Decrease in Skin Permeation and Antibacterial Effect of Parabens by a Polymeric Additive, Poly(2-methacryloyloxyethyl phosphorylcholine-co-butylmetacrylate)

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The inhibitory effect of poly(2-methacryloyloxyethyl phosphorylcholine-co-butylmetacrylate) (PMB) on the *in vitro* skin permeation of *p*-hydroxybenzoic acid and its esters (parabens; methylparaben, ethylparaben, *n*-propylparaben and *n*-butylparaben) as model compounds was evaluated. Solubility of the parabens in distilled water was increased by addition of PMB, and the increasing ratio was dependent on the concentration of PMB. The increment of the ratio was more marked in lipophilic parabens than hydrophilic ones. Skin permeability of parabens from their aqueous suspensions was decreased by addition of PMB, and the decreasing ratio was dependent on the PMB concentration. Then, antibacterial effect of parabens of their aqueous solutions was determined with and without PMB by measuring minimum inhibitory concentration (MIC) against *E. coli* and *S. aureus*. As results, MIC and antibacterial effect were increased and decreased, respectively, by addition of PMB. The contribution of PMB was more marked in butylparaben than methylparaben. These decreases in skin permeation and antibacterial effect of parabens by PMB are probably due to a higher solubility in water and a lower partition to the skin and bacterial membranes of parabens by addition of PMB.

Key words skin permeation; polymeric additive; paraben; antibacterial effect; poly(2-methacryloyloxyethyl phosphorylcholine-co-butylmetacrylate)

Topical formulations applied on skin can be divided mainly into semisolid formulations such as ointment, cream and lotion and dermal patches such as pressure sensitive adhesive tapes and cataplasma. These topical formulations contain many kinds of pharmaceutical additives. Alcohols, glycols, *l*-menthol, mineral and vegetable oils and surfactants as well as polymeric additives are prescribed for skin penetration enhancers, drug solubilizers, drug stabilizers, preservatives, excepients, and so on. Polymeric additives sometimes have unique characteristics in the topical formulations. They were used for increase in the compatibility, sustaining of the drug release from the formulations,[1)] and stabilization of the active drugs and formulations. Some polymeric additives may be added into a topical formulation for achievement of supersaturate system of drugs.[2—5)] In spite of the increased application of the polymeric additives, their effects and mechanism on the skin permeation of compounds have not been cleared yet.

Poly(2-methacryloyloxyethyl phosphorylcholine-co-butylmetacrylate) (PMB) is an amphipathic copolymer of 2-methacryloyloxyethyl phosphorylcholine and butylmethacrylate, as shown in Fig. 1. PMB solution seems to be a promising additive of topical formulations, since these polymers were reported to have a preventing effect on the skin roughness and moisturizing action due to their high affinity to the skin surface.[6,7)] In the present study, this unique polymer, PMB, was selected as a candidate polymeric additive for topical formulation.

On the other hand, *p*-hydroxybenzoic acid esters (parabens) have been used broadly as a preservative in many topical formulations. Therefore their low skin permeations are ideal after topical application, whereas their high antibacterial effects are expected in the formulations during the shelf life. *p*-Hydroxybenzoic acid (*p*-HBA) and its esters (parabens) were selected as model compounds to evaluate the effect of PMB on their skin permeation because a series of these compounds has similar molecular weight (MW 138—194) and different *n*-octanol–water partition coefficients (K_{ow}). Physicochemical parameters of the compounds are shown in Table 1.

The present study is aimed at clarifying various effects of PMB on topical formulation from following examinations. Skin permeation of parabens usually correlates with their partition coefficients from the formulation to skin.[8)] Then, *in vitro* skin permeation experiments of parabens were done using different concentrations of PMB, and the effect of PMB on the skin permeation of parabens was evaluated. The site of action of PMB in skin barrier was also estimated by a confocal laser scanning microscopy (CLSM) using fluorescein isothiocyanate (FITC) labeled PBM. Additionally, the effect of PMB on the antibacterial effect of parabens was examined against *E. coli* and *S. aureus*.

