Preface

The roots of this thesis go back about twenty years. In the early 1990s, I was working at Ajinomoto Co., Inc. as a researcher in Fine Chemical Department. The primary responsibility assigned to me was “claim substantiation” of cosmetic raw materials derived from amino acids such as acylglutamates, which is a typical example of acyl amino acids.

Ajinomoto developed and introduced acylglutamates on the toiletry market in 1970s and had been earning a good reputation as a mild anionic surfactants manufacturer accumulating Draize data in house and human patch data.

In 1990s, new anionic surfactants such as AMT (acyl methyl taurine), MAP (mono alkyl phosphate) and SCI (sodium cocoyl isethionate) had been launched as alternative materials for alkyl sulfates and metal soaps. We needed to know the safety profiles of these surfactants including acylglutamates. In the same time, criticisms of the Draize test have been embraced by the groups supporting abolition of animal testing and the development/ introduction of alternative methods for assessing irritation potential of cosmetic materials had been strongly required in the world. Then, we started to develop alternative methods (cytotoxicity tests) using human keratinocyte and to develop a comparative data set on acylglutamates. Due to the early development for alternative methods prior to commercially available test kits, we, Ajinomoto Co., Inc., could participate in the validation program of “*in Vitro* Eye Irritation Tests for Cosmetic Ingredients” chaired by Japan Cosmetic Industry Association (JCIA) and National Institute of Health Science Japan (NIHS).

The toxic manifestations of topically applied substances may induce immediate phenomena such as corrosion or primary irritation, delayed phenomena such as sensitization, phenomena that require an additional vector such as phototoxicity, and systemic phenomena. Such reactions cannot occur unless the toxic agent reaches a viable part of the skin by going through the stratum corneum with accompanying intercellular lipid structure disruption. If the toxicant can be stored in or absorbed by a skin layer without any alteration in intercellular lipid structure, it may not reach the viable tissues at all. Accordingly, we developed electron paramagnetic resonance (EPR) spectroscopic method employing nitroxide spin probe in order to investigate the mechanism of skin irritation focusing on the structural alteration/ fluidization of intercellular lipid bilayers in human stratum corneum. All the fundamental experiments were conducted with Dr. Howard Maibach, Dr. Cheol Heon Lee, Dr. Danyi Quan, Dr. Kazutami Sakamoto and Dr. Jun-ichi Mizushima at University of California San Francisco. We successfully established the basic EPR spin probe method on cadaver stratum corneum for characterizing the structural change in

intercellular lipid bilayers by anionic surfactants with a discussion on the correlation between spectral data and *in vivo* results by human patch testing.

In 2000s, Mizushima J, et al., Nakagawa N, et al., Yagi E, et al. has improved and advanced this EPR spin probe method by introducing “stripping method” for stratum corneum preparation, designing a “new EPR cell” and “computer simulation”, and now it becomes a robust method for monitoring the structural change in intercellular lipid bilayers induced by topically applied chemicals.

I am grateful to those advancements in EPR spin probe method and hope that EPR spin probe method would also aid in investigating the irritation potential of general chemicals, effects of topical penetration enhancers, drug delivery systems and skin diseases such as xerosis and atopic dermatitis and would be further improved and advanced.

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Lastly, I would like to thank my wife, Naoko and daughter, Marina. Without their support, I would not have been able to complete this writing.

Contents

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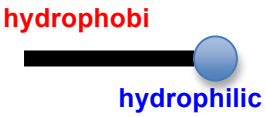
1. Introduction

1.1 Surfactants (Anionic Surfactants) and their behavior in solution

Surfactants are amphiphilic molecules, meaning that they possess two opposing moieties: hydrophilic (water-loving) and hydrophobic (water-hating) in their structure. (Fig-1.1) The surfactant, which has “negatively charged part(s)” as a hydrophilic part, is called as “anionic surfactant”.



Type	Charge	Chemical moieties
Ionic	Anionic (-)	carboxyl group sulfate group, etc.
	Cationic (+)	ammonium group, etc.
	Amphoteric (±)	betaine, etc.
Non-ionic (zero charge)		polyoxyethylene group, etc.

Fig-1.1: Schematic structure of surfactants and the categories

In general, dissolution phenomenon of chemicals into liquid is a process of which free energy of the entire system reduces thermodynamically. This means that the free energy change of the Gibbs equation (ΔG) is negative.

$$\Delta G = \Delta H - T\Delta S$$

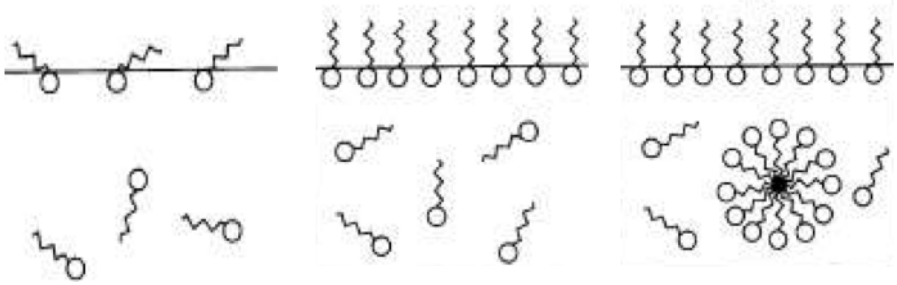
(ΔH: enthalpy change, ΔS: entropy change, T: absolute temperature)

Thus, in the system that is exothermic and its entropy increases, dissolution reaction spontaneously proceeds. On the other hand, even though dissolution process is endothermic, dissolution proceeds until Δ G reaches “zero” due to the greater entropy increase than the enthalpy ($\Delta H < T\Delta S$). Accordingly, the solute is dissolved uniformly as a molecule in the liquid until it reaches the saturation solubility.

However, the surfactant solution assumes a quite different complexion from the general solution due to the amphiphilic structure of the surfactant. The hydrophilic moiety in the surfactant promotes hydration with water due to its affinity to water, which is exothermic ($\Delta H < 0$). The hydration to the hydrophilic moiety spontaneously

proceeds. Contrarily, the hydrophobic moiety tries to minimize the contact with water, which means that hydrocarbon is less soluble to water and endothermic ($\Delta H > 0$) due to the hydrogen bonding amongst water molecules. (It cannot unequivocally said that ΔH is greater than "zero", because hydrocarbons is slightly soluble into water.) In other words, Van der Waals force between hydrophobic chains and between hydrophobic chain and water, is greater than the hydrogen bonding amongst water molecules. As the results, water molecules around the hydrophobic chains forms the structure like "ice", which decreases enthalpy and increases free energy of the system. The surfactant monomer absorbs at the air/water interface with the hydrophobic part trying to avoid the water environment.

Water solubility of surfactants as a single molecule (monomer) is very small as compared with the hydrophilic substances because of its amphiphilic nature. As the concentration of the surfactant increases and the surface is saturated, the surfactant forms molecular aggregates called "micelles" (Fig-1.2, Tominaga T, (2005)), which means entropy (ΔS) increases because of the destruction of water structure surrounding hydrophobic chains. The concentration of the surfactant forming "micelles" is called "critical micelle concentration (CMC)", which is a specific value assigned to each surfactant and implies the saturation concentration of monomer in bulk water phase.



(a) (b) (c)

Fig-1.2: Surfactant behavior in solution; (a) Surfactant molecule in aqueous solution absorbs at the air/water interface with hydrophobic moiety oriented toward the air side. (at very low concentration) (b) When the concentration of surfactant increases, the interface becomes saturated with surfactant molecules. (c) To minimize their interaction with water, the hydrophobic moieties of the surfactants interact together and form "micelles" in solution (at high concentration). The monomers and micelles are under equilibrium.

1.2 Development history of mild anionic surfactants

As listed in Table 1.1, anionic surfactants are mainly used in cleansing products, which consumers are exposed on a daily basis. Cleansing products are undoubtedly some of the irritating products, particularly because they are applied to the body, which is sensitive. Accordingly, evaluation of the irritation potential to human skin of anionic surfactants is a must.

Table 1.1: Surfactant containing products

Market type	Product categories (examples)
Cosmetics/ toiletries	Body cleansing liquid (shower gel, facial cleansers, etc.)
	Body cleansing solid (bar soaps, syndet bars, etc.)
	Hair shampoos
	Shaving products
	Toothpastes
	Deodorant
Household products	All purpose cleaners
	Window cleaners
	Hand dishwashing detergents
	Automatic dishwashing detergents
	Fabric detergents
	Fabric softeners

Table 1.2: Detergent Market in Japan (Year 2012)

2012年1月～12月洗浄剤等の製品販売統計

品目				販売量 (t)	販売金額 (百万円)	前年同期比（%） 数量	前年同期比（%） 金額
身体洗浄剤	皮膚用	浴用固形石鹸		42,498	24,618	87	86
		手洗い用液体石鹸		62,272	28,009	103	99
		洗顔・ボディ用身体洗浄剤		128,517	110,426	103	112
		計		233,287	163,053	100	105
	頭髪用	※シャンプー		144,990	104,603	96	93
		※ヘアリンス		43,954	29,010	96	99
		※ヘアトリートメント		56,091	74,939	94	92
		計		245,035	208,616	95	94
衣料用台所用住宅・家具用その他洗浄剤	石けん（洗濯用石けんなど）			32,621	6,543	93	93
	合成洗剤	洗たく用	粉末	311,174	73,113	89	87
			液体 中性	261,810	63,194	107	106
			液体 中性以外のもの	169,158	51,396	111	111
			液体 計	430,968	114,590	108	108
			計	742,142	187,703	99	99
		台所用		204,521	50,352	100	98
		住宅・家具用		125,918	33,624	99	99
		計		1,072,581	271,680	99	99
	柔軟仕上げ剤			260,332	71,459	103	102
	漂白剤	酸素系		143,623	27,704	104	109
		塩素系		147,142	20,522	99	96
		計		290,765	48,226	101	103
	酸・アルカリ洗浄剤			65,482	11,014	104	97
	クレンザー			11,350	1,797	93	91
合計				2,211,453	782,324	100	99

(注)
（1）業務用を含む
（2）※印は『化粧品月報』の調査による。
※印以外は、洗顔・ボディ用身体洗浄剤を除いて『油脂製品、石けん・合成洗剤等及び界面活性剤月報』の調査による。
（3）洗顔・ボディ用身体洗浄剤とは、『油脂製品、石けん・合成洗剤等及び界面活性剤月報』の「洗顔・ボディ用身体洗浄剤」と『化粧品月報』の「洗顔クリーム・フォーム」の計である。
（4）石けんとは、洗濯用（固形・粉末）・工業用・台所用・特殊用途用石けんなどの計である。
（5）数字の単位は四捨五入しているので、合計と内訳は必ずしも一致しない。
（6）金額は消費税込みである。

●2013年5月補正済みデータ

※2011年から、柔軟仕上げ剤の濃度の区分が廃止されました。

資料：経済産業省鉱工業動態統計調査室
作表：日本石鹸洗剤工業会

According to the market information for the year 2012 by Japan Soap and Detergent Association (http://jsda.org/), total 2.2 million tons/y of detergent products containing surfactants were sold on Japanese market. Body cleansing products, such as bar soaps, hair shampoos and so on, containing anionic surfactants as a primary surfactant, were sold at approx. half million tons/y. Household products like laundry detergents were sold at more thant 1million tons/y. Comsumption of surfactants has been increasing for last several decades.

From 1950s through 1970s, some new surfactants were developed and launched on the market as a soap alternative pursuing “detergency” and “formulation aids” such as AS (alkyl sulfate), AOS (alpha-olefin sulfonate), ABS (alkyl benzene sulfonate), etc., which created the problems of skin disorders and environmental issues.

In order to resolve social problems such as skin trouble and environmental issues, research and development of new anionic surfactant was further advanced after 1980s. (Kawasaki Y, et al (1991), Takino Y, et al (1994))

Now we have several types of anionic surfactants; (1) AES (alkyl ether sulfates), which is introduced polyoxyethylene (POE) between the hydrophobic (alkyl) group and a hydrophilic (sulfate) group of AS (alkylsulfates), (2) AGS (acylglutamate), AMT (acylmethyltaurine), LBA (Lauroyl-beta-alanine) which possess “amino acids” or similar chemical structure as a hydrophilic group, (3) MAP (mono alkyl phosphate), which is phosphoric acid ester type, (4) AEC (alkyl ether carboxylates), which is introduced polyoxyethylene (POE) between the carboxyl group and the alkyl group of the fatty acids, (5) SCI (cocoyl isethionate) and SS (salkylsulfosuccinate), which has “sulfonate” as a anionic part and also possesses “ester” bond in their structure.

The anionic surfactants in Table 1.3 were developed balancing performance as anionic surfactants and safety to human skin.

Table 1.3: Anionic surfactants structures and their usage

Anionic surfactants		Chemical Structure	Usage
Soap		R-COO M	body soap hand soap
AS	alkyl sulfate	R-O-SO_3 M	hair shampoo toothpaste laundry detergent
AES	alky ether sulfate	R-O-$(CH_2CH_2O)_n$-SO_3 M	hair shampoo dish wash laundry detergent
AGS	acylglutamate	RCONHCHCOOH CH_2CH_2COO M	body soap shampoo emulsifier for formulations
AMT	acyl methyl taurate	R$CON(CH_3)CH_2CH_2SO_3$ M	body soap shampoo
LBA	lauroyl-beta-alaninate	R$CON(CH_3)CH_2CH_2COO$ M	body soap
MAP	monoalkyl phosphate	O ‖ R-O-P-O M | OH	body soap emulsifier for formulations
AEC	alky ether carboxylate	R-O-$(CH_2CH_2O)_n$-CH_2-COO M	shampoo
SCI	cocoyl isethionate	R-COO-CH_2CH_2-SO_3 M	body soap
SS	monoester sulfossuccinate	R-O-$(CH_2CH_2O)_n$-$COCHCH_2$-COO M SO_3 M	body soap shampoo

[Note] M: counter ions such as Na^+, $(HOC_2H_4)_3N^+$, etc)

From the viewpoint of application of surfactants to detergent systems, which many people use on their skin by their own ways, "minimal damaging effect to the skin" is a critical requirement for surfactants. However, reducing significantly the function / performance as a detergent is fatal from its essential purpose. Accordingly, it is an important issue to establish both the performance as a detergent and the safety of the surfactant in the development of low irritation (mild) surfactants.

1.3 Test methods for evaluating irritation potential

Table 1.4 shows currently available test methods, which were established and have been studied for evaluating irritation potential of chemicals.

Table 1.4: Test Methods of Irritation Potential Evaluation

Test Methods			Advantages/ Disadvantages
In vivo	(1) Animal	• Draize test	• Accumulated data available • Good for harsh tests such as eye irritation, sensitization, mutation, etc. • Good for screening purposes • Costly and requiring time
	(2) Human	• Use test • Image analysis of skin surface • Closed patch test • Open patch test • Repeat patch test, etc.	• Best test to confirm safety to human • Too dangerous for harsh tests such as eye irritation, sensitization, etc. • Costly and requiring time
In vitro	(1) Biophysical approach (2) Biochemical approach	• Absorption test onto stratum corneum • Stratum corneum swelling test • Lipid removal test • Permeation test through membrane/ skin (human or animal) • Intercellular lipid structure analysis (model lipids) • Partitioning test, etc. • Protein denaturation • Hemolysis test, etc.	• Good for elucidating mechanism of adverse reaction • Good for screening purposes
	(2) Cell Biological Approach (cytotoxicity assay)	• Cell viability test • Cell proliferation test • Cell adhesion test • Cell metabolism, etc.	

To predict irritation on human induced by anionic surfactants, it should be ideal that we conduct tests on “human”. However, it is impossible to do it from a humanitarian point of view for all the compounds, especially new compounds. Accordingly, Draize test employing “animal (rabbit)”, of which procedure was developed

in 1944 by Draize and his colleague to assess primary irritation and corrosion induced by chemicals, has been utilized for a long time.

However, the reproducibility of Draize test procedure has been questioned, and numerous modifications have been examined and proposed to improve its prediction of human experience (Weil et al. (1971), Edwards (1972), Nixon et al (1975), MacMillan et al (1975), Guilot et al (1982)). Criticisms of the Draize test have been embraced by the groups supporting abolition of animal testing as demonstrating that use of the method is unwarranted. In Europe, the animal welfare movement got so active that the development of alternative methods was accelerated targeting 100% abolition of animal testing by the end of 1990s.

As summarized in Table 1.4, numerous in vitro/ ex vivo studies have been conducted, from 1980s through 2000s, to predict irritation potential of anionic surfactants considering the multiplicity of ways. Surfactants first act on the surface of the skin, then penetrate the stratum corneum and perhaps beyond it. Within the stratum corneum, potential target sites of action are intercellular lipid, keratin in corneocytes, and desmosomal intercorneocyte connections. If stratum corneum has been permeated, surfactants can affect living cell metabolism in the epidermis or even elicit a cytotoxic action. Penetration past the living epidermis into the dermis can elicit an inflammatory response. The surfactant may not even have to act directly on the dermis. Communication via production of cytokines that can elicit a response from dermal components also occurs. (Polefka TG (1990)). All these interactions may lead to irritation. Whatever the mechanism of interaction between the surfactant and the skin, the free monomeric form will be the key driver to initiate irritation because micelles of which surfactant molecules aggregates have more than 3 kDa and cannot go through stratum corneum.

From 1990s to 2000s, cell biological approach for assessing toxicity of chemicals has significantly evolved as “cytotoxicity assays”, employing human viable cells and the other equivalent. Numerous studies were conducted and have been extensively examined and now some methods have been validated and accepted as “non-animal test methods” by OECD (Organization for Economic Co-operation and Development), USDA (United States Department of Agriculture), FDA (Food and Drug Administration) and JaCVAM (Japanese Center for the Validation of Alternative Methods) (http://www.alttox.org/ttrc/ validation-ra/validated-ra-methods.html).

Contrarily, although physicochemical approach has not been validated/ accepted by regulations, it has been continued steadily as a fundamental approach to understand the mechanism of skin irritation reaction.

