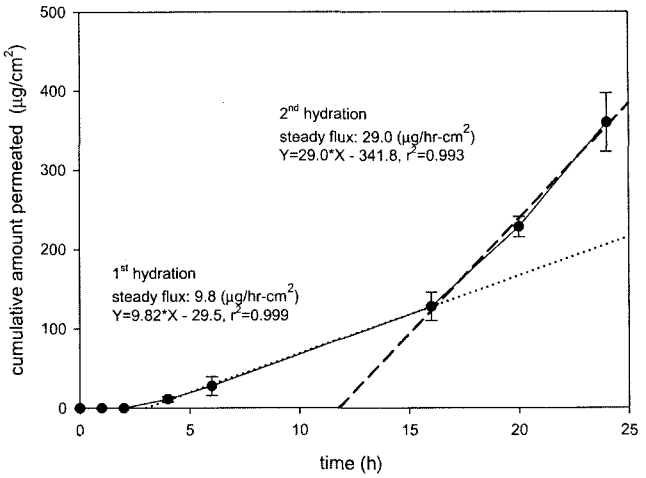

Fig. 5. Skin permeation profile of NA from saturated solution of NA (Cs: 13 mg/mL) in the presence of 0.5% pMPC in the donor solution. The significant decrease in the initial steady state flux (9.8 μg/cm²/h) and the 2nd phase steady state flux (29.0 μg/cm²/h) as compared with those without pMPC suggests the possible protective role of pMPC against the skin damage due to skin hydration.

coworkers reported that water itself disrupted the stratum corneum intercellular lipid lamellar bilayer and induced the weakening of the stratum corneum corneocyte cohesion with a strong hydration effect. And this could cause a significant enhancement of skin permeation (Warner *et al.*, 1999).

Effect of pMPC on the barrier properties of the stratum corneum

Fig. 5 shows the permeation profile of NA in the presence of 0.5% pMPC in the donor solution. A significant decrease of skin flux in initial 1st phase and 2nd hydration phase were observed. But the permeation profile still shows biphasic pattern suggesting the partial protection from water activity. More than 18-fold decrease in 2nd hydration phase skin flux with 0.5% pMPC suggests the protective role of pMPC against the skin damage due to significant skin hydration. The significant decrease in the initial steady state flux (9.8 μg/cm²/h) and the 2nd phase steady state flux (29.0 μg/cm²/h) as compared with those without pMPC suggests the possible protective role of pMPC against the skin damage due to skin hydration.

The permeation profile of NA with 3% pMPC (Fig. 6) in the donor solution shows monophasic pattern: the steady state flux (10.9 μg/cm²/h) without abrupt increase of the flux. The degree of NA permeation rate decreased in a concentration-dependent manner of pMPC. This may be due to the higher molecular density of pMPC film on skin surface at higher concentration of pMPC in donor solution. The reduced permeation rate may be attributed to the denser pMPC film constituted with higher pMPC concentration. The structural alterations in the stratum corneum (SC), inter-lamellar bilayer (ILB) and dermis layers in pMPC-treated and -untreated skin sections were investigated with transmission electron microscopy (TEM). Fig. 7 shows the TEM of the stratum corneum and dermis layer from a 360 μm hairless mouse skin sections perfuse for 12 h with water (A) and 2% pMPC (B). Corneocytes are widely separated and focal dilations within intercellular spaces are observed in (A). While in pMPC treated skin specimen (B) showed that the majority of corneocytes are tightly cohesive and no significant alterations in ILB.

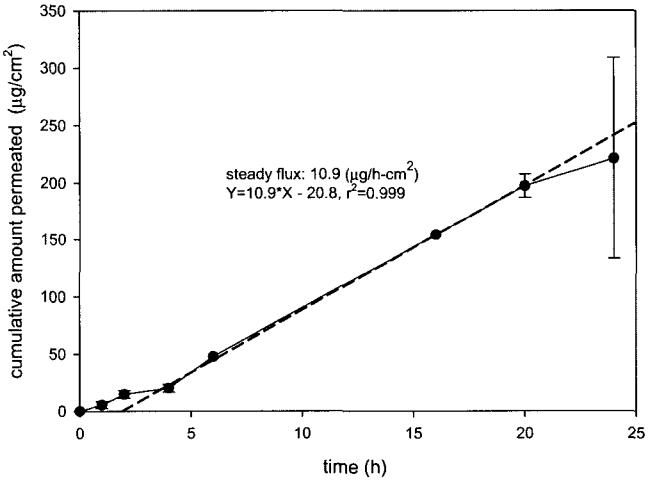

Fig. 6. Skin permeation profile of NA from saturated solution of NA (Cs: 13 mg/mL) in 3% pMPC aq. solution. The steady state flux (10.9 μg/cm²/h) was determined from the slope of the straight line represents the best linear fit to the data.