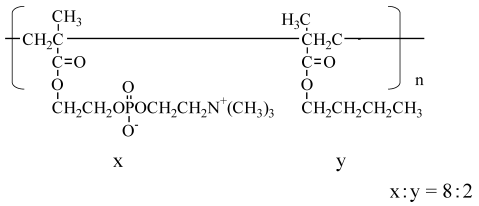

Fig. 1. Chemical Structure of PMB
x and y show 2-methacryloyloxyethyl phosphorylcholine and butylmethacrylate.

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Table 1. Physicochemical Parameters of *p*-Hydroxybenzoic Acid and Parabens

	p-Hydroxybenzoic acid (*p*-HBA)	Methyl paraben (MP)	Ethyl paraben (EP)	*n*-Propyl paraben (PP)	*n*-Butyl paraben (BP)
MW	138.12	152.15	166.18	180.20	194.23
log K_{ow} [a)]	0.94[9)]	1.93[8)]	2.27[8)]	2.81[9)]	3.53[9)]
Solubility[b)] (mM)	70.21±18.96	19.68±1.50	8.82±2.26	2.55±0.14	1.60±0.10

a) *n*-Octanol–water system at 37 °C. *b*) In water at 32 °C.

Experimental

Materials PMB and FITC labeled PMB were obtained by Tsukuba Research Laboratory, NOF Corporation (Tsukuba, Ibaragi, Japan). *p*-HBA and its esters (methyl (MP), ethyl (EP), *n*-propyl (PP) and *n*-butyl (BP) paraben) were purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan). Fluorescein Na and rhodamine B were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of HPLC grade or reagent grade and used without further purification. Muller–Hinton broth for microbiological assay was obtained by Difco Laboratories (Detroit, MI, U.S.A.). *Escherichia coli* (*E. coli*, O111:B4) and *Staphylococcus aureus* (*S. aureus*, 209P) were supplied by Microbiology Laboratory, Josai University (Sakado, Saitama, Japan).

Animals Male hairless rats (WBM/ILA-Ht, 230—280 g) were obtained from Life Science Research Center, Josai University (Sakado, Saitama, Japan) or Ishikawa Experimental Animal Laboratories (Fukaya, Saitama, Japan). All animal experiments were done under the guidelines of Life Science Research Center, Josai University.

Determination of Solubility of Parabens Excess amount of *p*-HBA and parabens (MP, EP, PP, BP) were added to different concentration of PMB solutions (0.1, 0.5, 1.0, 2.0, 5.0, 10.0%) and stirred at 32 °C overnight. The resulting *p*-HBA and parabens suspensions were filtered with a membrane filter (pore size 0.45 μm, Advantec, Tokyo) to obtain the supernatant. The supernatant was adequately diluted to determine the *p*-HBA and parabens solubilities by HPLC. No adsorption of *p*-HBA and parabens onto the filter was observed.

***In Vitro* Skin Permeation Experiments** Abdominal skin was excised from hairless rats under anesthesia of pentobarbital (50 mg/kg i.p.) and the debris and excess fat were trimmed off from the excised skin. The skin was then set on the Franz type diffusion cell (effective diffusion area of 3.14 cm^2). Aqueous suspension of *p*-HBA and parabens in different concentrations (0, 0.5, 1.0, 2.0, 5.0, 10.0%) of PMB (2.0 ml) was applied on the stratum corneum side, and pH 7.4 phosphate buffered saline (PBS) was to the dermis side (*ca.* 17 ml). 10 mM MP and 1 mM BP aqueous solution with or without 5% PMB were also used as donor solution. Those suspension used were prepared by addition of an excess amount of *p*-HBA or parabens to PMB solution and stirring at 32 °C overnight. The additive amounts of *p*-HBA or parabens in those suspension were large enough to keep suspended condition during the skin permeation experiments. The receiver solution was stirred by a magnetic stirrer and a bar, while whole set was kept at 32 °C. At predetermined intervals, 500 μl of the receiver solution was sampled and the same volume of the fresh PBS was added to continue the experiment. *p*-HBA and paraben concentrations in the samples were assayed by HPLC to determine the skin permeation profiles.