The skin is exposed to chemicals in cosmetics and toiletry products and it is thought that they interact in some way with the lipid structure of the stratum corneum after migrating into the stratum corneum. The intercellular lipid layers in stratum corneum are the first gates controlling the penetration of surfactants. Unless surfactants disrupt the lipid structure of the stratum corneum, we do not have worry about the potential reactions with the viable cells under stratum corneum. Qualitative and quantitative characterization of structural change in the intercellular lipid layers induced by surfactants is very important to discuss their irritation potential. Various techniques are used to study lipid structure (both inter- and intra-molecular) and action of chemical constituents with these lipids.

Some of these techniques are (1) X-ray diffraction study the effects on interlayer spacing and water penetration into the lipid lamellar structure using liquid crystal model of stratum corneum lipids and allowing conclusions on structural rearrangements (Blaurock, AE (1982); Friberg S, (1988)); (2) Differential scanning calorimetry (DSC) to study the phase transitions and various thermodynamic properties associated with those transitions allowing conclusions regarding the effects of agents on physico-chemical state of barrier (Goodman M, et al. (1986); Inoue T, et al. (1986)); (3) Fourier transform infrared spectroscopy, which measures the vibrational energies of molecules by light absorption, the frequency and intensity of which is greatly affected by neighboring molecular environments (Golden GM, et al. (1987); Mak FHW, et al. (1990); Takeuchi Y, et al., (1992)); (4) nuclear magnetic resonance spectroscopy (NMR) and electron paramagnetic resonance spectroscopy (EPR) techniques, which can determine mobility of molecules and interrelationships with another molecules in the lipid membrane and effects of other chemical constituents on the interactive parameters (Gay CL, et al. (1989); Gay CL, et al. (1990), Eagle SC, et al. (1992)); (5) optical microscopy technique under regular or polarized light, which provides information about phases present in barrier lipid mixtures and sensitivity of phase changes to environmental and chemical constituents.

Considering the sensitivity of measurement, sample shape limitation, future modification of the method, electron paramagnetic resonance spectroscopy (EPR) technique was selected to use in this study.

1.4 Study Objectives

As mentioned above, surfactants are widely used in cleansers for several areas of the human body. The mildness of surfactants to the skin is a great concern to consumers because of the high frequency of usage and possible presence of several defective skin conditions such as xerosis, atopic dermatitis and so on. Thus, it is worthwhile to develop surfactants with minimal damaging effects on the skin. Identification of the mechanisms underlying surfactant damage to the skin should be very helpful in reducing irritancy of surfactants.

Surfactants contained in cosmetics and toiletry products interact in some way with the lipid structure in the stratum corneum. The intercellular lipid layers in stratum corneum are the first gates controlling the penetration of surfactants. Unless surfactants disrupt the lipid structure of the stratum corneum, we do not have to worry about the potential reactions with the viable cells under stratum corneum. Qualitative and quantitative characterization of structural change in the intercellular lipid layers induced by surfactants is very important to discuss their irritation potential.

In this study, mechanism of anionic surfactants irritation is to be characterized focusing on their interaction with human intercellular lipid layers using electron paramagnetic resonance spectroscopy (EPR) as a function of surfactant structure, and the correlation between EPR spectral data (order parameter) and the clinical/ in vitro assay data; patch tests on human, Draize tests and several techniques of in vitro cytotoxicity assay, is also being discussed.

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2. Characterization of Irritation Potential of Anionic Surfactants *in vitro* testing; Acylglutamates as Anti-Irritant for Mild Detergent System

副論文（１）

Kanari M, Kawasaki Y and Sakamoto K (1993) Acylglutamates as Anti-Irritant for Mild Detergent System (マイルド洗浄剤におけるアシルグルタミン酸塩の刺激緩和効果), *JSCCJ, 27, p.498-505*

[Synopsis]

Aclyglutamate is an anionic surfactant synthesized from two natural occurring moieties, glutamic acid and fatty acid. The pH of its aqueous solution is around 5.5, which is nearly equal to that of normal human skin. Aclyglutamate, of which mildness for skin and eyes has been proven through laboratory and clinical tests, has been used as a primary surfactant for various types of cleansing products on the market.

In this chapter, sodium lauroyl glutamate (SLG), sodium laurylether sulfate (SLES), sodium laurate (SL), sodium monolauryl phosphate (SMLP), sodium lauroyl methyl taurate (SLMT), sodium lauryl sulfate (SLS) and some other surfactants utilized in toiletry products on the market, were selected as test articles. We discuss irritation potential of these anionic surfactants with *in vitro* methodologies; (1) cytotoxicity using human epidermal keratinocytes (MTT assay, NR assay) and SIRC (rabbit cornea) cell (colony formation assay with Crystal Violet), and (2) SKINTEX^{TM} and EYTEX^{TM} system (*In Vitro* International, Irvine, CA), and also discuss their correlation with the conventional Draize score (PII for skin irritation).

SLG showed lowest irritation potential by all the tests and demonstrated as an anti-irritant for a surfactant mixture systems containing salt of acylated glutamic acid at more than 50%.

2.1 Introduction

Anionic surfactants are mainly used in cleansing products which consumers are exposed on a daily basis. Cleansing products are undoubtedly some of the irritating products, particularly because they are applied to the body, which is sensitive. Therefore, it is not surprising that one of the most desirable claims for cleansing products is “mild”.

Although the properties of detergency and mildness seem to be contradictory, Goldemberg RL, et al. (1977) and Cade P (1990) demonstrated that it is possible to reconcile these opposites by careful choice of surfactants that are known to show the reduction of potential irritation.

Acylglutamate is an anionic surfactant composed of two naturally occurring moieties, L-glutamic acid and fatty acid. The pH of its solution is around 5 to 6, which is nearly equal to that of human skin. Extreme mildness of acylglutamate, which was developed more than 30 years ago, was proven by extensive animal and human study at the time. Since then, acylglutamate has been used mainly as a primary surfactant for mild cleansing products. Throughout the consumers’ daily use of products containing acylglutamate over these 30 years, we have convinced the usefulness of acylglutamate. Further more, acylglutamate contributed to the people who are suffering from the use of regular/ ordinary products. For example, the pigmented cosmetic dermatitis or Riehl’s melanosis, which was once a serious problem in Japan, was almost swept away by the joint effort of dermatologists and cosmetic manufacturers. Development of allergen control cosmetics by Nakayama H, et al. (1977) was a key concept to resolve this problem where aclyglutamate played an important role as a mild, hypoallergenic and non-comedogenic surfactant.

Primary irritation and corrosion are usually evaluated by the method described by Draize and his colleagues (1944a, 1944b). The test was widely used in late 1940s and 1950s even though it was not mandated by regulatory agencies until enactment of the Federal Hazardous Substance Act (FHSA).

Results of the tests are usually presented in a tabular form showing erythema and edema response for each animal and the Primary Irritation Index (PII) is calculated. The PII values are calculated by averaging values for erythema from all test sites, averaging values for edema from all test sites, and adding the averaged values. A substance producing PII of <2 is considered “mildly irritating”, 2-5 is “moderately irritating” and >5 is “severely irritating”. The material producing PII of greater than 5 requires precautionary labeling.

Table 2-1: Grading scale used in Draize Tests in rabbits

Description		Score assigned
Erythema	No erythema	0
	Very slight erythema (barely perceptible)	1
	Well defined erythema	2
	Moderate to severe erythema	3
	Sever erythema (beet redness) to slight eschar formation	4
Edema	No edema	0
	Very slight edema (barely perceptible)	1
	Well defined erythema (edge of area well defined by definite raising)	2
	Moderate to severe edema (raised approx. 1 mm)	3
	Sever erythema (raised more than 1 mm & extending beyond the area of exposure)	4

Note: The scale is defined by Draize and adopted various agencies. Primary Irritation Index (PII) is calculated by averaging values for erythema from all test sites, averaging values for edema from all test sites, and adding the averaged values. (Maximum PII = 8)

The reproducibility of the test procedure (Weil et al. (1971)) and the relevance of test results to human experience (Edwards (1972), Nixon et al (1975), MacMillan et al (1975), Guilot et al (1982)) have been questioned, and numerous modifications to the procedure have been examined and proposed to improve its prediction of human experience. One criticism that is often repeated is that the test is not sensitive enough to separate mild to moderate irritants. We should remember that the purpose of Daize test is basically to identify chemicals that posed a severe hazard to the public and difficult to use it for comparing products mildness. Criticisms of the Draize test have been embraced by the groups supporting abolition of animal testing as demonstrating that use of the method is unwarranted. This overlooks the tremendous value of the test in warning consumers and manufacturers of potential dangers associated with specific chemicals so that appropriate precautions could be taken.

Due to the animal welfare movement and needs for prediction of chemicals irritancy to human based on biochemical standpoint, *in vitro* alternative methodologies, which is “cytotoxicity assays” employing viable cells, have been extensively studied and developed for assessing toxicity of chemicals.

Cytotoxicity (cell toxicity) assays can be categorized into three groups: (1) adhesion/ cell proliferation, (2) membrane integrity, and (3) cell metabolism. Several assays have been developed in each of three groups. The advantages of cell toxicity assays include their technical simplicity and the fact that endpoints are mostly quantitative and objective. SIRC (rabbit cornea) cell (colony formation assay with Crystal Violet) is an example of the test that requires both adhesion and proliferation of cells.

For the detection of cytotoxicity or cell viability following exposure to toxic substances, LDH leakage assay, protein assay, neutral red and MTT assay are now the most commonly employing various viable cells like human keratinocytes, fibroblast, and so on.

LDH (lactate dehydrogenase) assay is based on the measurement of leaked LDH activity in the extracellular medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage. Reliability, speed and simple evaluation are some of the characteristics of this assay (Decker and Lohmann-Matthes, 1988). It has been employed as an indicator of cytotoxicity in HepG2 cells following exposure to cadmium chloride (Dong et al., 1998) as well as in toxicity studies using rat renal proximal tubular cells (Fukumoto et al., 2001).

MTT assay is another cell viability assay often used to determine cytotoxicity following exposure to toxic substances. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltet- razolium bromide) is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells. MTT assay has been used in HepG2 cells (Tully et al., 2000) and in rat lung epithelial cells after exposure to cadmium chloride (Hart et al., 1999) and was examined its validity in various cell lines (Mossmann, 1983).

Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride; NR) assay can be also used to measure cell viability. It has been used as an indicator of cytotoxicity in cultures of primary hepatocytes (Fautz et al., 1991) and other cell lines

(Morgan et al., 1991). Living cells take up the neutral red, which is concentrated within the lysosomes of cells.

In this study, we selected several kinds of anionic surfactants as test articles from commercially available ones, which are commonly used in toiletry products. we discuss irritation potential of the anionic surfactants with *in vitro* methodologies; (1) cytotoxicity using human epidermal keratinocytes (MTT assay, NR assay) and SIRC (rabbit cornea) cell (colony formation assay with Crystal Violet), and (2) SKINTEX® and EYETEX® system *(In Vitro* International, Irvine, CA), and also discuss correlation between conventional Draize score (PII for skin irritation). We also examined anti-irritant potential of acylglutamates to other anionic surfactants for establishing mild detergent systems.

2.2 Experimental

2.2.1 Materials

We selected several kinds of anionic surfactants (Table 2-2) as test articles from commercially available ones, which are commonly used in toiletry products. As for monosodium N-lauroyl-L-glutamate (SLG), sodium monolaurylphosfate (SMLP) and sodium N-lauroyl-N-methyltaurate (SLMT), they were re-crystallized and confirmed that each surfactant showed single spot with thin layer chromatography.

Table 2-2: Test Articles (anionic surfactants)

#	Abbreviations	Chemical Name	Lipophilic moiety	Hydrophilic moiety
1	SLS	Sodium lauryl sulfate	C12	sulfate
2	SLMT	Sodium N-lauroyl-N-methyltaurate	C11	methyltaurate (sulfonate)
3	SLES	Sodium laurylethersulfate POE(3)	C12	ethersulfate
4	SL	Sodium laurate	C11	carboxylate
5	SMLP	Sodium monolaurylsphosfate	C12	phophate
6	SLG	Sodium N-lauroyl-N-glutamate	C11	glutamate (carboxylate)
7	SCI	Sodium N-cocoyl isethionate	C11~13	sulfonate
8	SCG	Sodium N-cocoyl-N-glutamate	C11~13	glutamate (carboxylate)
9	TCG	Triethanolamine N-cocoyl-N-glutamate	C11~13	glutamate (carboxylate)
10	SCMT	Sodium N-cocoyl-N-methyltaurate	C11~13	methyltaurate (sulfonate)
11	SSS	Sodium monolaurylsulfosuccinate	C12	Sulofonate + carboxylate

2.2.2 MTT assay

MTT is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells.

Normal human epidermal keratinocyte (NHEK) were cultured according to the procedure defined by Greene (1979) under the condition of 37^{o}C, 5% CO_2, FAD medium (Ham-F2/Dulbecco's modified Eagle's medium / adenine / penicillin / NaHCO3 / fetal calf serum / insulin / transferrin / T3 / cholera toxin / EGF / hydrocortisone) was used for cultivation of NHEK growth. After 80 ~ 90% confluent in a culturing flask, NHEK were removed from the flask by trypsin / EDTA treatment and transferred into 96-well plate at 10000 cells/well with FAD medium. The test articles were dissolved in FAD medium at several different concentrations and added to the designated wells. The plates were incubated until the control wells, which were treated with FAD media not containing test articles, became confluent (5~6 days). Then, MTT assay was conducted with the following procedures.

The medium was removed from the wells and 0.1mL of 0.05% MTT solution dissolved in FAD was added to the well. The plates were incubated at 37^{o}C for 5 hours. After the incubation, 0.1mL of 10% SLS (sodium lauryl sulfate) 0.04N HCl/ PBS was added to the wells. The plates were left for 24 hours and the absorbance at 570nm (A_{570}) and at 630nm (A_{630}) for each well were measured with a Microplate Reader (Bio-Rad). The value of "$A_{570} - A_{630}$" of the control and each test article with different concentration of test articles were calculated as $\delta A_{control}$ and δA_{sample}, respectively. Then, the "% of control" for each article at each dose was calculated in accordance with following formula.

$$\% \text{ of control} = \delta A_{sample} / \delta A_{control} \times 100 \ (\%)$$

Based on the dose response curve, the concentration of each test article's causing 50% reduction of control (MTT_{50}) was determined.

2.2.3 Neutral Red (NR) assay

The NR assay was conducted in accordance with the protocol described in the book edited by Goldberg AM (1987, 1989) Borenfreund et al. (1984, 1988) and determines the accumulation of the NR dye in the lysosomes of viable, uninjured keratinocytes.

Normal human epidermal keratinocyte (NHEK) were cultured in the serum-free medium. After 80 ~ 90% confluent in a culturing flask, NHEK were removed from the flask by trypsin / EDTA treatment and transferred into 96-well plate at 2500 cells/well with FAD medium. The test articles were dissolved in the culture medium at several different concentrations and added to the designated wells. The plates were incubated until the control wells, which were treated with the medium not containing test articles, became 30~50% confluent (2 days). The test articles were dissolved in the medium at several different concentrations and added to the designated wells. The plates were incubated until the control wells, which were treated with FAD media not containing test articles, became confluent (2~3 days). Then, NR assay was conducted with the following procedures.

The medium was removed from the wells and 0.25mL of 0.05% NR solution dissolved in the culture medium was added to the well. The plates were incubated at 37^{o}C for 3 hours. Cells were then washed with Phosphate Buffered Saline (PBS) followed by gentle shaking with 0.1mL solvent for approx. 10 minutes so that complete dissolution was achieved. The resulting solutions were transferred to 96-well plates and absorbance at 540 nm (A_{540}) was recorded using the Microplate Reader (Bio-Rad). Then, % of control of each test article at each designated concentration was calculated by the following formula.

$$\% \text{ of control} = A_{540}\,[\text{sample}] / A_{540}\,[\text{control}] \times 100\ (\%)$$

[for each concentration of test articles]

Based on the dose response curve, the concentration of each test article's causing 50% reduction of control (NR_{50}) was determined.

2.2.4 SIRC cell toxicity assay

SIRC colony forming assay, reported by North-Root et al. (1982 & 1985), is an example of a test that requires both adhesion and proliferation of cells to obtain the endpoint. SIRC cells are fibroblast like cells and an established line (ATCC No.CCL-60) derived from rabbit cornea available from American Type Culture Collection (ATCC).

In the assay, a relatively small number (approx. 400) of SIRC cells are plated in 60 mm culture dishes in Ham's F12S medium. After 18 hours incubation at 37^{o}C and cells attach the plates, cells were washed with the medium and exposed to 4mL fresh medium containing the test articles at different concentrations. After 1 hour of additional incubation at 37^{o}C, cells were washed, provided fresh medium and incubated for 7 days to allow surviving cells to form visible colonies. Cells are fixed, stained by crystal violet and colonies were counted and compared with the number of colonies in untreated control plates.

% of control = # of colonies treated sample/ # of colonies control x 100 (%)

[for each concentration of test articles]

Based on the dose response curve, the concentration of each test article's yielding 50% reduction of control (LC_{50}) was determined.

This test was fully conducted at Microbiological Associates, Inc. (Bethesda, MD, USA)

2.2.5 SKINTEX® and EYTEX® Test

SKINTEX® and EYTEX® Test, on which Kelly CP (1989) and Kruszewski FH (1990) reported, were fully conducted by ROPACK Laboratory (Irvine, CA, USA) based on their test protocols. Test solutions were prepared at 5%wt for all test articles for comparing to Draize scores.

2.3 Results and Discussion

2.3.1 Assessment of anionic surfactants by MTT assay

MTT_{50} results of anionic surfactants having C_{12} carbon chain and sodium (Na^+) as a counter ion are summarized in Table 3 including PII values by Draize test.