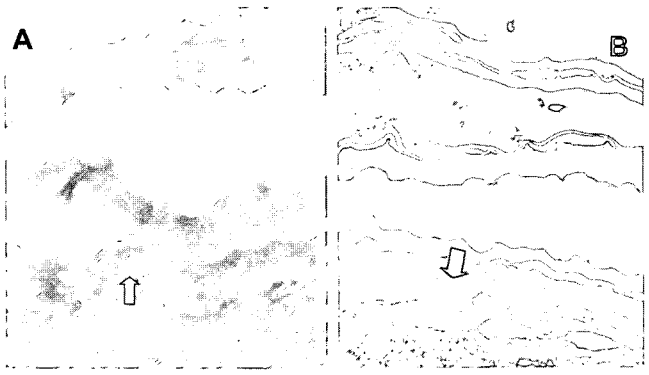

Fig. 7. Transmission Electron Micrograph of Stratum corneum equilibrated with water (A) and 2% pMPC (B) for 12 h. A: Corneocytes are widely separated and focal dilation within the intercellular space is observed. B: The majority of corneocytes are tightly cohesive. There are no significant alterations in Intercellular Lamellar Bi-layers.

DISCUSSION

In this study, we investigated the barrier-like properties of pMPC against the skin penetration of foreign materials. *In vitro* skin permeation study showed that the flux of a model drug, NA, was significantly decreased in the presence of pMPC (0.5-3%) in the donor solution. Reduced flux of NA could be attributed by two possibilities. First, NA, negatively charged in neutral condition, can interact with positively charged quaternary ammonium ion in pMPC molecule; NA tends to bind with pMPC on skin surface, which may prevent the permeation of NA across the skin. Second, pMPC (Mw=940K) has about 3186 functional residues per molecule, and each residue has a nature of zwitter ion which has both positive and negative charges in a molecule. Zwitter ionic nature of the residues can make pMPC molecules interact each other to form a cross-linked conformation, so that the surface may be covered with pMPC network which may function as a barrier against the penetration of drugs across skin.

In this study, the stabilization of membrane by pMPC was investigated using TEM. TEM of skin equilibrated with water or 2% pMPC for 12 h showed that corneocytes are still cohesive and epidermis is tightly bound to dermis in 2% pMPC treated skin while wider separation among corneocytes and focal dilations in the intercellular spaces were observed in water only treated skin. Previously we studied the lamellar structure change by added pMPC with small angle X-ray crystallography and observed the presence of 4.2-4.4 nm peak attributed to the long periodicity phase (LPP) in pMPC treated SC, which indicates the stabilization of LPP. And the relationship between stabilization of inter-lamellar structure and the water holding capacity of pMPC was reported. pMPC increased the water holding capacity of the skin up to 3 fold to its original weights as compared with untreated skin. DSC-thermograms focusing on the phase-transition temperature of lipid domain of SC show that the presence of pMPC stabilizes the ILB. The heat of lipid transition (ΔH) in the absence and presence of pMPC were 6.05 and 7.02 mJ/mg, respectively. The ratio of ΔH of the lipid transition with the pMPC to that without pMPC was 1.12 (Cho Lee *et al.,* 2001).

pMPC possesses very unique water-holding properties derived from its hygroscopic nature and its effective promotion of intercellular lipid function (Kanegura, 2002; Shaku *et al.,* 2000). Over the past decade, a great progress has been made toward elucidating the structure and function of the stratum corneum, the outermost layer of the epidermis (Golden *et al.,* 1987). The stratum corneum cells (corneocytes) protect against desiccation and environmental challenge by regulating water flux and retention. Maintenance of an optimal level of hydration by the SC is dependent on intercellular lamellar lipids, organized predominantly in an orthorhombic gel phase, provide an effective barrier to the passage of water through the tissue (Rawlings and Harding, 2004). The stratum corneum of the patients with various forms of dermatitis has functional deficiency in water-holding capacity result in decreased hydration state of the stratum corneum (Tanaka *et al.,* 1998). Thus, the role of pMPC for maintaining a proper SC lipid bi-laminate structure is very important to balance the skin hydration state and skin barrier property.

In conclusion, the presence of small percent (<1-3%) of pMPC in water can help to maintain the barrier property of the skin by preventing disruption of ILB structure. pMPC could function as a barrier-like membrane to prevent the skin penetration of toxic substance. Based on the experimental results, pMPC may take an important role of the stabilization on skin surface to prevent the permeation of NA through skin barrier. Modulation of the skin permeability by pMPC may be useful for other compounds such as neutral or cationic drugs.

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