Assay Methods The same volume of acetonitrile (5 μg/ml) containing an internal standard (different paraben from that in the sample) was added to the samples and thoroughly mixed. The supernatant after centrifugation was injected into HPLC. The HPLC system consists of pump (LC-6A), UV detector (SPD-6A), chromatopac (C-R6A), system controller (SCL-6B) and auto injector (SCL-6B) (whole parts, Shimadzu, Kyoto, Japan). Mobile phases were 0.1% phosphoric acid : acetonitrile=75 : 25 for *p*-HBA, MP and EP and 0.1% phosphoric acid : acetonitrile=55 : 45 for PP and BP and their flow rate was 1.0 ml/min. Detection was done at UV 260 nm.

Observation of Permeation Pathway in the Skin Barrier by a CLSM Excised hairless rat skin was set on the Franz type diffusion cell as shown in the permeation experiments. PMB solution at a concentration of 5% containing adequate amount of fluorescent dyes or 5% FITC-labeled PMB solution (2.0 ml) was applied on the stratum corneum, and pH 7.4 PBS (*ca.* 17 ml) was applied into the dermis side. Fluorescein Na (Ex 493.5 nm, Em 520 nm) and rhodamine B (Ex 554.0 nm, Em 577.0 nm) were selected as a model hydrophilic and lipophilic fluorescent dye, respectively. *n*-Octanol–water partition coefficients, K_{ow}, of these compounds were determined as below. Skin surface was washed with distilled water after 6 h-permeation experiment and fluorescence (from the skin surface to 0 μm thickness) was observed under a CLSM (MRC-600 confocal system, Bio-Rad, Hercules, CA, U.S.A.).

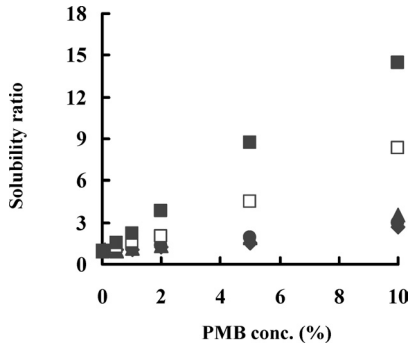

Fig. 2. Effect of PMB and Its Concentration on the Solubility Ratio of Parabens

Symbols: *p*-HBA (◆), MP (●), EP (▲), PP (□), BP (■). Solubility ratio of parabens: solubility in PMB solution against control solution.

Determination of Partition Coefficient of Fluorescein Na and Rhodamine B The same volume of *n*-octanol and distilled water were mixed and saturated each other. Aqueous solution of fluorescein Na and rhodamine B at a concentration of 50 mM was prepared with *n*-octanol-saturated distilled water, and the same volume of distilled water-saturated *n*-octanol was added, thoroughly mixed and kept overnight in a dark room. Fluorescent level in the resulting aqueous phase was determined by a fluorescent spectrophotometer (RF-5300PC, Shimadzu) to calculate partition coefficients, K_{ow}.

MIC Assay of Parabens Minimum inhibitory concentration (MIC) of parabens against the bacteria, *E. coli* and *S. aureus*, was determined by microdilution method.[10,11)] A hundred microliter of serial two-fold dilution of MP or BP were prepared in 96-wells plates with Muller–Hinton broth containing 5% PMB. To prepare an inoculum, the bacteria were cultured with the broth at 37 °C overnight, and were then used to inoculate in fresh Muller–Hinton broth. The bacteria in the logarithmic phase of growth were diluted with Muller–Hinton broth in approximately 1×10^7 colony forming unit (cfu)/ml and 5 μl of the inoculum was added into each well. The plates were incubated at 37 °C for 20—24 h and finally observed for transparency or opacity. MIC of parabens was determined as the minimum concentration showing no opacity.

Results and Discussion

Effect of PMB on the Solubility of *p*-HBA and Parabens in Water Solubility of drugs may be increased by addition of some polymers in the drug solutions. In the present study, solubility change of parabens in distilled water was measured by addition of PMB. Solubility increase was observed in all parabens by addition of PMB. The solubilities of all compounds were increased, and the increase in solubility was proportional with an increase in PMB concentration at least until 10%. Figure 2 shows a relation between solubility ratio and PMB concentration, where the solubility ratio is a ratio of solubility of *p*-HBA and parabens without PMB against that with PMB. The solubility of every compound with PMB was higher than that without it. The solubility ratio was higher in a more lipophilic paraben. These phe-