Table 2-3: MTT_{50} results of anionic surfactants

#	Test Articles	MTT50 (ppm)	Draize score Skin irritation (5%)
1	SLS	80	7
2	SLMT	108	2.5
3	SLES	114	3.5
4	SL	263	3
5	SMLP	643	3.3
6	SLG	904	1.3

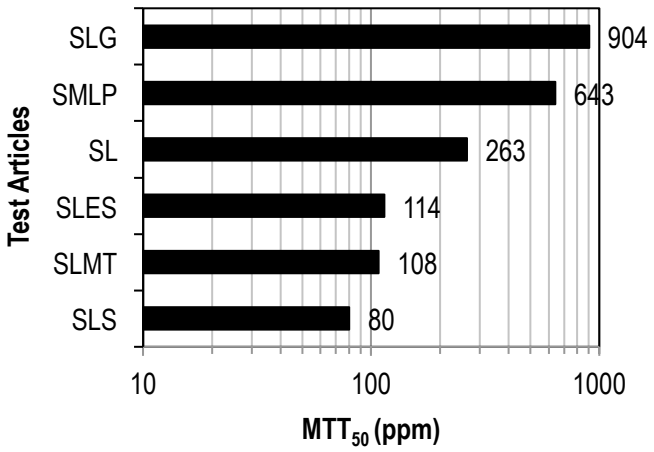


Fig 2-1: MTT_{50} results of anionic surfactants

All the test articles are popular surfactants used in the current toiletry products as either primary surfactant or co-surfactants. Draize grading system showed that SLG is “mild” (PII < 2), SLS is “severe” (PII > 5) and other surfactants

are “moderate” (2 ≦ PII ≦ 5). SL (sodium laurate), which is one of the fatty acids salt and has been used since ancient time, represents “moderate” surfactant. SLES (sodium lauryl ether sulfate), SLMT (sodium lauroyl methyltaurate), SMLP (sodium monolauryl phosphate), which were introduced on the market with balancing detergency and mildness, represent “moderate” surfactants as well. It is interesting that SLG (sodium lauroyl glutamate), in which glutamic acid structure is introduced as an ionic dissociation moiety, showed lowest PII value amongst the test articles.

As shown in Fig 2-1, MTT_{50} for SLG was 904 ppm, which is the highest number and implies the “mildest” surfactant amongst the articles. Contrarily, SLS showed 80 ppm as MTT_{50} that means the “harshest” surfactant.

SLS, SLMT and SLES, which possess “sulfate” or “sulfonate” structure as an ionic dissociation moiety in their structure, showed MTT_{50} in the range from 80 to 114 ppm. SLMT and SLES originally should have been developed in order to balance detergency and mildness to the skin, however, MTT_{50} results does not support it. SL had 263 ppm as MTT_{50}, which means SL is milder than “sulfate” based surfactants and SMLP is indicated as a mild surfactant close to SLG.

Irritation potential of anionic surfactants may depend on the chemical structure of ionic dissociation moieties, which relates to pKa, dissociation constant in water. Under the fixed condition with dodecyl (C_{12}) hydrocarbon chain, it may be concluded that strong acid “sulfate” gives irritation to the skin and weak acid “carboxylate”, “glutamate” does moderate to mild irritation to the skin. However, “phosphate” does not show irritation potential same as glutamate even though “phosphate” is a strong acid. It is well known that phosphatidyl choline, having a phosphate moiety, forms lipid bilayer structure. SMLP, possessing the same “phosphate”, does not destroy either the skin structure or cell membrane, but is incorporated as a part of their component.

Fig 2-2 shows the correlation between MTT_{50} and PII value by Darize score. The correlation coefficient (r^2) between them is “0.41”, which implies MTT_{50} is not good parameter for predicting skin irritation potential of anionic surfactants.

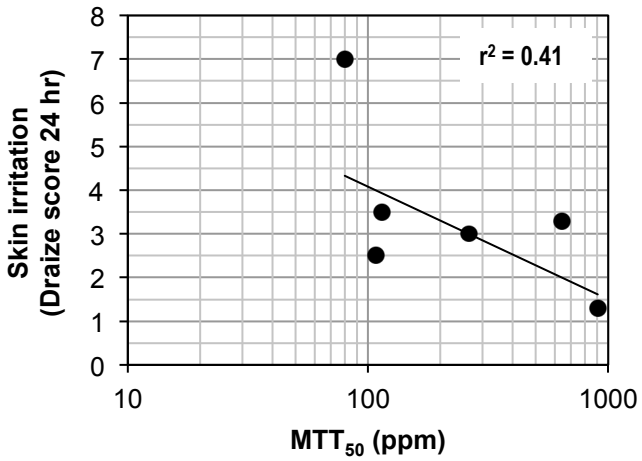


Fig 2-2: Correlation between MTT_{50} and PII value by Draize test

2.3.2 Assessment of anionic surfactants by NR assay

NR_{50} results of anionic surfactants having C_{12} carbon chain and sodium (Na^+) as a counter ion are summarized in Table 2-4 including PII values by Draize test and Fig 2-3.

Table 2-4: NR_{50} results of anionic surfactants

#	Test Articles	NR_{50} (ppm)	Draize score Skin irritation (5%)
1	SLS	4.1	7
2	SLMT	35.8	2.5
3	SLES	3.4	3.5
4	SL	9.9	3
5	SMLP	7.4	3.3
6	SLG	60.2	1.3

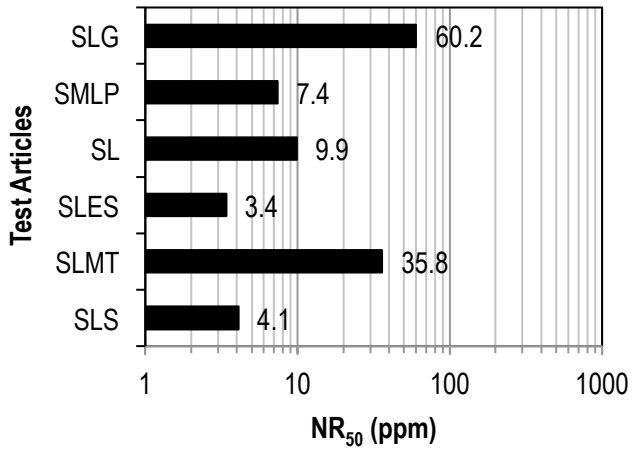


Fig 2-3: NR_{50} results of anionic surfactants

NR_{50} for SLG and SLMT was 60.2 ppm and 35.8 ppm respectively, which imply “mildest” surfactant amongst the articles. SLG is derived from glutamic acid and possesses a “peptide (-CO-NH-)” bond in the structure. SLMT is derived from methyl taurine and also possesses a “quasi-peptide (-CO-N(CH_3)-)”. SL has a carboxyl group like SLG, however, the NR_{50} is one order small number. SLMT has sulfonate group like SLES and SLS, however, the NR_{50} is one order larger number contrarily. It is suggested that surfactants having “peptide” bond in the structure like SLG and SLMT may have different interaction with human keratinocytes from other type of surfactants and induce less irritation.

Only viable cells can take NR in their nucleus, however, dead cells do not. MTT is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan as the metabolic reaction in mitochondria. The formazan compound is impermeable to the cell membranes and therefore it accumulates in healthy cells.

Fig 2-4 shows the correlation between NR_{50} and PII value by Draize test. The correlation coefficient (r^2) between them is “0.54” including all test articles, however, r^2 becomes “0.87” excluding SLS. It may indicate that SLS has different interaction with human keratinocytes or induces a different type of total skin reaction from others. We need further study by increasing test articles.

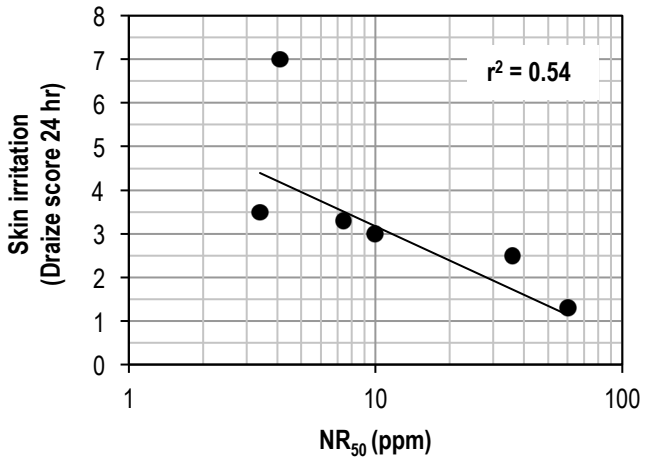


Fig 2-4: Correlation between NR_{50} and PII value by Draize test

2.3.3 Assessment of anionic surfactants by SIRC cell toxicity assay

LC_{50} results of anionic surfactants having C_{12} carbon chain and sodium (Na^+) as a counter ion are summarized in Table 2-5 including PII values by Draize test and Fig 2-5.

Table 2-5: LC_{50} results of anionic surfactants

#	Test Articles	LC_{50} (ppm)	Draize score Skin irritation (5%)
1	SLS	17.5	7
2	SLMT	204	2.5
3	SLES	15.3	3.5
4	SL	619	3
5	SMLP	151	3.3
6	SLG	2540	1.3

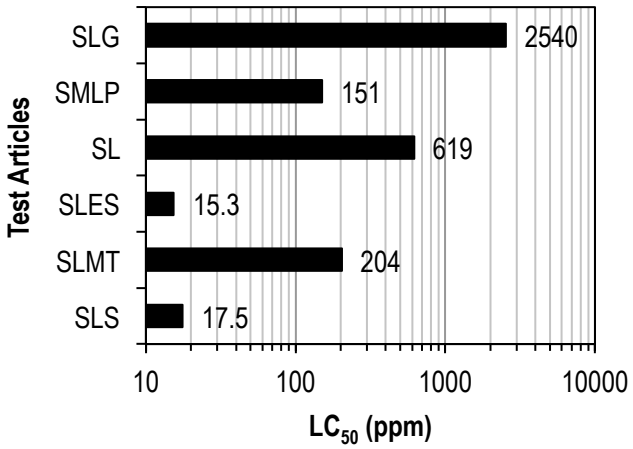


Fig 2-5: LC_{50} results of anionic surfactants

LC_{50} assay results were similar to those of NR_{50}. LC_{50} for SLG was 2540 ppm, which was the highest number amongst the test articles. SLS and SLES having “sulfate” as a hydrophilic moiety showed the lowest LC_{50} as 17.5 ppm and 15.3 ppm, respectively, which means SLS and SLES are the harshest surfactants. SL, SLMT and SMLP showed 619 ppm, 204 ppm and 151 ppm, respectively, which seems to form a surfactant group between “the mildest” and “the harshest”.

SLG, which is derived from glutamic acid and possesses “peptide (-CO-NH-)” bond in the structure, exhibited “mild”. SLMT, which is derived from methyl taurine and also possesses “quasi-peptide (-CO-N(CH_3)-)”, has a sulfonate group like SLES and SLS, however, the LC_{50} is significantly high. LC_{50} assay results also suggested that surfactants having a “peptide” bond in the structure like SLG and SLMT may have the least interaction with human keratinocytes from other type of surfactants and eventually induces less/ minimal irritation.

Fig 2-6 shows the correlation between LC_{50} and PII value by Draize test. The correlation coefficient (r^2) between them is “0.60, which is similar to that of NR_{50}.

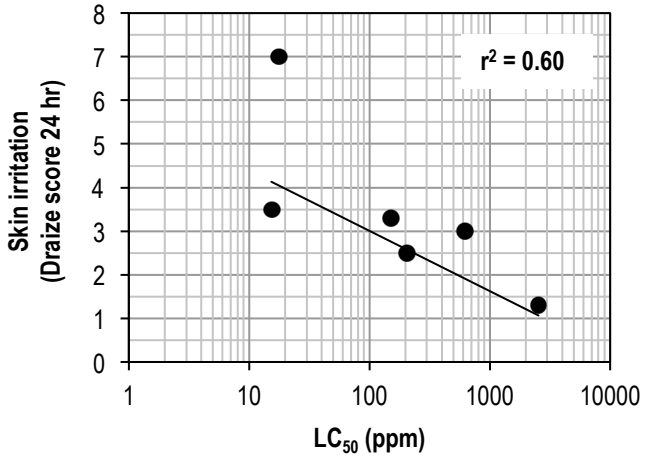


Fig 2-6: Correlation between NR_{50} and PII value by Draize test

2.3.4 Assessment of anionic surfactants by SKINTEX® and EYETEX® assay

SKINTEX® and EYETEX® results of anionic surfactants are summarized in Table 2-6 including PII values of Draize test and Fig 2-7.

Table 2-6: SKINTEX® and EYTEX® results of anionic surfactants

#	Abbreviations	SKINTEX® (5%)	EYETEX® (5%)	Draize score Skin irritation (5%)	Draize score Eye irritation (5%)
1	SLS	5	38.9	7	19.5
2	SMLP	2.1	14.2	3.3	22.5
3	SCI	0.1	19.8	2.8	14
4	SSS	0.1	12.3	na	na
5	SCMT	0	20.8	2.5	14.5
6	SCG	0	8.5	na	na
7	SLG	0	10.5	1.3	4

Note: “na” means “data is not available”

SKINTEX® and EYESTEX® showed that acylglutamates, SCG and SLG, exhibited the least irritation potential amongst the samples tested.

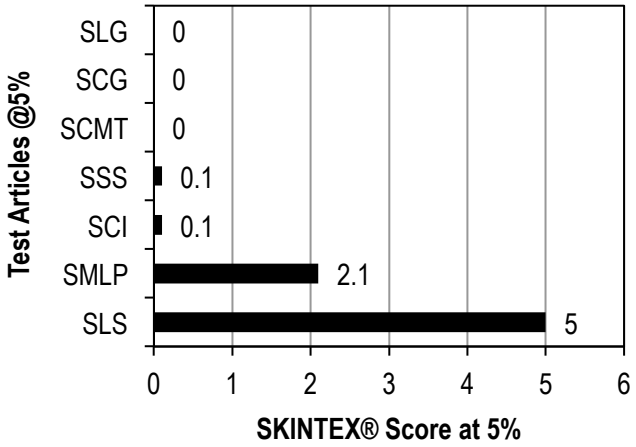


Fig 2-7: SKINTEX® score of anionic surfactants at 5%wt

As shown in Fig 2-8, the correlation coefficient between SKINTEX® score and PII value by Draize score is “0.90”, which is significantly better correlation than the other *in vitro* studies. The SKINTEX® system was fairly sensitive in its ability to predict irritation potential of chemicals in human based on its dose response.

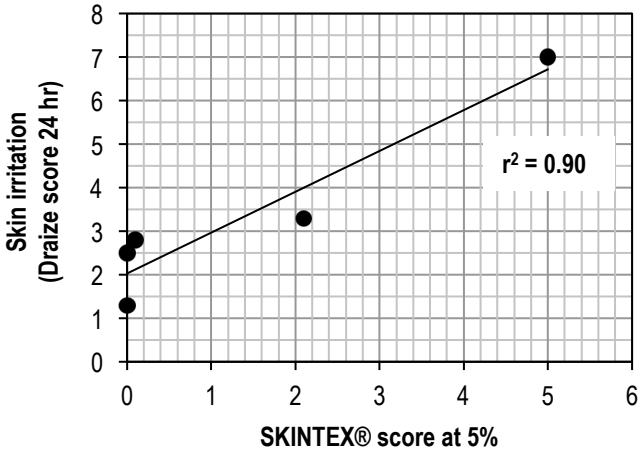


Fig 2-8: Correlation between SKINTEX® score and PII value by Draize test

SKINTEX®, one of the systems utilizing non-human substrates, can be described as a membrane barrier/ protein matrix (MB/PM) system (Gordon et al., 1990). This method detects changes in the intact barrier matrix with an indicator dye attached to the matrix that is released with exposure to an irritant. The amount of dye released is quantified and correlated with expected protein disruption and denaturation. A second compartment is a reagent system that responds to irritants by producing turbidity. This response provides an internal detection for materials that perturb conformations after permeating the membrane. This provides a quantitative response to materials that may produce irritation by membrane damage. Protein binding, enzyme inactivation and a variety of other pathways where macromolecule conformation is altered, take place in the initiation of dermal irritation.

Bason et al (1992) reported that the *in vitro* dose response for benzarkonium chloride and phenol with SKINTEX® were strikingly similar to those generated *in vivo*. SKINTEX® systems are the unique *in vitro* method allowing to discuss dose response characteristics of chemicals although other *in vitro* methods based on viable cells just focus on the evaluation of toxicity threshold of the chemicals.

2.3.5 Assessment of anti-irritant potential of SLG addition to other surfactant

As explained in the previous section, SLG, SMLP, SLMT and SLES were developed balancing the detergency and safety to human, and now are widely used in various finish goods on the market. For product development such as shampoos, body soaps and so on, we actually formulate multiple surfactants in the formulation for delivering cleansing performance and less irritancy to human. It would be very important to select surfactants. The test results above, from Draize test to *in vitro* methodologies, consistently implied that acylglutamates such as SLG and SCG are the mildest surfactants among the tested articles. In this section, anti-irritant effect of acylglutamates was examined with MTT assay on the binary combinations of aclylglutamate and others.

Several binary combination mixtures of aclylglutamate and others (ex. 100/0, 75/25, 50/50, 25/75, 0/100; SLG/SL or SLES/SLES) were prepared and tested with MTT assay for assessing and Draize test method. The results were summarized in the following tables and figures.

Table 2-7. (a) anti-irritant effect of SLG to SL by MTT assay

SLG / SL	MTT50 (ppm)	Predict Score
100/0	875	
75/25	575	726.3
50/50	490	577.5
25/75	460	428.8
0/100	280	

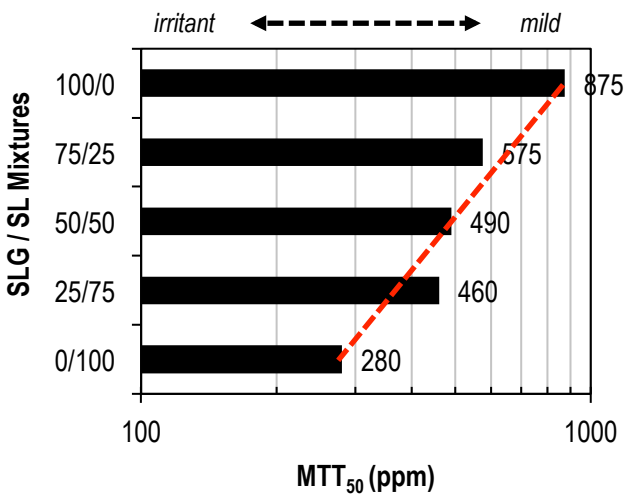
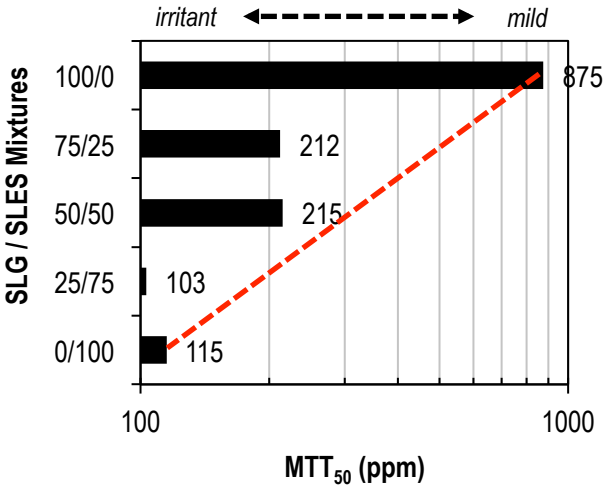


Table 2-7. (b) anti-irritant effect of SLG to SLES by MTT assay

SLG / SLES	MTT50 (ppm)	Predict Score
100/0	875	
75/25	212	685.0
50/50	215	495.0
25/75	103	305.0
0/100	115	



Irritation score profile against the binary combination mixtures depends on what surfactants are mixed. Fig 2-7 (a) is the MTT_{50} assay results of SLG/ SL mixtures and irritation score proportionally decreased as more SLG was added. However, the results of SLG/SLES mixtures were significantly smaller than the calculated values as shown in Fig 2-7 (b). It suggests that addition of SLG may change the physico-chemical behavior of micelles SL and the biological interaction to the human keratinocytes was shifted to that of SLG. However, SLG cannot change the interaction of SLES with keratinocytes. This information and method would be valuable for selecting surfactant and designing formulas of consumer products.

2.4 Conclusion

Irritation potential of anionic surfactants were assessed as function of their chemical structure employing several types of *in vitro* testing. All the *in vitro* methods used in this study; MTT assay, NR assay, SIRC colony formation assay, SKINTEX®, EYETEX®, showed consistent results concluding **SLG** (sodium lauroyl glutamate), which is derived from glutamic acid and has “peptide (-CO-NH-)” bond in the structure, exhibited “extremely mild surfactant” behavior, which corresponds well to the results of Draize test.

Throughout the study here, we may be allowed to conclude that the “sulfate” group surfactants like SLS (sodium lauryl sulfate) and SLES (sodium lauryl POE ether sulfate) are “irritating surfactants”. However, SLMT (sodium lauroyl methyltaurate), which also has “sulfate” group, may have less irritation potential than SLS and SLES due to the “quasi-peptide (-CO-N(CH_3)-)” in its structure.

Anti-irritating effect of SLG to other anionic surfactants was also demonstrated although detail insight was not defined. This could be beneficial in order to formulate consumer products balancing “cleansing performance” and “skin mildness”.

The correlation between Draize test data and SKINTEX® data was found to be fairly “high” as “r^2=0.90” compared to other correlations. It may be due to the uniqueness of SKINTEX® methods, which detects the changes in the intact barrier matrix system considering a variety of other pathways where macromolecule conformation is altered in the initiation of dermal irritation.

In order to validate alternative methods predicting irritation potential of chemicals, we need to examine more samples selected from different chemical groups and have further discussion on mechanism of skin irritation.

After this set of studies, it was our honorable experience (Ohuchi J., et. al, 1999) that we as a team at Ajinomoto Co., Inc., could participate in the validation program of “*in Vitro* Eye Irritation Tests for Cosmetic Ingredients” chaired by Japan Cosmetic Industry Association (JCIA) and National Institute of Health Science Japan (NIHS).

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3. Effect of surfactant mixtures on irritant dermatitis potential in man: sodium lauroyl glutamate and sodium lauryl sulfate

主論文（4）

Lee CH, Kawasaki Y, and Maibach HI (1994) Effect of Surfactant Mixtures on Irritant Dermatitis Potential in Man Sodium Lauroyl Glutamate and Sodium Lauryl Sulfate, Contact Dermatitis, 30, p.205-209

[Synopsis]

The purpose was to ascertain the irritant contact dermatitis potential of sodium lauroyl glutamate (SLG) and access its possible anti-irritating potential in surfactant mixture on human skin by using visual scores and measurement of transepidermal water loss (TEWL). 15 healthy adult volunteers free of skin disease and with no history of atopic dermatitis were treated with sodium lauryl sulfate (SLS) solutions (1%, 0.75%, 0.50% and 0.25%), 1% SLG solution and 1% surfactant mixture solutions (M1 (75/25), M2 (50/50), M3 (25/75) (SLG/SLS)). We applied 0.1 ml of solution using a polypropylene chamber for 24 hours. Application sites for each solution were randomized to minimize anatomical bias. We measured baseline TEWL before test solution application. After removal of the patches, each site was visually graded and TEWL recorded with Evaporimeter EP-1 daily for 4 days. The visual scores and TEWL values of 1% SLG solution were lower than those of the other test solutions, except the vehicle control (deionized water). 1% surfactant mixture solutions showed lower visual scores and TEWL values than 1% SLS solution. Increase of SLG concentration decreased the visual scores and TEWL values. Comparing the visual scores and TEWL values of M1 and 0.25% SLS solution, M2 and 0.50% SLS solution, and M3 and 0.75% SLS solution, M1. M2 and M3 showed lower values although not statistically different. These findings suggest that SLG is a mild surfactant and its utilization can decrease irritation potential in SLS and possibly other mixtures. This model may offer a facile system for screening the surfactant and other mixtures for decreased iritation potential.

3.1 Introduction

Alkaline surfactants such as salt of fatty acids, have irritation potential, partially based on their pH. There is interest in weakly acidic surfactants, more closely resembling the skin pH. Acylglutamate is an amino acid surfactant synthesized from glutamic acid and fatty acid. The pH of its solution is approximately from 5 to 6. It has been used as a primary surfactant for cleansing products. Sodium lauroyl glutamate (SLG) is the sodium salt of lauric acid amide of glutamic acid and its empirical formula is $C_{11}H_{30}NO_5Na$ [1-4]. Nakayama et Al. [5] evaluated its irritation potential in animal and human studies.

Kanari et al. [6] showed that acylglutamates, like SLG, exhibited extremely low irritation potential amongst the anionic surfactants and an anti-irritating effect on other anionic surfactants in *in vitro* testing such as cytotoxicity tests.

The present study delineates the validation for the mildness of SLG and assesses its irritation potential in surfactant mixtures on human skin, using visual scoring and transepidermal water loss (TEWL) measurement.

3.2 Materials and Methods

3.2.1 Subjects

15 healthy volunteers, 6 male and 9 female, aged from 24 to 46 years, provided informed consent. They were all free of skin disease and had no history of atopic dermatitis.

3.2.2 Materials

Test surfactants were sodium lauryl sulfate (SLS; 96% purity, from Fischer Scientific Co.) and sodium N-lauroyl-L-glutamate (SLG; 95% purity, from Ajinomoto Co.). 1.00% SLG aqueous solution was prepared and the following solutions of SLS were also prepared and coded; 1.00%, 0.75%, 0.50% and 0.25%. Deionized water was used as a vehicle control. We also prepared three 1% solutions, M1: 75.25, M2: 50/50, M3: 25/75 (1% SLS solution/ 1% SLG solution), to define the effect of SLG in mixtures.

3.2.3 Methods

After 30 minutes acclimatization (temperature 16.5 – 20.8°C; 52.0 – 65.4%RH), we marked the test sites, measured baseline of TEWL, and applied 0.1mL of each solution using a polypropylene chamber (Hiltop®, Cincinnaci, Ohio) secured with paper (Scanpor®, Norgesplaster, Oslo, Norway) and plastic tape (Tegaderm®, 3M, St Paul, Minnesota)for the protection of patches. Application sites were randomized to minimize anatomical functional bias [7]. Patches were removed after 24 hour and the test sites exposed for at least 30 minutes to allow deconvolution of excess water.

Each site was visually examined and the results were recorded with a visual grading system; 0 = normal skin or no reaction, 0.5 = faint, barely perceptible erythema or slight dryness, 1 = definite erythema, 2 = erythema and induration, 3 = vesiculation.

TEWL was measured quantitatively with an Evaporimeter® EP-1 (Servo Med, Stockholm, Sweden), Tewameter TM 210® (Courage + Rhazaka, Cologne, Germany), at 30 minutes after patch removal. Readings were performed at a stable level, 30 seconds or more after application of the probe on the skin. Room temperature and relative humidity were recorded to document environmental fluctuations. TEWL values were expressed as $g/m^2/h$.

3.3 Results

Mean daily visual scores and TEWL values of each test are shown in Table 3-1 and 3-2 and the time course of visual scores and TEWL values in Fig 3-1 and Fig 3-2.

The average daily time course of visual scores showed somewhat different from patch to patch. Four solutions, 0.50% SLS, 0.75% SLS, 1% SLS and M2 solutions, showed their maximum value at D2. M3 at D1 and D2 (i.e. D1 = D2), and the remaining four solutions at D1. Maximum visual scores were shown at D1 in 48 patches (35.6%), at D2 in 7 patches (5.2%), at D1 and D2 in 33 patches (24.4%), and at D1, D2 and D3 in 47 patches (34.7%)

The average daily time course of TEWL values showed a similar pattern at all sites. The maximum TEWL value appeared at D1 and slowly declined thereafter at most sites (115 patches; 85.2%). The maximum value appeared at D2 in 18 patches (13.3%) and at D3 in 2 patches (1.5%).

The visual score of 1% SLG solution were significantly lower than those of four different dose SLS alone solutions ($p<0.05$). Comparing the visual scores of 1% SLG solution, three surfactant mixture solutions at total 1% (M1, M2, M3) and 1% SLS solution, 1% SLG solution showed lower values than M1 ($p=0.002$) and 1% SLS solution showed higher values than M3 ($p=0.002$). Amongst three kinds of solution of surfactant mixture at 1% total (M1, M2 and M3), M3 showed higher values than M2 ($p=0.009$). In general, the increase of SLG concentration decreased the visual scores (Fig 3-3), although there was no significant difference in visual scores between M1 and M2 ($p>0.05$).

Although the visual scores were increased linearly by increasing SLS concentration, no statistical differences were observed between them ($p>0.05$). (Fig 3-5)

TEWL values at the sites 1% SLG solution applied were significantly lower than those of four different dose SLS alone solutions ($p<0.05$). Comparing the 1% SLG solution, three surfactant mixture solutions at total 1% (M1, M2 & M3) and 1% SLS solution, TEWL values of 1% SLG applied sites were lower than those of three mixture solutions (M1, M2 & M3) applied sites ($p<0.01$). On the contrary, TEWL values of 1% SLS applied sites were higher than those of three kinds of mixture solutions (M1, M2 & M3) applied sites ($p<0.01$). Amongst three kinds of mixture solutions (M1, M2 & M3), M3 applied sites showed the highest TEWL values and M1 applied sites showed the lowest. Although the increase of SLG decreased the TEWL values, no statistical differences were observed in TEWL values between M1 and M2, M2 and M3 ($p>0.05$ for both cases)

To determine the possible anti-irritating effect of SLG, we compared the observed TEWL values of surfactant mixtures (M1, M2 & M3) with predicted values calculated from those of 1% SLG and SLS solution applied values expecting a synergistic TEWL reduction. For M1, the mean value of the observed TEWL was higher than the predicted value. For M3, the observed value was lower than the predicted value. (Table 3-3) Also statistically analyzed the visual scores and TEWL values of following three data sets: M1 and 0.25% SLS solution, M2 and 0.5% SLS solution, M3 and 0.75% SLS solution. Although M1, M2 and M3 respectively showed lower values in visual and TEWL than those of each SLS solution at 0.25%, 0.5% and 0.75%, no statistical difference was observed.

Even though TEWL values increased linearly by increasing SLS concentration as shown in Fig 3-6, no significant difference was observed in TEWL values of SLS applied sites between 0.75% and 1%.

Table 3-1: Results of Visual Grading

Test solution	D1	D2	D3	D4
1% SLG	0.666 ± 0.080	0.300 ± 0.082	0.100 ± 0.072	0.133 ± 0.076
1% M1	0.733 ± 0.083	0.566 ± 0.083	0.433 ± 0.107	0.300 ± 0.095
1% M2	0.666 ± 0.080	0.678 ± 0.082	0.500 ± 0.109	0.366 ± 0.091
1% M3	0.866 ± 0.114	0.866 ± 0.114	0.666 ± 0.105	0.566 ± 0.083
1.00% SLS	1.033 ± 0.150	1.233 ± 0.120	1.000 ± 0.119	0.733 ± 0.067
0.75% SLS	0.866 ± 0.142	1.033 ± 0.150	0.800 ± 0.118	0.633 ± 0.076
0.50% SLS	0.700 ± 0.095	0.866 ± 0.103	0.733 ± 0.067	0.433 ± 0.096
0.25% SLS	0.733 ± 0.082	0.700 ± 0.082	0.533 ± 0.091	0.333 ± 0.093
water	0.533 ± 0.076	0.100 ± 0.053	0.033 ± 0.033	0.000 ± 0.000

Note: D1 value mean the visual score 30 minutes after patch removal and values are expressed as the mean ± SEM (D = day)

Table 3-2: Results of TEWL values ($g/m^2/h$)

Test solution	D0	D1	D2	D3	D4
1% SLG	5.9 ± 0.51	15.8 ± 1.07	8.8 ± 0.82	8.7 ± 0.95	8.5 ± 0.77
1% M1	5.8 ± 0.41	20.0 ± 1.74	12.9 ± 2.03	12.5 ± 1.67	10.9 ± 0.97
1% M2	5.7 ± 0.41	20.7 ± 1.92	15.1 ± 1.69	14.1 ± 1.72	12.7 ± 1.13
1% M3	5.9 ± 0.49	21.2 ± 2.56	18.2 ± 2.33	15.8 ± 1.87	13.6 ± 1.23
1.00% SLS	5.9 ± 0.54	25.4 ± 2.56	23.7 ± 2.85	20.7 ± 1.82	16.1 ± 1.03
0.75% SLS	5.7 ± 0.51	22.7 ± 1.54	20.4 ± 2.82	18.0 ± 2.26	14.1 ± 1.56
0.50% SLS	5.9 ± 0.51	22.3 ± 1.67	16.2 ± 2.00	16.3 ± 1.72	13.2 ± 1.23
0.25% SLS	5.9 ± 0.62	22.3 ± 1.67	12.9 ± 1.33	12.9 ± 1.38	11.3 ± 0.90
water	5.2 ± 0.23	13.0 ± 0.95	5.8 ± 0.21	5.5 ± 0.28	5.6 ± 0.26

Note: D0 values mean the baseline of TEWL values and values are expressed as the mean ± SEM (D = day)

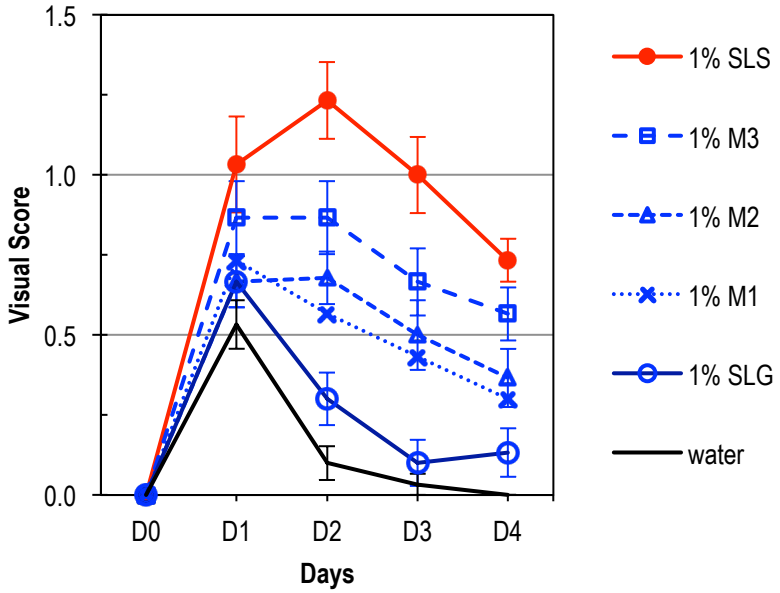

Fig 3-1. Average daily visual scores of 5 test solutions (1% SLG, M1, M2, M3, 1% SLS) and control (deionized water)

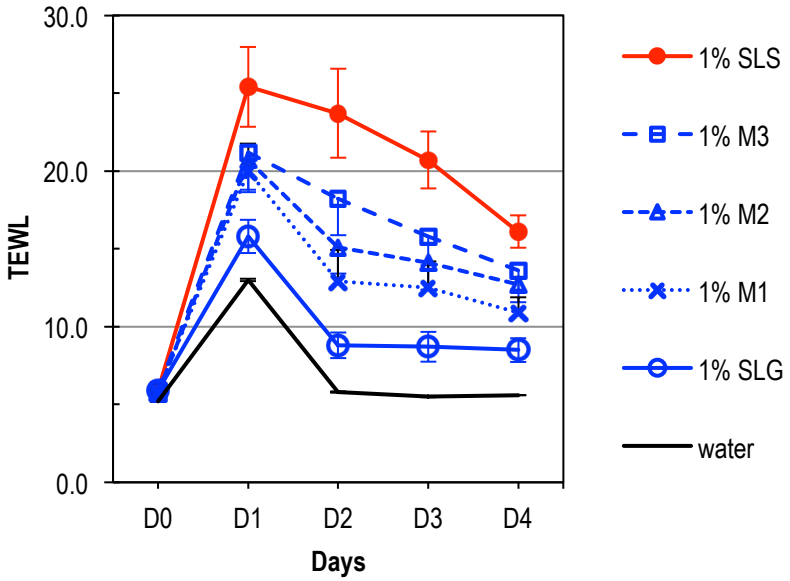

Fig 3-2. Average daily TEWL values of 5 test solutions (1% SLG, M1, M2, M3, 1% SLS) and control (deionized water)