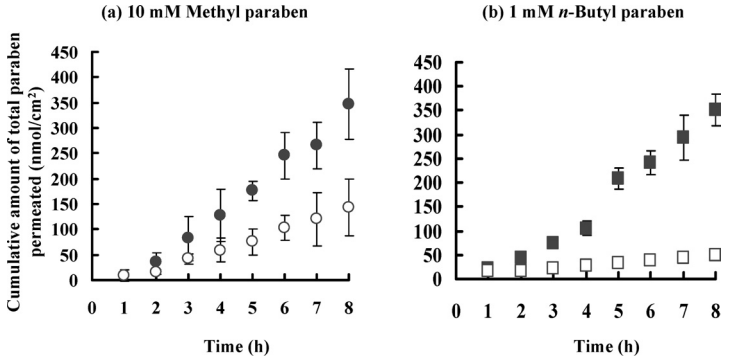

Fig. 3. Cumulative amount of MP and BP Permeated through the Excised Hairless Rat Skin

Each point represents the mean±S.D. (n=3—6). Closed symbol, control; open symbol, 5% PMB solution.

nomena may be explained as follows. Addition of PMB in water may shift the aqueous solution more lipophilic to dissolve lipophilic compounds. Interaction between parabens and PMB to increase their solubilities may be more marked in a more lipophilic paraben. The following experiments were done to evaluate this assumption.

Effect of PMB on the Skin Permeation of *p*-HBA and Parabens Skin permeation experiment of *p*-HBA and parabens was done with and without PMB in the donor solution. The resulting permeation profiles were evaluated using *n*-octanol–water partition coefficient, K_{ow}. Figure 3 shows typical time courses of the cumulative amount of MP and BP that permeated through excised hairless rat skin after application of 10 mM MP and 1 mM BP (these are solutions) with and without 5.0% PMB. *p*-HBA, a metabolite of MP and BP, was also found in the receiver cell as well as the esters. Each value in the figure shows the sum of the esters and *p*-HBA. Addition of 5.0% PMB decreased the skin permeation of both parabens. When compared using the cumulative amount permeated over 8 h, 54.8% and 85.6% decreases by addition of PMB were found for MP and BP permeations, respectively, suggesting that the inhibitory effect of PMB on the skin permeation of parabens was more marked for a more lipophilic compound.

To explain these profiles with PMB in detail, skin permeation profiles of *p*-HBA and parabens (MP, EP, BP) from suspension in different concentrations of PMB solutions were evaluated. Since skin barrier can be considered as one homogenous layer, the steady-state flux (J) of compound in *in vitro* skin permeation is usually expressed by Eq. 1. Figure 4 shows the effect of PMB concentration on J and permeability coefficient (P) of these compounds. The P value was determined as follows:

$$J = \frac{D \cdot K \cdot Cd}{L} = P \cdot Cd \quad (1)$$

where D, K and Cd are diffusion coefficient, partition coefficient (skin/donor solution) and donor concentration (determined by the solubility experiments for Fig. 3.) of penetrant and L is thickness of the skin barrier. Little difference was observed in the steady state flux over 0.5—10% PMB, in spite of several PMB concentrations were used. Thus, no obvious change of supersaturation by PMB was found for the flux of parabens. As the P value is calculated to be J/Cd, on the other hand, the P value was decreased with an increase in PMB concentration.

The P value is a function of D and K values, as shown in Eq. 1. Since the molecular weight of PMB is large enough to ignore its skin permeation, PMB probably does not affect to the diffusivity of *p*-HBA and parabens in the skin barrier. Thus, the inhibitory effect of PMB on the skin permeation of *p*-HBA and parabens must be related to partition coefficient of the compounds, K. Addition of PMB shifts the paraben solution to more lipophilic than that before addition, and decreases the partition of parabens from the solution to the skin barrier. This assumption is related to that a greater increase in the K value (lipophilic parabens) showed a more marked decrease in the P value. As results, a moderately lipophilic paraben, MP ($\log K_{ow}$=1.93) showed 0.42 and 0.37 times skin permeation for 5 and 10% PMB, respectively, compared without PMB, whereas a lipophilic paraben, BP ($\log K_{ow}$=3.24) showed 0.12 and 0.06 times skin permeation for 5 and 10% PMB. It is clear from the results that the PMB effect on the skin permeation is marked for the lipophilic parabens and that the PMB effect is closely related to the increasing effect on the lipophilicity in the paraben solution. Though the D may change according to the moisturizing action of PMB,[6,7] the above-mentioned assumption will be appropriate, because the changes of skin permeation of lipophilic paraben by PMB was more affected than that of moderately lipophilic one.

Estimation of Action Site of PMB in the Skin Barrier Primary permeation route through skin for most low molecular compounds is the stratum corneum not the appendages such as hair follicles and sweat ducts.[12] Hydrophilic compounds mainly permeate through the paracellular aqueous rich domain, whereas the lipophilic compounds permeate through the lipid rich transcellular and paracellular routes of the corneocytes in the stratum corneum. Then, skin surface was observed by a CLSM to evaluate the effect of PMB on the skin permeation of *p*-HBA and parabens.

Figure 5 shows CLSM images 6 h after application of FITC labeled PMB on skin. The fluorescent dye was found evenly on the skin surface. This was much different from that after application of free FITC which was distributed mainly to the corneocytes (data not shown). Figures 6a and b show CLSM images 6 h after application of hydrophilic fluorescence Na ($\log K_{ow}$=−0.625) and lipophilic rhodamine B ($\log K_{ow}$=2.379) with and without 5% PMB on skin, although fluorescent intensity was not directly determined. No significant change was observed between two groups with and without PMB for both fluorescent dyes. These results suggest no or little selectivity for the PMB effect on the *p*-

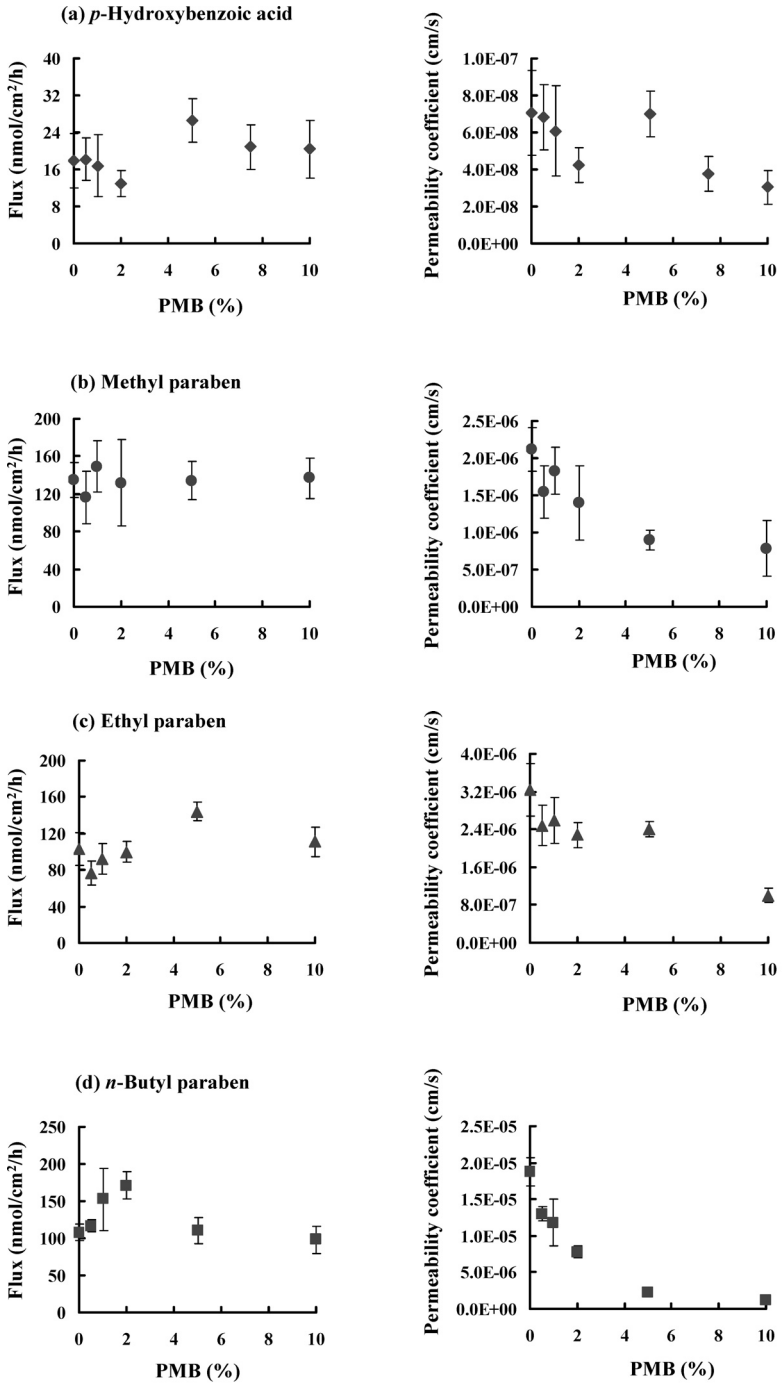

Fig. 4. Effect of PMB and Its Concentration on the *p*-HBA and Paraben Flux and Permeability Coefficient

Each point represents the mean±S.D. (n=4—7). Left side, steady-state flux (J); right side, permeability coefficient (P).

HBA and parabens permeations through different routes of skin pathway.