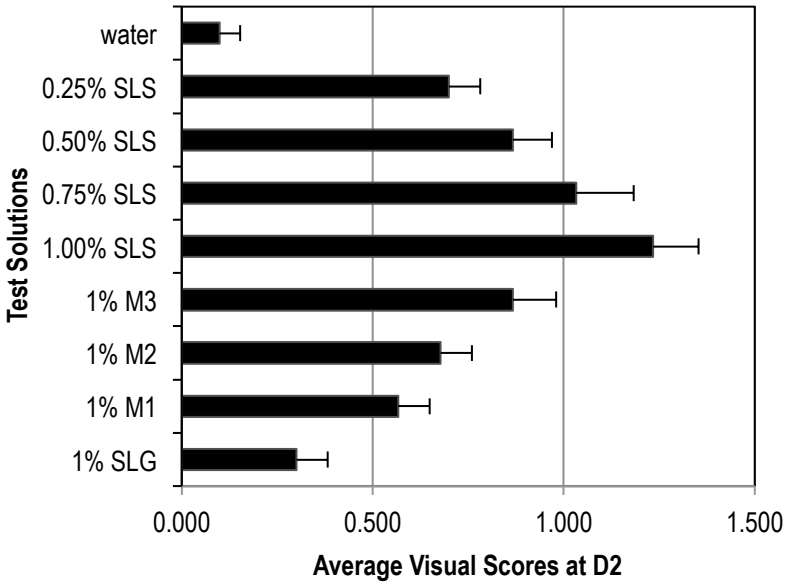

Fig 3-3. Average visual scores of 8 test solutions (1% SLG, M1, M2, M3, 0.25% SLS, 0.5% SLS, 0.75% SLS, 1% SLS) and control (deionized water). Note the SLS dose response and decrease in response of mixtures with increasing amount of SLG

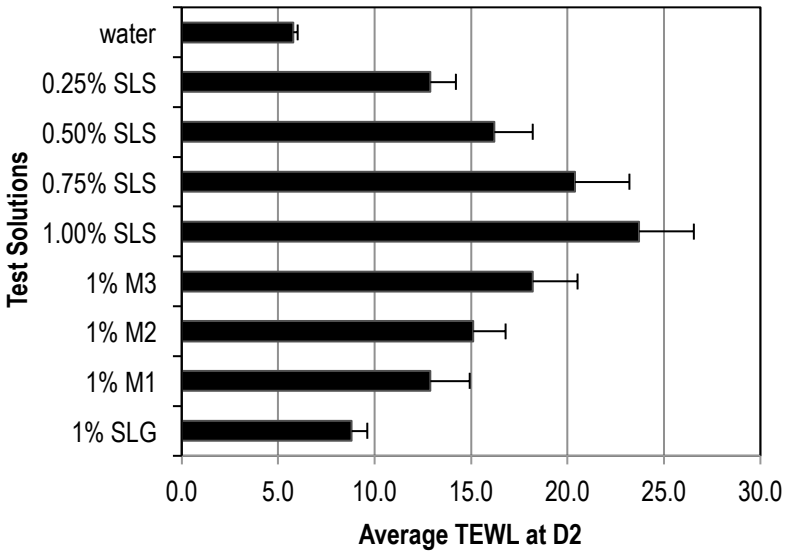

Fig 3-4. Average TEWL values of 8 test solutions (1% SLG, M1, M2, M3, 0.25% SLS, 0.5% SLS, 0.75% SLS, 1% SLS) and control (deionized water). Note the SLS dose response and decrease in response of mixtures with increasing amount of SLG

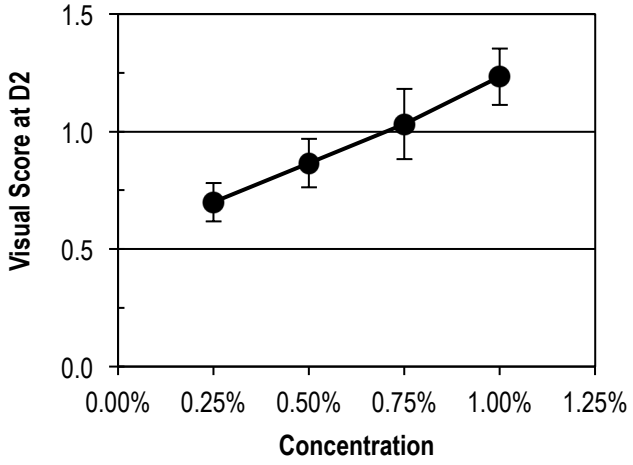

Fig 3-5. Average visual scores of four SLS solutions (0.25%, 0.5%, 0.75%, 1%) at day 2.

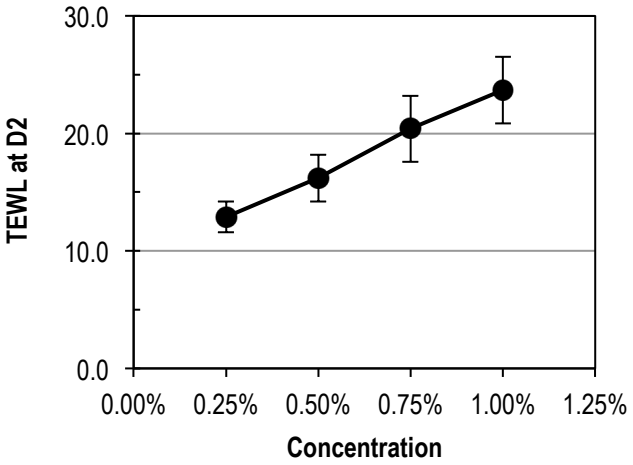

Fig 3-6. Average TEWL values of four SLS solutions (0.25%, 0.5%, 0.75%, 1%) at day 2.

Table 3-3. Observed and predicted TEWL values of surfactant mixtures

	D1	D2	D3	D4
1% SLG	15.8	8.8	8.7	8.5
1% M1	20.0 *(18.2)*	12.9 *(12.5)*	12.5 *(11.7)*	10.9 *(10.4)*
1% M2	20.7 *(20.6)*	15.1 *(16.3)*	14.1 *(14.7)*	12.7 *(12.3)*
1% M3	21.2 *(23.0)*	18.2 *(20.0)*	15.8 *(17.7)*	13.6 *(14.2)*
1% SLS	25.4	23.7	20.7	16.1

[Note] Values in parentheses mean the "predicted" TEWL values from those of 1% SLG and 1% SLS. The observed values of M1 are higher than the predicted ones, and the observed values of M3 are lower than the predicted ones. No statistic significance was observed ($p>0.05$). "D" means "day".

3.4 Discussion

Products containing surfactants may have skin irritating potential. In view of the universal exposure of human skin to soap or detergents containing surfactants, it is important to choose one that is "mild".

Nakayama et al. [5] studied the safety of acylglutamate and showed that irritancy of acylglutamate is low. Kanari et al. [6] demonstrated with other surfactants such as sodium monolaurylphosphate, sodium acylmethyltaurate, sodium acylisethionate and sodium lauryl POE(3) sulfate in various in vitro tests including cytotoxicity tests. Our study compared the visual scores and TEWL values of 1% SLG solution with those of 1% SLS, 0.75% SLS, 0.5% SLS, 0.25% SLS solutions and deionized water. 1% SLG solution showed significantly lower values than 0.25% SLS solution and higher values than deionized water.

Kanari et al. [6] conducting dose response tests on binary combinations of 1% surfactant solutions; 100/0, 75/25, 50/50, 25/75 and 0/100 (SLG / other anionic surfactants), determined the anti-irritating effect of acylglutamate, and showed that the increase of acylglutamate decreased the irritation potential of other anionic surfactants in cytotoxicity tests. In the study of the anti-irritating effect of SLG, our results were similar to those of Kanari et al. [6] 1% SLG solution showed lower visual scores and TEWL values than 1% surfactant mixture solutions (M1, M2 and M3), and visual scores and TEWL values of 1% SLS solution were higher than

those of 1% surfactant mixture solutions (M1, M2 and M3). 1% surfactant mixture solutions (M1, M2 and M3) showed some lower values in TEWL and visual scores than those of 0.25%, 0.5% and 0.75% SLS solutions, however, there were no significant differences.

The increase of SLG in the mixture with SLS, decreased the visual scores and TEWL values. Comparing the observed TEWL values with predicted values, the observed TEWL value of M1 was higher than the predicted one, however, the observed TEWL value of M3 was higher than the predicted one. Those findings suggest that SLG has possible anti-irritating potential of SLS.

The mechanisms by which surfactants cause skin irritation are incompletely understood. Factors such as protein denaturation, lipid removal, inhibition of proliferative capacity and mediator release can contribute to the surfactant inducing irritation [7-12]. The presence of by-products in commercially available surfactants was also suggested as being an important factor [13, 14].

Additionally, a factor controlling the physico-chemical behavior of surfactants in solution has been considered to play a major role in reducing irritation. Surfactants form micelle in the solution at or above their CMC (critical micelle concentration). Assuming micelles are not the likely species to act on keratin, then a factor controlling the level of monomers remaining in the solution may be important in moderating the skin response. The hypothesis that monomer is responsible for eliciting irritation was proposed by the observation that mixtures of SLS and milder surfactant interact synergistically to reduce surfactant's binding to stratum corneum [8, 15, 16]. Gracia-Dominguez et al. [15] demonstrated the formation of complexes between anionic SLS and cocamidopropylbetaine. These interactions reduce the ocular irritation below that caused by individual surfactant alone.

Rhein et al. [11] demonstrated that stratum corneum denaturation caused by SLS can be ameliorated by adding a milder co-surfactant, C_{12}-C_{14} ether 6-POE sulfate (AEOS-6EO); Rhein et al. [17] studied the in vivo human response using 21 day cumulative irritation test to 2 surfactants, SLS and C_{12}-C_{14} ether 7-POE sulfate (AEOS-7EO), and showed a significant reduction in erythema. They proposed several possible explanations for decreased skin response in the presence of mild co-surfactant. The mild surfactant may compete with SLS for surface area on the skin. The second possibility is that the skin reacts with the surfactant monomers. Surfactants begin to form larger aggregates (micelles) in solution at or above CMC and surfactant monomers level off at the CMC and higher doses.

There is several possible explanations of some reduced skin response of 1% surfactant mixture solutions noted here. The reduced skin response might be caused by replacement of irritating SLS with the milder surfactant SLG. And the mild surfactant SLG may compete with SLS for the surface area of the skin. The 3rd possibility is that the CMC level of SLS was lowed by the addition of SLG and the level of surfactant monomer decreased, resulting in reduced skin response.

3.5 Conclusions

SLG is less irritating than SLS; adding SLG to SLS mixture decreased irritation. Either the anti-irritant or decreased biological effect is due to the addition of SLG. This model system may provide a facile method for screening the effect of individual component and mixture for their irritation potential.

3.6 References

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4. Electron paramagnetic resonance (EPR) studies on the influence of anionic surfactants on intercellular lipid fluidity in human cadaver skin

主論文（1）

Kawasaki Y, Quan D, Sakamoto K and Maibach HI (1999) Influence of surfactant mixtures on inter cellular lipid fluidity and skin barrier function, Skin Research and Technology, 5, p.96-101

主論文（2）

Kawasaki Y, Quan D, Sakamoto K and Maibach HI (1997) Electron resonance study on the influence of anionic surfactants on human skin, Dermatology, 194, 238–242

主論文（3）

Kawasaki Y, Quan D, Sakamoto K and Maibach HI (1995) 電子スピン共鳴（ESR）によるアニオン界面活性剤の角質層に及ぼす影響の解析, 粧技誌, *29, p.252-257*

主論文（6）

Mizushima J, Kawasaki Y, Kitano T, Sakamoto K, Kawashima M, Cooke R, and Maibach HI (2000) Electron Paramagnetic Resonance Study Utilizing Stripping Method on Normal Human Stratum Corneum, Skin Research and Technology, 6, p.108-111

主論文（7）

Mizushima J, Kawasaki Y, Sakamoto K, Kawashima M, Cooke R, and Maibach HI (2000) Electron Paramagnetic Resonance: A New Technique in Skin Research, Skin Research and Technology, 6, p.100-107

[Synopsis]

An electron paramagnetic resonance (EPR; which is also called as “electron spin resonance (ESR)”) spectroscopy employing a nitroxide spin probe is a valuable method to obtain microscopic information on skin lipid structure. The stratum corneum, which is the most outer layer of the human skin, is composed of corneocytes embedded in lipid domains consisting of alternately hydrophilic and lipophilic layers. When the skin exposed to surfactants in cosmetics and toiletry products, it is thought that they interact in some way with the lipid structure in the stratum corneum.

In this study, we conducted EPR study to investigate the effect of anionic surfactants (SLS; sodium lauryl sulfate, SLG; sodium lauroyl glutamate, SL; sodium

laurate, SLES; sodium lauryl POE (3) ether sulfate, SLEC; sodium lauryl POE (3) ether carboxylate, and mixtures of SLS and SLG) on the lipid bilayers of human stratum corneum obtained from cadaver skin. 5-doxyl stearic acid (5-DSA) was used as a spin probe and order parameter S, which represents the fluidity of the lipid bilayer structure, was calculated from the EPR spectra. 1% SLS aqueous solution induced a temporary conformational modification of the lipid bilayers structure of which fluidity increases. On the other hand, almost no change in EPR spectra was found in the stratum corneum treated with 1% SLG aqueous solution. SLES, SL and SLEC induce fluidization of intercellular lipids at intermediate level between SLS and SLG. We also conducted human patch test on the same anionic surfactants and examined the correlation between EPR spectral data and clinical observables (visual score and TEWL; transepidermal water loss, respectively).

An increase of fluidity of the intercellular lipid bilayers suggests a decrease of skin barrier function, which is represented by increase of TEWL in clinical testing. EPR technique may provide a facile and robust method to define subclinical irritation potential of anionic surfactants and other chemicals

4.1 Introduction

Anionic surfactants are used in cleansing products to which consumers are exposed daily. Cleansing products may be irritating, particularly when applied to so-called sensitive skin: thus, it is not surprising that a desirable claim for cleansing product is “mild to the skin”. The stratum corneum, a major barrier, is composed of corneocytes embedded in lipid domains consisting of alternately hydrophilic and lipophilic layers (Barry BW, 1983; Elias PM, 1983).

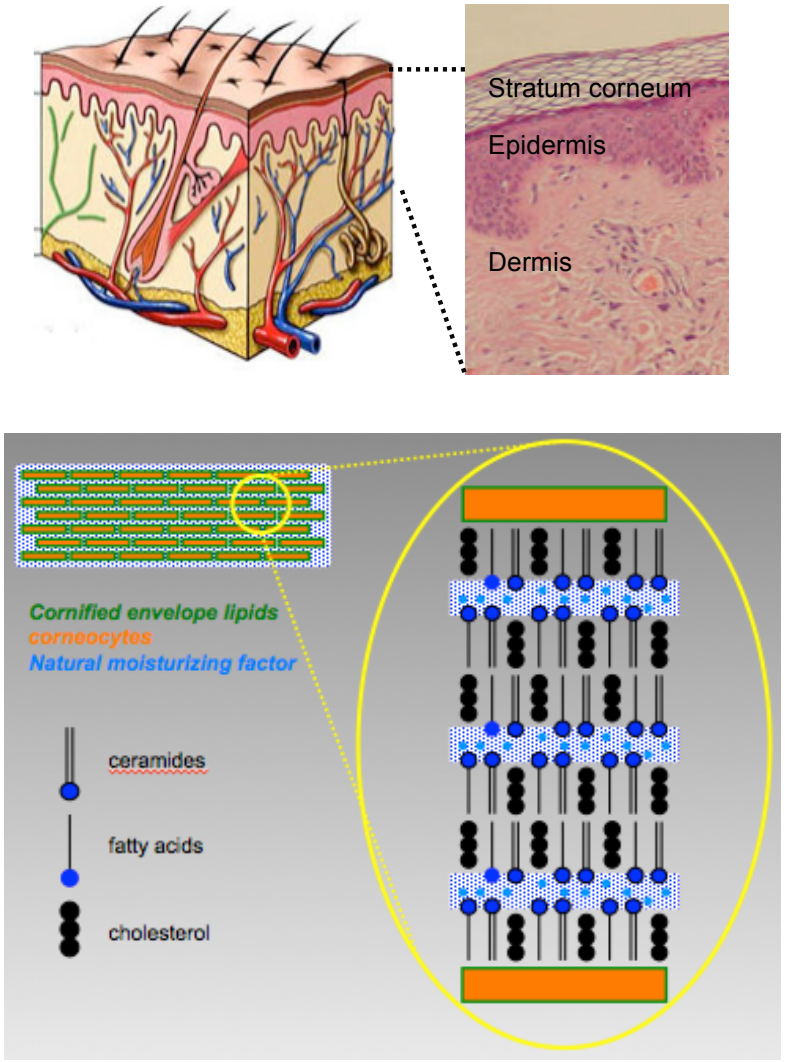


Fig 4-1. Schematic representation of “brick and mortal” model of the stratum corneum.

The skin is exposed to chemicals in cosmetics and toiletry products and it is thought that they interact in some way with the lipid structure of the stratum corneum after migrating into the stratum corneum. Various techniques are used to study lipid structure (both inter- and intra-molecular) and action of chemical constituents with these lipids. Some of these techniques are (1) X-ray diffraction to study the effects on interlayer spacing and water penetration into the lipid lamellar structure using liquid crystal model of stratum corneum lipids and allowing conclusions on structural rearrangements (Blaurock, AE (1982); Friberg S, (1988)); (2) Differential scanning calorimetry (DSC) to study the phase transitions and various thermodynamic properties associated with those transitions allowing conclusions regarding the effects of agents on physico-chemical state of barrier (Goodman M, et al. (1986); Inoue T, et al. (1986)); (3) Fourier transform infrared spectroscopy, which measures the vibrational energies of molecules by light absorption, the frequency and intensity of which is greatly affected by neighboring molecular environments (Golden GM, et al. (1987); Mak FHW, et al. (1990); Takeuchi Y, et al., (1992)); (4) nuclear magnetic resonance spectroscopy (NMR) and electron paramagnetic resonance spectroscopy (EPR) techniques, which can determine mobility of molecules and interrelationships with another molecules in the lipid membrane and effects of other chemical constituents on the interactive parameters (Gay CL, et al. (1989); Gay CL, et al. (1990), Eagle SC, et al. (1992)); (5) optical microscopy technique under regular or polarized light, which provides information about phases present in barrier lipid mixtures and sensitivity of phase changes to environmental and chemical constituents.

EPR spectroscopy of nitroxide spin label provides a valuable method in the study of the structure of biological membranes, membrane properties and drug-membrane interaction (Curtain CC, et al., 1984; Sauerheber RD, et al., 1977). Spin labels are specifically incorporated with the lipid or lipid part of biological membranes. Thus, each label reflects properties of a different membrane region. EPR spectra of membrane-incorporated spin labels are sensitive to the rotational mobility of the labels, the polarity of the environment surrounding the labels and their orientation. These effects have been used to provide advantage in numerous studies of membranes and skin. Some physical conditions (e.g. temperature and chemical compounds) can perturb biological membranes to yield characteristic changes in their respective EPR spectra. (Cannon B, et al., 1975; Hubbell WL, et al., 1969; Keith AD, et al., 1973; Seeling J, 1970; Quan D, et al., 1994; Quan D, et al., 1995)

In this chapter, an EPR study was conducted to investigate the effect of anionic surfactants (SLS; sodium lauryl sulfate, SLG; sodium lauroyl glutamate, SL; sodium laurate, SLES; sodium lauryl POE (3) ether sulfate, SLEC; sodium lauryl POE (3) ether carboxylate, and mixtures of SLS and SLG) on the lipid bilayers of human stratum corneum. We also conducted human patch test of the anionic surfactants and examined the correlation between EPR spectral data and the clinical observables; visual score and TEWL (transepidermal water loss), respectively.

4.2 What is Electron Paramagnetic Resonance (EPR) spectroscopy?

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), is the name given to the process of resonant absorption of microwave radiation by paramagnetic ions or molecules, with at least one unpaired electron spin in the presence of a static magnetic field. EPR was discovered by Zavoisky in 1944. It has a wide range of applications in chemistry, physics, biology, and medicine: it may be used to probe the "static" structure of solid and liquid systems, and is also very useful in investigating dynamic processes.

Most biological systems give no intrinsic EPR signal because they have no unpaired electrons. Therefore, if EPR is to be used in studying these systems such as lipid membranes or macromolecules, one or more radicals known as spin labels must be coupled to the system under investigation. The spin label thus is an extrinsic probe or reporter group providing information that reflects the state of the biological system.

4.2.1 Principles

The detailed principles of EPR are explained in the book written by Wertz J. E., et al. (1972) Here, EPR principles are introduced in brief.

The principles of EPR are similar to those of NMR (Nuclear Magnetic Resonance). The magnetic moment of an unpaired electron is given by

$$m = -g_e (eh / 4\pi m_e) m_s \text{ (SI)}$$

in which g_e is the electronic g-factor (a number very nearly equal to 2). $-e$ and m_e are respectively the electronic charge and mass, and m_s is the spin quantum number (equal to ±1/2). The quantity in parentheses in the first equation is called the

Bohr magnetrons, and has the value of 9.2732-x 10^{-24} J T^{-1} in SI units (9.2732 x 10^{-21} erg G^{-1}). In an applied magnetic field of strength *B*, the transition of an electron from ground to the excited state requires energy

$$\Delta E = g_e \, (eh / 4\pi m_e) \, B \text{ (SI)}$$

In a magnetic field of 2T (20 kG), this energy corresponds to the absorption of radiation of the frequency

$$\nu = \Delta E / h = 2 \text{ x } (9.273\text{x } 10^{-24} \text{ JT}^{-1})(2 \text{ T}) / 6.62 \text{ x } 10^{-34} \text{ Js} = 5.6 \text{ x } 10^{10} \text{ Hz}$$

which is in the microwave region of the spectrum.

Paramagnetic substances are detected readily by EPR. About 10^{-13} mole of a substance gives an observable signal, so this technique is one of the most sensitive of all spectroscopic tools. (Eisenberg D, et al. (1979))

The interaction of an electron spin in resonance with a neighboring nuclear spin in a molecule is called “hyperfine coupling”. In the case of nitroxide spin probe, 14N of the probe has three quantum states: +1, 0, -1. Each quantum state interacts with an electron spin and further splits into two sets of energy states (Fig 4-2) the selection rules for transitions in hyperfine coupling are $\Delta m_s = 1$ and $\Delta m_I = 0$. Thus, one can observe three transition (resonance) lines for fast-tumbling nitroxide spin probe in a spectrum. The interval of the resonance lines is called the hyperfine coupling constant. The EPR spectra are usually recorded as the first derivative of the absorption spectrum as shown in lower part of Fig 4-2.

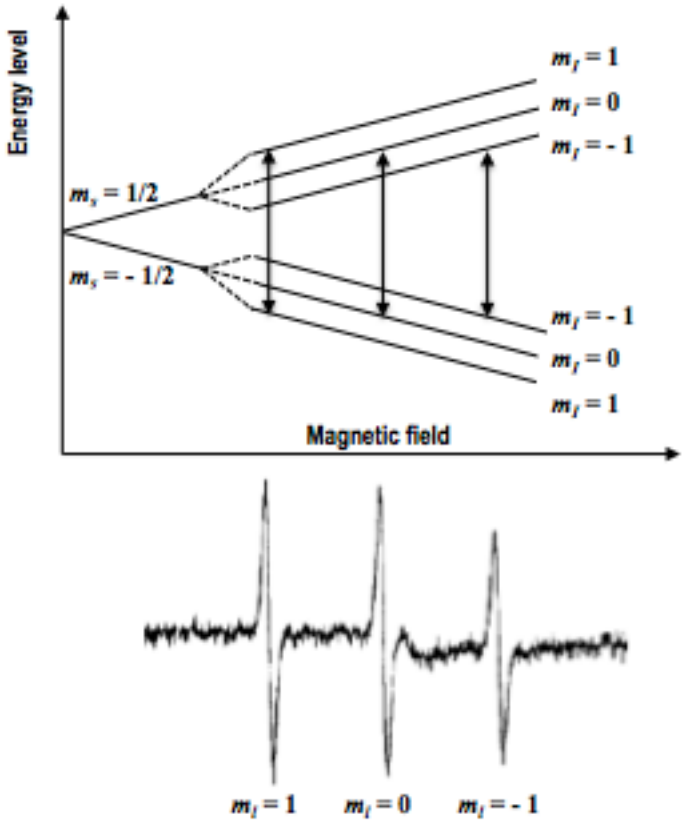


Fig 4-2. Hyperfine levels and transitions for nitroxide nitrogen nucleus (^{14}N) of I = 1 with positive coupling constant

4.2.2 Electron Paramagnetic Resonance Spectrometer

A modern EPR instrument consists of three basic units: (a) a microwave bridge and resonator, (b) a variable field magnet and (c) signal amplification circuitry (Fig 4-3).

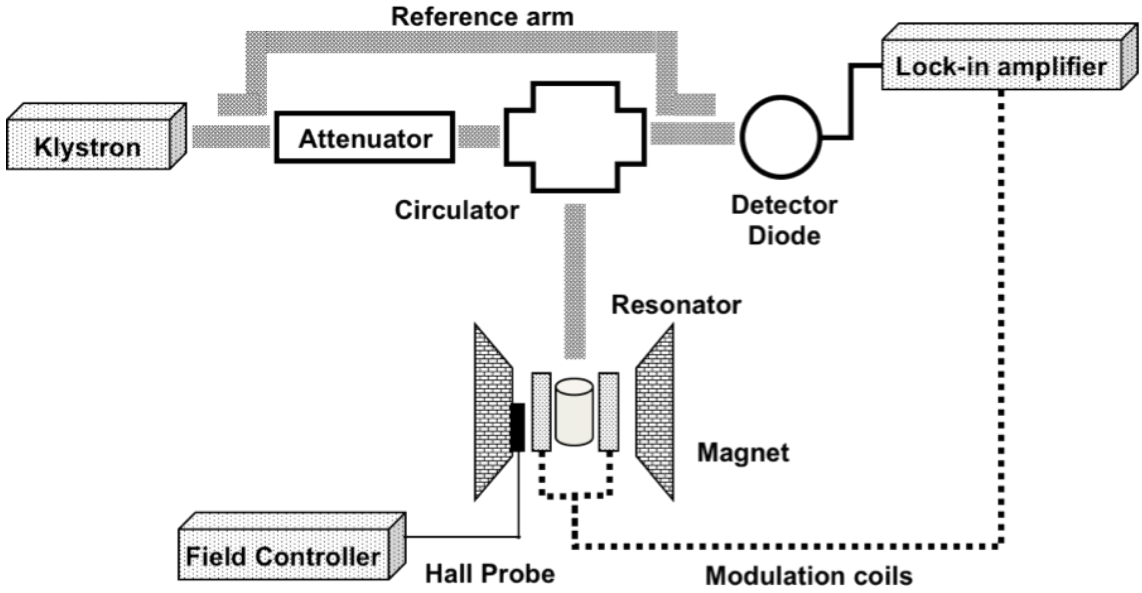


Fig 4-3. Block diagram of a typical EPR spectrometer

Microwaves of the desired frequency are generated by either a klystron or Gunn diode. Their intensity is adjusted by an attenuator and transmitted via a waveguide to the sample chamber/resonator. During resonance, a small amount of microwaves is reflected from the resonator and detected by a Shottky diode. To separate the reflected and incident microwaves, a circulator is placed between the attenuator and resonator. The circulator channels the microwaves in a forward direction: incident microwaves to the resonator and reflected microwaves to the detector. The bridge often contains an additional pathway – a reference arm which taps off a small fraction of the microwaves from the source – which bypasses the resonator and falls on to the detector to ensure its bias for the optimal detection of small intensity changes during resonance.

A static magnetic field is provided by an electromagnet stabilized by a Hall probe. The field is slowly swept by varying the amount of current passing through the electromagnet.

4.2.3 Spin Labeling Method; Paramagnetic Nitroxide Molecules that Serve as Probes in Membranes

McConnell et al showed that significant information could be derived about macromolecules and membranes from the EPR spectra of bound nitroxide molecules (McConnell HM, et al. (1972)). These are stable molecules that possess an unpaired 2*p* electron. The unpaired electron endows the molecules with strong EPR spectra. 5-Doxyl Stearic Acid (5-DSA) is one of the most commonly used spin probes and its structure is shown in Fig 4-6. A nitroxide molecule bound to a macromolecule is called a spin label.

Because the ^{14}N nucleus in a nitroxide molecule is near the unpaired electron, there is an interaction between them, thereby producing hyperfine splitting in the EPR spectrum. The ^{14}N nucleus has a spin of one, and consequently three absorption bands appear in the EPR spectra. The EPR spectra are usually recorded as the first derivative of the absorption spectrum, so instead of three bands there are three rise-and-dip spikes, which are the derivatives of the three bands. Triplet signals, which are sharp, can be observed when the spin-probe moves freely, as shown in Fig 4-4. However, the spectrum becomes broader when spin probe mobility is restricted by interaction with other components. When the spin probe is incorporated into the highly-oriented intercellular lipid structure of normal skin, the probe cannot move freely due to the rigidity of the lipid structure, and its EPR spectrum represents the broad profile as seen in Fig 4-5 (a). Once the normal structure is destroyed by chemical and/or physical stress, there is nothing to inhibit probe mobility, and the EPR spectrum profiles become sharp, as in Fig 4-5 (b). The EPR spectral profile represents the rigidity of the environment of the spin probe. To express the rigidity quantitatively, an order parameter S is calculated from the EPR spectrum.

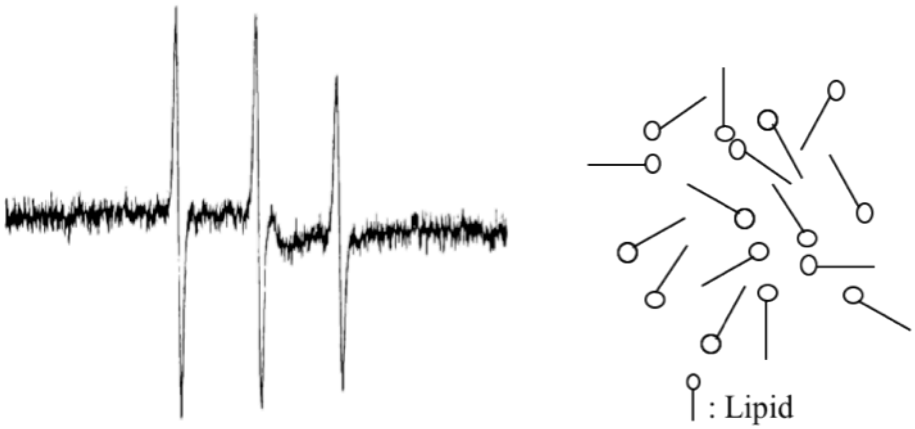


Fig 4-4. EPR spectrum of 5-DSA in aqueous solution

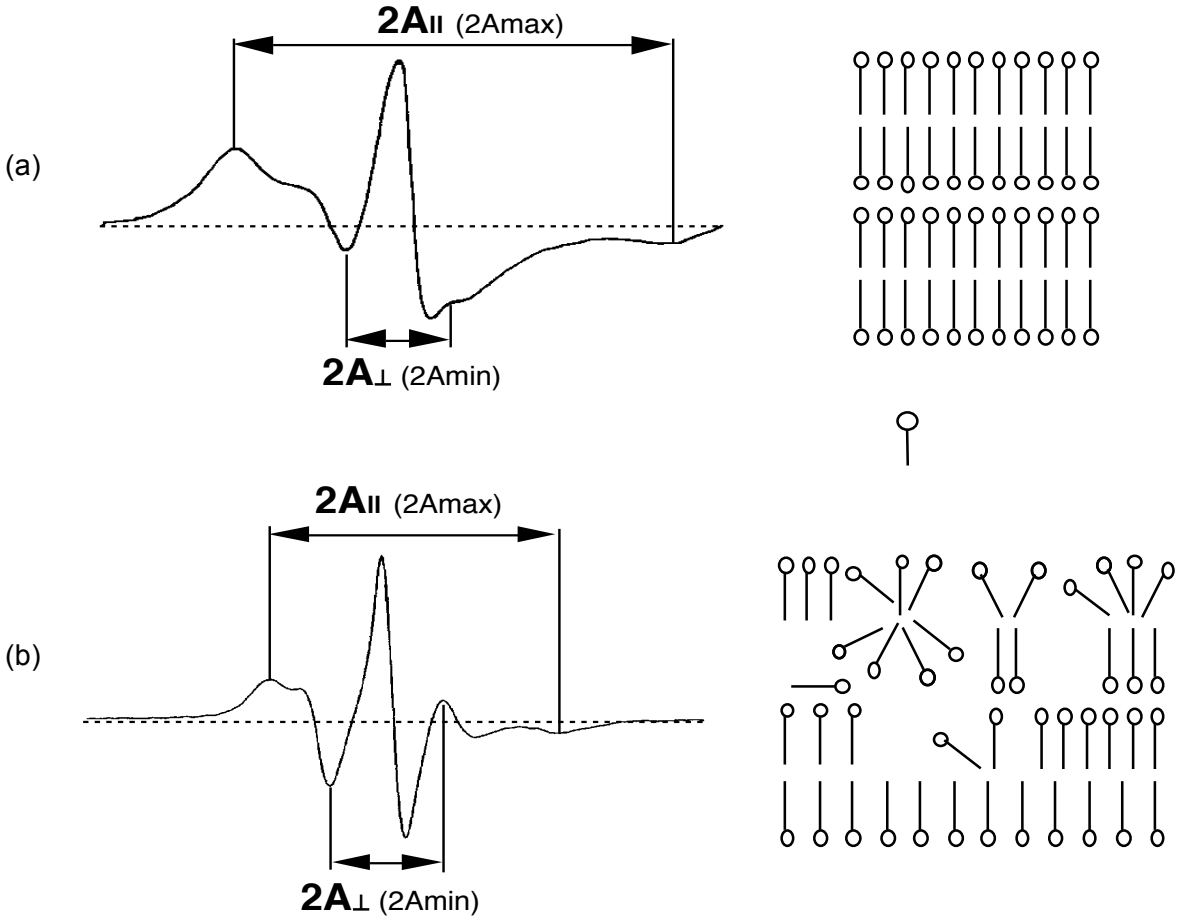


Fig 4-5. EPR spectrum of 5-DSA labeled stratum corneum from cadaver,
(a) non treatment (control), (b) treated with 1%wt SLS (sodium lauryl sulfate)

Spin labels provide information about the molecules to which they are bound. They can report the rate of motion of the molecule to which they have been covalently bound, or the amount of thermal motion in a membrane into which they have been inserted. The principle is that the bands of the EPR spectrum are broadened when the spin label is immobilized and narrowed when it is tumbling rapidly. The narrowing comes from the more rapid relaxation of the spin when neighboring groups are moving rapidly with respect to the spin label.

A second type of information is the polarity of the local environment surrounding the spin probe. The extent of splitting of the side bands from the central band depends on the dielectric constant of the medium in which the spin label is dissolved. Solvents of high dielectric constant augment the polarity of the N-O bond and increase the splitting. By measuring the splitting, an estimate can be made of the polarity of the surroundings of the spin label. This is of interest, for example, when a spin label is bound to a membrane, since it allows one to determine if the label is bound near the polar head groups or near the non-polar hydrocarbon chains (Mehlhorn RJ, et al. (1972))

4.2.4. Calculation of Order Parameter *S*

Order parameters were calculated according to Griffith OH, et al. (1976), Hubbell WL, et al. (1971), and Marsh D (1981):

$$S = (A_{||} - A_{\perp}) / [A_{ZZ} - 1/2 (A_{XX} + A_{YY})](a_0/a_0')$$

where $2A_{||}$ is identified with the outer maximum hyperfine splitting A_{max}, and $A_{\perp}$ is obtained from the inner minimum hyperfine splitting A_{min} (Fig 4-5).

a_0 is the isotropic hyperfine splitting constant for nitroxide molecules in the crystal state.

$$a_0 = (A_{XX} + A_{YY} + A_{ZZ}) / 3$$

The values used to describe the rapid anisotropic motion of membrane-incorporated probes of the fatty acid type are:

$(A_{XX}, A_{YY}, A_{ZZ}) = (6.1, 6.1, 32.4)$ Gauss

Similarly the isotropic hyperfine coupling constant for the spin label in the membrane (a_0') is given by:

$$a_0' = (A_{||} + 2 A_{\perp}) / 3$$

a_0' values are sensitive to the polarity in the environment of the spin labels since increases in a_0' value reflect an increase in the polarity of the medium.