The Effect of PMB on the Preservative Effect of Parabens Site of action of parabens is the cell membranes of bacteria. The cell membranes are found to be disordered by parabens.[13—15] Therefore, the antimicobial action of parabens may be related to their characteristics to distribute from the drug formulations to cell membranes of bacteria. The distribution phenomena to the cell membrane are closely related to that to the skin barrier membrane, because both the cell membrane and skins are very lipophilic than PMB solution. Change in skin permeability of parabens may influence their preservative effects. Fukahori *et al.*[16] demonstrated that the uptake to bacterial cells and the antibacterial activity of parabens were logarithmically proportional to the carbon number of the alkyl group from MP to BP. Then the effects of PMB on the skin permeation and on the preservative effect of parabens were evaluated. Antimicobial effect of parabens was evaluated by the MIC against *E. coli* and *S. aureus*. Figure 7 shows the results.

MIC and antibacerial effect of parabens increased and decreased, respectively, by addition of PMB against *E. coli* and *S. aureus*. Increase in MIC was not significant for MP, but significant for BP. This tendency was the same to the effect

of PMB on their skin permeations. Addition of PMB probably decreased the partition of parabens into the cell membrane and decreased antibacterial effect, since site of action of parabens is cell membrane of bacteria. Results for the antibacterial test of parabens supported the decrease in paraben partition into the skin membrane as well as the bacterial cell membrane.

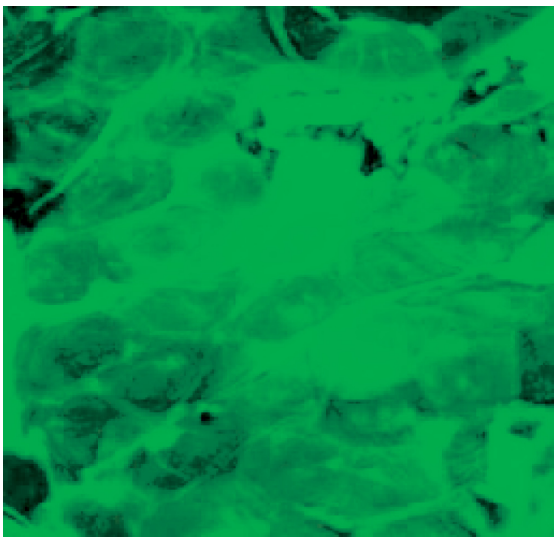

Fig. 5. CLSM Image of FITC-PMB on Excised Hairless Rat Skin

Conclusion

It became clear from the present study that the addition of PMB suppresses undesirable parabens permeation, but decreased the antibacterial activity of parabens. The inhibitory effect of PMB on the skin permeation of parabens was dependent on its effect as a solubilizer. Increase in solubility of parabens decreases the partition into the skin and cell mem-