The order parameter provides a measure of the flexibility of the spin labels in the membrane. It follows that S = 1 for highly oriented (rigid) states and S = 0 for completely isotropic motion (liquid). Increases of order parameter reflect decreases in the segmental flexibility of the spin label, and conversely decreases in the order parameter S reflect increases in the flexibility (Curtain CC, et al. (1984)).

4.3 Material and Methods

4.3.1 Materials

5-doxyl stearic acid (5-DSA; FW=384.6) purchased from Aldrich was used as a spin-labeling reagent without further purification. Sodium lauryl sulfate (SLS ; Sigma Chemical Co., USA ; purity 99%up), sodium laurate (SL ; Junsei Chemical Co., Japan ; purity >98%), sodium lauroyl glutamate (SLG ; Ajinomoto Co., Inc., Japan ; purity >98%), sodium lauryl POE (3) ether carboxylate (SLEC ; Sanyo Kasei, Ltd. , Japan ; commercial grade), and sodium lauryl POE (3) ether sulfate (SLES ; Kao Co., Japan ; commercial grade) were used as anionic surfactants without further purification. Purity of all materials were as stated by the suppliers. The purity of all materials was as stated by the suppliers. All chemical structures are described in Fig-6 and Fig-7.

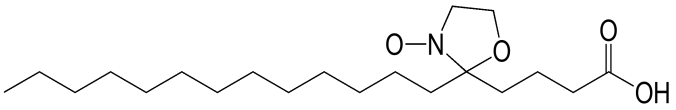


Fig 4-6. Chemical structure of spin label 5-DSA

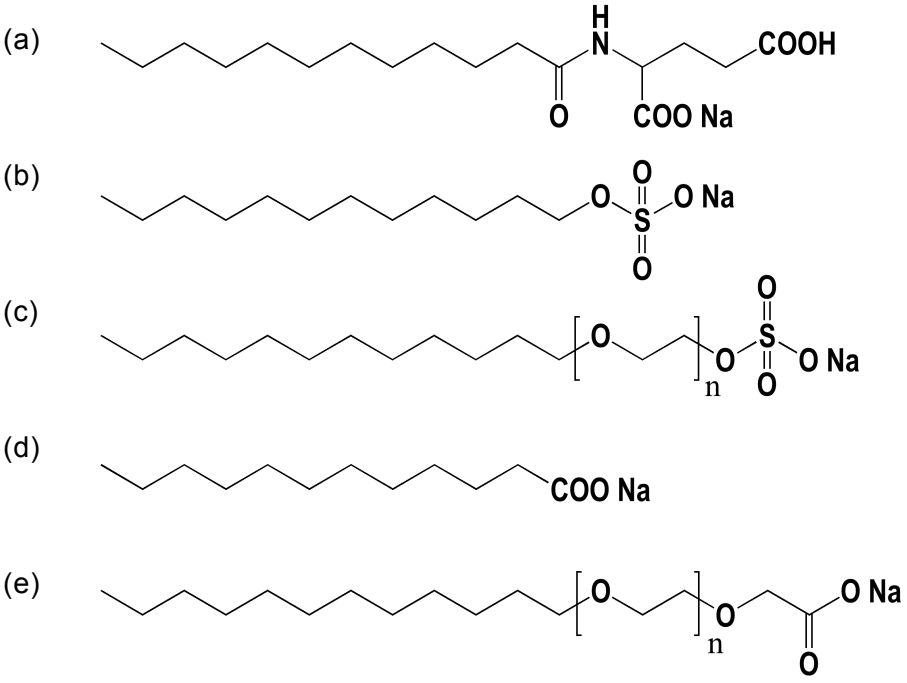


Fig 4-7. Chemical structure of anionic surfactants; (a) Sodium Lauroyl Glutamate: **SLG**, (b) Sodium Lauryl Sulfate: **SLS**, (c) Sodium Lauryl POE(3) Ether Sulfate: **SLES,** (d) Sodium Laurate: **SL**, (e) Sodium Lauryl POE(3) Ether Carboxylate: **SLEC**

4.3.2 Preparation of human stratum corneum from cadaver skin

Human abdominal skin was obtained from fresh cadaver skin with a dermatome (Northern California Transplant Bank; San Rafael, CA, USA). The epidermis was separated from the dermis by immersing the dermatomed skin in 60^{o}C water bath set for 2 minutes followed by mechanical removal. Then, the epidermis was placed stratum corneum side up, on filter paper and floated on 0.5% trypsin (type II; Sigma), in a Tris-HCl buffer solution pH 7.4, for 2 hours at 37^{o}C. After incubation any softened epidermis was removed by mild agitation of stratum corneum sheets. Stratum corneum was dried and stored in a desiccator at -70^{o}C for 3~4 days. Details of the method are described in Quan and Maibach. (1994)

4.3.3 Spin-labeling procedures and treatment with anionic surfactants

5-DSA was used as a stearic acid spin labeling agent. Some slices of dry stratum corneum sheets (approx. 0.5 cm^{2}, approx.0.5 cm x approx. 1 cm) were incubated in Tris-HCl buffer solution (pH 7.4) dissolving spin labeling reagents at 10 mg/L (2.6x10^{-5} M) for 2 hour at 37^{o}C and then dried under flow of nitrogen gas for 1 hour at approx. 25^{o}C over silica gel for laboratory convenience. (Gay CL, et al., 1990).

Treatment with anionic surfactants was performed as follows: spin-labeled stratum corneum (5-DSA) was immersed in the surfactant aqueous solutions prepared at designated concentration, and incubated at 37^{o}C for 1 hour. After rinsing with purified water, they were dried by the same procedure as described the above. The spin-labeled (5-DSA) stratum corneum treated with purified water instead of surfactant solution was used as a control.

4.3.4 EPR spectral measurement

Three slices of stratum corneum samples previously labeled with 5-DSA were mounted on the flat surface of modified quartz cell (Wilmad Glass Co., Buena, NJ, USA). The cells are 21 cm in length with an outer diameter of 1 cm. The EPR measurement was carried out under the condition controlled at approx. 25^{o}C.

ESR spectrum measurements were carried out by an ER200 Series ESR spectrometer (Brucker Inc., MA, USA) with microwave power out of 25mW and

spectrum data were collected by an IBM-PC system with PC/FORTH (ver. 3.2 Laboratory Microsystems, CA, USA). The hyperfine splittings of labeled skin samples were determined with 100 gauss scan width, 2 x 10^5 receiver gain, 2 gauss modulation amplitude and 0.1 second time constant. Each sample was scanned several times and ESR parameters from each spectrum were averaged to give a single estimate for the example.

4.3.5 *in vivo* human patch test

72 healthy volunteers, 32 male and 40 female, age 45 ± 14 years, were recruited for patch testing. They were all free of skin disease, had no history of atopic dermatitis.

After 30 min acclimatization (temperature : 16 - 23 ^{o}C, relative humidity : 50 - 62 %), test sites were marked on the subject's back and baseline of TEWL was measured. 0.1 ml of each test solution was applied using polypropylene chambers (Hilltop ; Cincinnati, Ohio, USA) secured with paper tape (Scanpor, Norgesplaster, Oslo, Norway). Application sites were randomized to minimize anatomical bias.(Van der Valk et al., 1989) Patches were removed after 24 hours and the test sites were exposed to air at least 30 min in order to allow deconvolution of excess water.

Each site was visually graded in accordance with the following system; 0 = normal skin or no reaction, 0.5 = faint, barely perceptible erythema or slight dryness, 1 = definite erythema or dryness, 2 = erythema and induration, 3 = vesiculation. TEWL was measured quantitatively with an Evaporimeter EP-1 (Servo Med, Stockholm, Sweden) at 30 min after patch removal. Reading was performed at a stable level, 30 seconds or more after application of the TEWL probe on the skin. TEWL values were expressed as g/m^2/h.

4.4 Results & Discussion

4.4.1 Anionic surfactant effect on the EPR spectra of human stratum corneum

Three concentrations of anionic surfactants were used. The EPR spectra of human stratum corneum treated with 1.0% SLS and SLG solutions were measured at 25^{o}C as a function of drying time.

Clearly, EPR spectra of surfactant treated stratum corneum were totally different from those of control (5-DSA treated stratum corneum) as shown in the Fig 4-8 ~ 4-10.

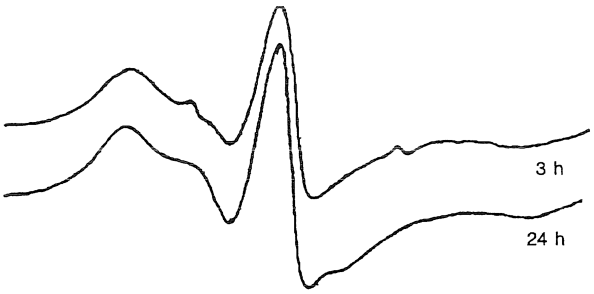


Fig 4-8. Drying time dependence of EPR spectra of 5-DSA labeled human stratum corneum

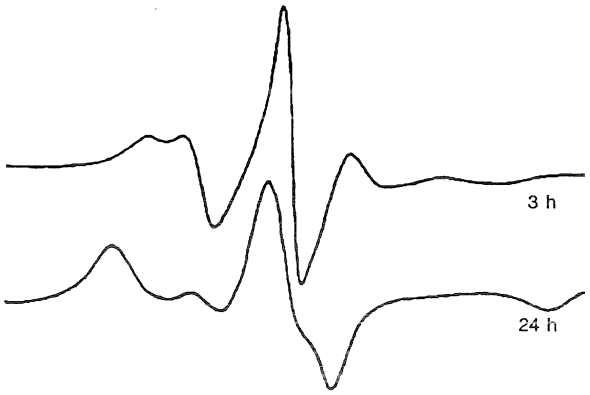


Fig 4-9. Drying time dependence of EPR spectra of 5-DSA labeled human stratum corneum treated with 1.0% SLS solution (incubation time in SLS solution = 1 hour)

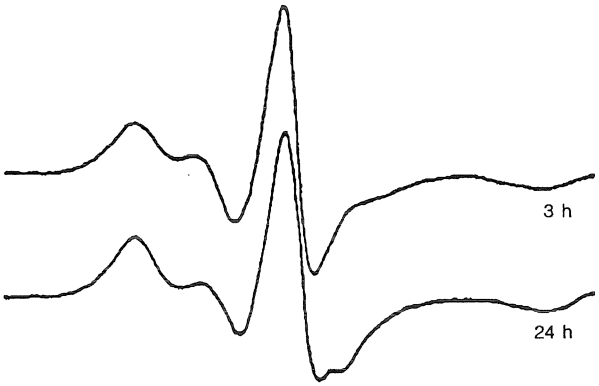


Fig 4-10. Drying time dependence of EPR spectra of 5-DSA labeled human stratum corneum treated with 1.0% SLG solution (incubation time in SLG solution = 1 hour)

With the diffusion of anionic surfactants into the spin labeled stratum corneum, EPR spectra indicates that the spin label binds to the lipids or to the lipid binding protein, and the motion of the spin label shows some level of anisotropy. (Quan D, et al., 1994)

The corresponding order parameter S obtained from the EPR spectra of 5-DSA labeled human stratum corneum treated with different concentrations of SLS and SLG is summarized in Fig 4-11 and 4-12.

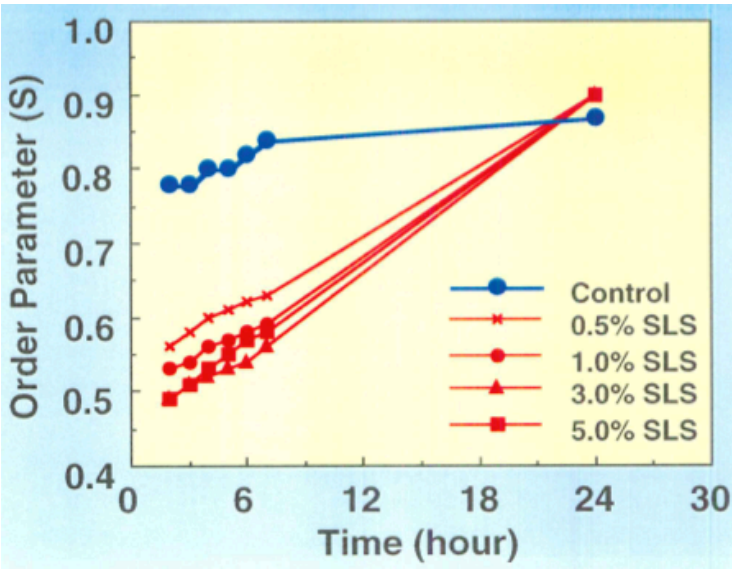


Fig 4-11. Drying time dependence of order parameter S of 5-DSA labeled human stratum corneum treated with four concentrations of SLS (incubation time in SLS solution = 1 hour)

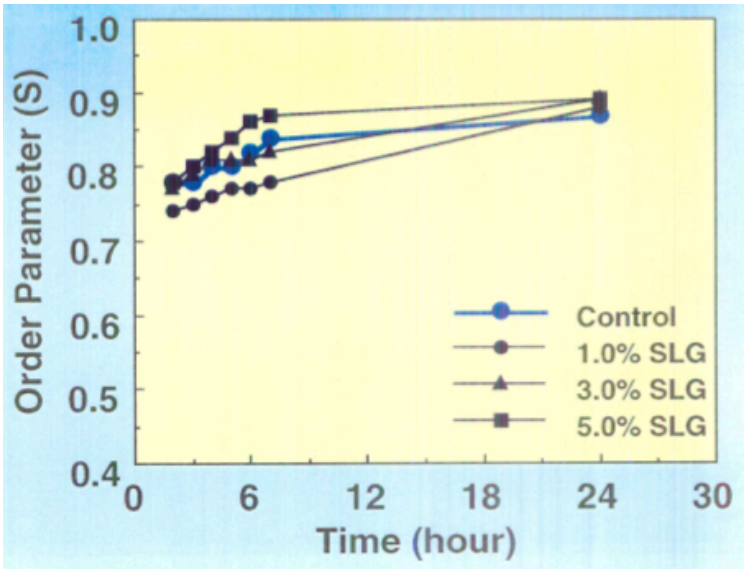


Fig 4-12. Drying Time dependence of order parameter S of 5-DSA labeled human stratum corneum treated with four concentrations of SLG (incubation time in SLG solution = 1 hour)

Anionic surfactants possess a polar head group (“sulfate” for SLS and “glutamic acid” for SLG) and a long hydrocarbon chain, that potentially incorporate into structured lipid bilayers (lamellar structure). SLS disorders the lipid structure even at a low concentration such as 0.5%wt. Conversely; SLG does not significantly increase the fluidity of the lamellar structure of the skin lipids. The presence of anionic (hydrophilic = polar) structure such as sulfate forces apart the alkyl chain of the skin lipids yielding more free space in which to move, hence disordering the skin intercellular lipids.

It seems that SLS simply fluidizes lipid structure by solubilizing lipid and/or pushing water into the lipid bilayer structure. Increase of SLS concentration enhances these effects. However, it is indicated that SLG induces more rigid structure in accordance with the increase of concentration of SLG applied. SLG should have a unique property interacting the lipid bilayer. Although “glutamic acid” moiety in SLG can be thought to be larger than “sulfate” structure in SLS, the influence of SLG on lipid structure is smaller than that of SLS. SLG possesses “peptide” bond in its structure, which means SLG may absorb to protein (keratin and

cornified envelope) due to the attractive force. SLG would have minimal effect with intercellular lipids.

As for the dose response profiles of each surfactant, any concentration of SLS significantly decreases S; the higher concentration of SLS is applied, the more fluidized in the lipid structure is induced. However, SLG shows an opposite trend. The higher concentration of SLG is applied, the more rigid structure is indicated to form.

SLG possesses a peptide bond, which has potential forming hydrogen bonding. SLG may induce structured water in the lipid bilayer, which means SLG removes water molecules hydrated in the lipid structure and more packed structure is induced the distance between lipid molecules becomes shorter.

The role of water in the stratum corneum must be also considered for an understanding of the effects of surfactants on lipid layers. Takino Y, et al., (1996) demonstrated that application of anionic surfactants onto the skin might influence water penetration and/or skin swelling. Rhein et al (1986, 1990) examined the swelling of stratum corneum caused by surfactants and reported that the swelling effect of surfactants suggests a mechanism of action as the basis for *in vivo* irritation potential. Alonso et al. (1995 & 1996) reported that water increases the fluidity of intercellular lipids of rat stratum corneum at the region close to the hydrophilic area, but not in the lipophilic area, deep inside the intercellular lipid layer. Treatment with anionic surfactants may influence water penetration into stratum corneum. In case that the stratum corneum from a cadaver is treated with SLS and SLG for one hour, the order parameter decreases with the dose dependency of the surfactants.

When order parameter S is followed over time under dry conditions, those values increase. After 24 hours, the order parameter becomes higher than that of control (Fig 4-11, 4-12). It is suggested that the altering of water content in cadaver stratum corneum affects on the order parameter. This does not simply mean loss of water, because after the untreated control stratum corneum dries, the order parameter still shows a minimal order parameter change.

4.4.2 Effect of Anionic Surfactant Mixture on Intercellular lipid Fluidity of Human Stratum Corneum

A previous study (Kawasaki Y, et al (1995, 1997)) showed that sodium lauryl sulfate (SLS) caused a significant change in EPR spectra of 5-DSA labeled stratum corneum. However, sodium lauroyl glutamate (SLG) produced only minimal changes. The results suggest that the interaction between anionic surfactant and stratum lipids depends on the chemical structure of anionic surfactants. This observation agrees to the results of the *in vivo* human patch test study by Lee, et al. (1994). In this study, we extensively used EPR spin label technique to discuss: (1) influence on the fluidity of human stratum corneum intercellular lipid of SLG and SLS mixture and (2) the correlation between EPR spectral data and human skin irritation reaction induced by the surfactants mixture.