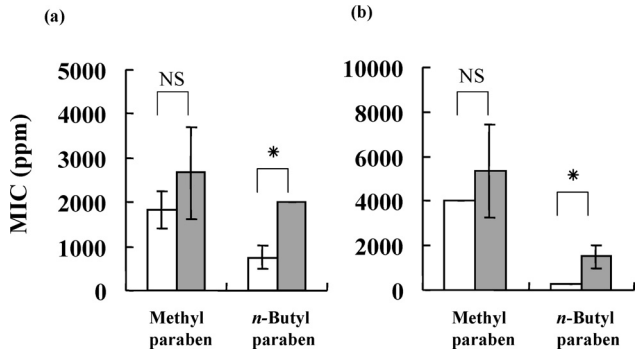

Fig. 7. Antimicrobial Activity of MP and BP to *E. coli* (a) and *S. aureus* (b)

Each column represents the mean±S.D. (n=6). Symbol: □; control, ■; 5.0% PMB (* p<0. 05, NS; p>0.05, Mann–Whitney-U test).

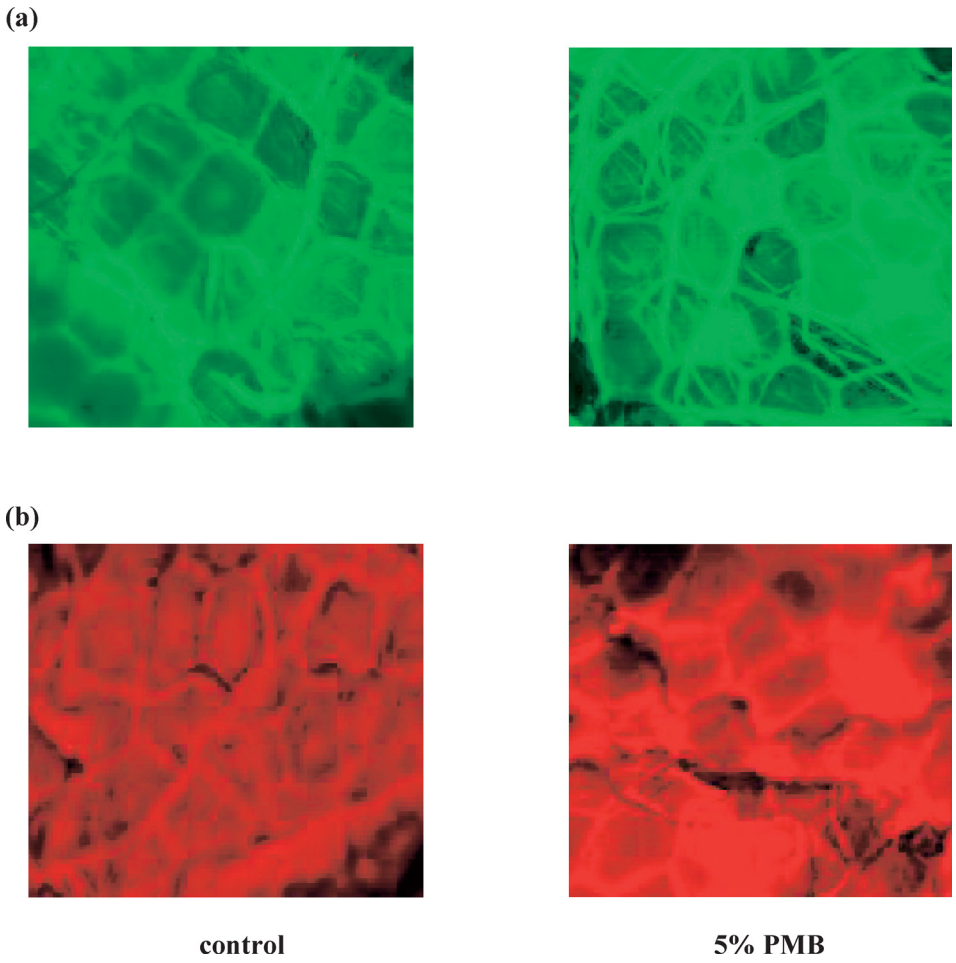

Fig. 6. CLSM Image of Fluorescein Na (a) and Rhodamine B (b) on Excised Hairless Rat Skin

Left side, control (without PMB); right side, 5% PMB.

brane. These are reasons why lower skin permeation and higher MIC of parabens were found with PMB. Effect of polymeric additives on the skin permeation may be so complex, that the effect of polymeric additives as a solubilizer must be very important to verify in the topical formulations. Solubility tests of penetrants may be used to screen formulation additives, especially for polymeric additives like PMB.

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