The order parameters ***S*** obtained from each EPR spectrum of stratum corneum treated with water, 0.25 %wt, 0.50 %wt, 0.75 %wt, 1.00 %wt SLS solutions, SLS/SLG mixtures (total concentration is constant at 1.00 %wt) and 1.00 %wt SLG solution were summarized in Table 4-1 and Fig 4-13.

The order parameter of water treated stratum corneum (vehicle control) was 0.86 ± 0.03. Anionic surfactants as an amphiphilic molecule might be incorporated into structured lipids (lamellar structure). Order parameter (***S***) calculated from 1.0%wt SLS treated stratum corneum was 0.56 ± 0.03, indicating lipid structure disordering. On the contrary, the high ***S*** value (0.82 ± 0.02 almost equals to water) for 1.0%wt SLG means less lipid structure disordering. Treatment with 0.25%wt, 0.50%wt and 0.75%wt SLS solutions revealed intermediate levels between 1.0%wt SLG and SLS.

Each order parameters of 5-DSA labeled stratum corneum treated with SLS/SLG mixtures (total concentration is constant at 1.00 %wt) showed higher values than that of 0.25 %wt, 0.50 %wt, 0.75 %wt SLS, respectively. There was no statistically significant difference between 0.50 %wt SLS and 0.50 %wt SLS / 0.50 %wt SLG, and between 0.75 %wt SLS and 0.75 %wt SLS / 0.25 %wt SLG. ($p>0.05$)

These results suggest that SLG inhibited SLS to fluidize lamellar lipid structure. To confirm the anti-fluidization of SLG, the SLS/SLG mixture solutions were prepared with making SLG concentration constant at 1.00 %wt and measured EPR spectra of 5-DSA labeled stratum corneum treated with them. The calculated order parameters ***S*** were also plotted in Fig.4-13

Table 4-1. Order parameters of stratum corneum treated with surfactants and clinical observations

Sample Name	Averaged Order Parameter ***S*** (mean ± SD; n=3)	Human Patch (mean ± SEM; n=15)	
		Visual Score	TEWL g/m^2/h
Control	0.86 ± 0.03	0.53 ± 0.08	13.0 ± 1.0
0.25 %wt SLS	0.70 ± 0.02	0.73 ± 0.08	22.3 ± 1.7
0.50 %wt SLS	0.66 ± 0.04	0.70 ± 0.10	22.3 ± 1.7
0.75 %wt SLS	0.64 ± 0.03	0.87 ± 0.14	22.7 ± 1.5
1.00 %wt SLS	0.56 ± 0.03	1.03 ± 0.15	25.4 ± 2.6
0.25 %wt SLS + 0.75 %wt SLG	0.81 ± 0.07	0.42 ± 0.30	20.0 ± 1.7
0.50 %wt SLS + 0.50%wt SLG	0.71 ± 0.00	0.08 ± 0.20	20.7 ± 1.9
0.75 %wt SLS + 0.25 %wt SLG	0.66 ± 0.04	0.04 ± 0.10	21.2 ± 2.6
0.25 %wt SLS + 1.00 %wt SLG	0.81 ± 0.05	**NA**	**NA**
0.50 %wt SLS + 1.00 %wt SLG	0.79 ± 0.05	**NA**	**NA**
0.75 %wt SLS + 1.00 %wt SLG	0.74 ± 0.04	**NA**	**NA**
1.00 %wt SLS + 1.00 %wt SLG	0.66 ± 0.05	**NA**	**NA**
1.00 %wt SLG	0.82 ± 0.02	0.67 ± 0.08	15.8 ± 1.1

[Note] Error bars : Mean ± SD, n=3 for order parameters, Mean ± SEM, n=15 for clinical data, NA : Not available

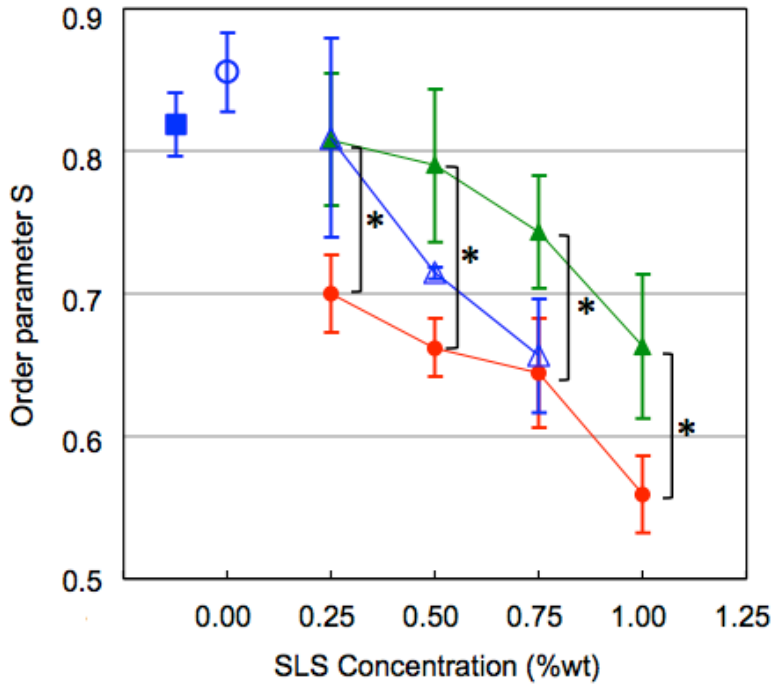


Fig 4-13. Order parameter of 5-DSA labeled stratum corneum treated with water, SLS, SLG, and SLS+SLG mixtures (total concentration 1%wt, 1%wt SLG addition to the SLS solutions). Error bar: mean ± SD for n=3, the result for 1%SLG is plotted on the left for comparison. * means P< 0.05. ●: SLS, ○: Control (water), ▲: SLS + 1%wt SLG, △: SLS + SLG (total 1%wt), ■: 1%wt SLG

Order parameters at each SLS concentration (0.25, 0.50, 0.75 and 1.00 %wt SLS) with 1.0%wt SLG showed higher values than those of SLS only solutions. There were statistically significant differences between with and without 1.0%wt SLG. ($P<0.05$), suggesting that the addition of 1.0%wt SLG inhibits the fluidization of intercellular lipid by SLS. It may be hypothesized that the direct interactions between SLS and intercellular lipids were interrupted by SLG. The log P (partition coefficient; $\log\{[SLS]_{lipid} / [SLS]_{bulk}\}$) of SLS into the intercellular lipid might be decreased.

The change of order parameter corresponds to the structural changes of lipid layers. Two phases can be speculated to increase lipid structure fluidity (decreasing the order parameter). (1) The first phase is an effect of surfactant incorporated into the lamellar structures. If surfactant interferes or decreases lateral interactions between lipids, mobility increases similar to the phase conversion from liquid crystal to gel in the lamellar layers. (2) The second phase is the destruction of lamellar structure by

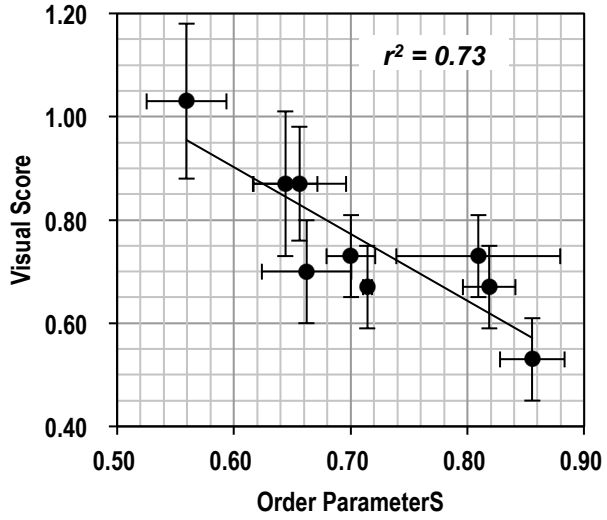
micellization or solubilization of lipid by surfactant. In this case, lipids no longer have dimensional restrictions and gain higher mobility.

To better understand this phenomenon, further investigation on the molecular interactions and structural effects between surfactant and lipid layers is needed. The role of water in the stratum corneum must be also considered for the effects of surfactant on lipid layers. Alonso et. al. (1996) reported that water increases the fluidity of intercellular lipid of rat stratum corneum. Treatment with anionic surfactants might influence water penetration or skin swelling. Rhein et. al. (1986) examined the swelling of stratum corneum caused by surfactants and reported that swelling effect of surfactants suggests another mechanism of action as the basis for *in vivo* irritation potential.

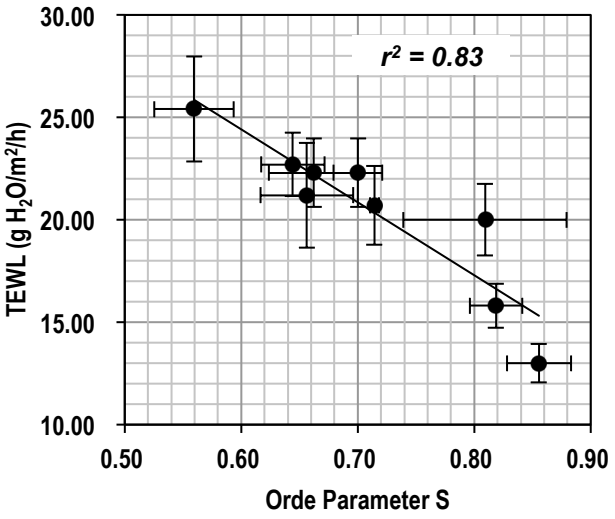
Correlation between order parameter and human clinical data

Order parameter ***S*** (EPR spectral data) correlated with the clinical readings (Fig 4-14 (a) and (b)). The correlation coefficients ($\mathbf{r^2}$) of visual score and TEWL values were 0.733 and 0.833, respectively. The order parameter ***S*** correlates to TEWL values better than to visual scores. This difference may be explainable that TEWL is a measure of water barrier function while visual scores represent total skin reactions including physical or structural changes and physiological or biological reactions with surfactant. The order parameter measurement represents the skin water barrier function.

The order parameters represent the disorder of stratum corneum induced by short-term contact with surfactant (1 hour incubation at 37^{o}C). However, clinical data represent the skin irritation reaction induced by 24 hour occlusive contact with surfactants. Order parameter measurement of stratum corneum may predict the minimal difference of irritating potential among various kinds of chemicals.



(a)



(b)

Fig 4-14. Correlation between clinical data of 24 hour patch test and order parameter of 5-DSA labeled cadaver stratum corneum incubated in surfactant solution for 1 hour at 37^{o}C: (a) correlation between order parameter and visual scores; (b) correlation between order parameter and TEWL (error bars: mean ± SD for order parameter n=3, mean ± SEM for clinical data n=15)

4.4.3 Correlation between order parameter and human clinical data on several kinds of anionic surfactants

We have studied the influence of anionic surfactants on intercellular lipid fluidity in human cadaver skin with focusing on two kinds of anionic surfactants, which are **SLG** (monosodium N-lauroyl-L-glutamate) and **SLS** (sodium lauryl sulfate), and successfully demonstrated that the S values, reflecting fluidity of intercellular lipids, correlate to both TEWL and visual scores.

In this chapter, we examined other anionic surfactants which are widely used in cosmetics and toiletries market addition to **SLS** and **SLG**; Sodium Laurate: **SL**, Sodium Lauryl POE (3) Ether Carboxylate: **SLEC**, and Sodium Lauryl POE (3) Ether Sulfate: **SLES** regarding their effect on the intercellular lipid fluidity and discuss the correlation between the order parameters and *in vivo* human skin irritation (24 hour patch test results).

The profiles of EPR spectra of 5-DSA labeled depend on the anionic surfactants treated. The corresponding order parameters S obtained from each EPR spectrum and *in vivo* human patch test results were summarized in Table 4-2.

Table 4-2. Order parameters of cadaver stratum corneum treated with anionic surfactants and their *in vivo* human patch test results

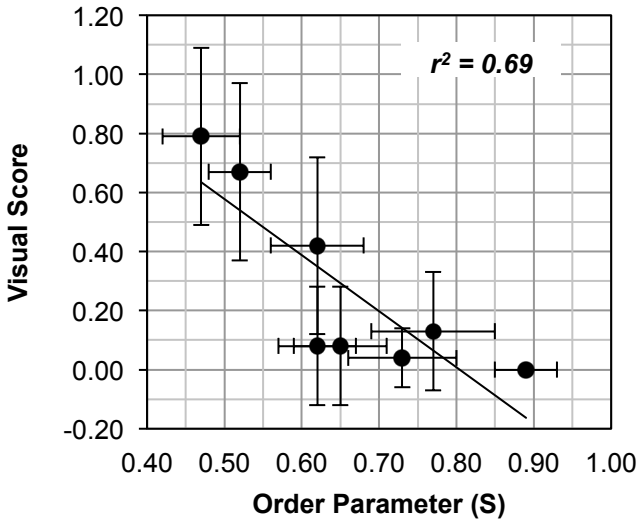
Sample	%wt	Order Parameter (S)	Human Patch Test	
			Visual score (mean ± SD)	TEWL g $H_2O/m^2/h$
Control (Water)		0.89 ± 0.04	0.00 ± 0.0	5.0 ± 1.1
SLG	(1.0%)	0.73 ± 0.07	0.04 ± 0.1	6.7 ± 3.5
	(5.0%)	0.77 ± 0.08	0.13 ± 0.2	5.5 ± 2.3
SL	(1.0%)	0.65 ± 0.06	0.08 ± 0.2	7.1 ± 3.9
	(5.0%)	0.52 ± 0.04	0.67 ± 0.3	13.3 ± 3.7
SLES	(1.0%)	0.62 ± 0.06	0.42 ± 0.3	7.6 ± 2.8
SLEC	(1.0%)	0.62 ± 0.05	0.08 ± 0.2	7.4 ± 2.9
SLS	(1.0%)	0.47 ± 0.05	0.79 ± 0.3	13.6 ± 3.1

(Note: mean ± SD for order parameter n=3, mean ± SD for clinical data n=15, "TEWL" was measured with Evaporimeter Servo Med EP-1 (Stockholm, Sweden))

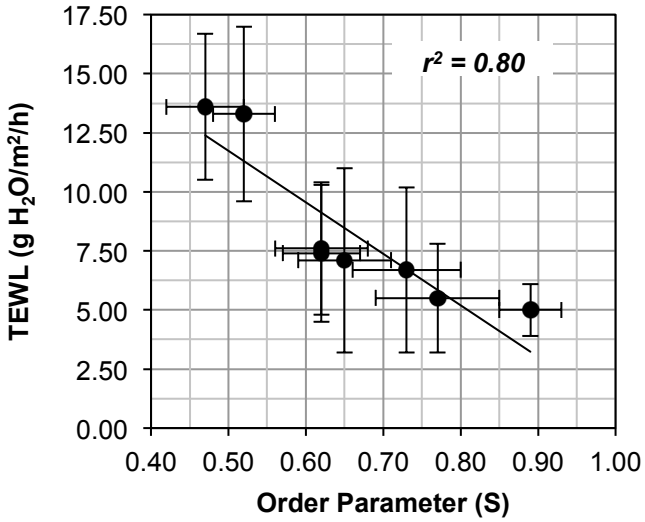
As discussed in the previous chapter, anionic surfactants as an amphiphilic molecule might be likely incorporated into structured lipids (lamellar structure). Order parameter (***S***) calculated from 1.0 %wt SLS treated stratum corneum was 0.47, indicating significant disordering of the lipid structure. On the contrary, the high S value (0.73) for 1.0%wt SLG means less effect to the structured lipid. Treatment with 1.0%wt solution of SL, SLES and SLEC revealed intermediate levels between SLG and SLS.

The clinical readings were plotted against the order parameter ***S*** corresponding each tested anionic surfactant as shown in Fig 4-15 (a) and (b). The correlation coefficients ($\mathbf{r^2}$) of visual score and TEWL values were 0.736 and 0.83, respectively. The order parameter ***S*** correlates to TEWL values better than to visual scores. This difference may be explainable that TEWL is a measure of water barrier function while visual scores represent total skin reactions including physical or structural changes and physiological or biological reactions with surfactant. The order parameter measurement represents the skin water barrier function.

The order parameters represent the disorder of stratum corneum induced by short-term contact surfactant (1 hour incubation at 37^oC). However, clinical data represent the skin irritation reaction induced by 24 hour occlusive contact with surfactants. Order parameter measurement of stratum corneum may predict the minimal difference of irritating potential among various kinds of chemicals.



(a)



(b)

Fig 4-15. Correlation between clinical data of 24 hour patch test and order parameter of 5-DSA labeled cadaver stratum corneum incubated in surfactant solution for 1 hour at 37°C: (a) correlation between order parameter and visual scores; (b) correlation between order parameter and TEWL (error bars: mean ± SD for order parameter n=3, mean ± SD for clinical data n=15)

The results shown in Table 3 indicate that mobility increase by SLG can be attributed to the phase one structural changes in the lipid layers and SLS might cause further distraction of the structures of lipid layers. To better understand the phenomena, further investigation on the molecular interactions and structural effects between surfactant and lipid layers are needed.

4.4.4 Correlation between clinical data and *in vitro* irritation test data

In order to examine the correlation amongst order parameter, clinical data and *in vitro* irritation test data, the data available in Chapter 3 was populated into the Table 4-3 and the correlation coefficient (r^2) were calculated amongst each observable attribute (Table 4-4).

Table 4-3. *in vivo* human patch test results and *in vitro* test results

		Human Patch Test		*in vitro* tests (cytotoxicity, etc)				
		Visual score (mean ± SD)	TEWL g $H_2O/m^2/h$	MTT_{50} (ppm)	NR_{50} (ppm)	SIRC LC_{50} (ppm)	Skintex®	Eyetex®
Control (Water)		0.00 ± 0.0	5.00 ± 1.1					
SLG	(1.0%)	0.04 ± 0.1	6.70 ± 3.5	904	60.2	2540	0	105
SL	(1.0%)	0.08 ± 0.2	7.10 ± 3.9	263	9.9	619		
SLES	(1.0%)	0.42 ± 0.3	7.60 ± 2.8	114	3.4	15.3		
SLEC	(1.0%)	0.08 ± 0.2	7.40 ± 2.9		38.4			
SLS	(1.0%)	0.79 ± 0.3	13.60 ± 3.1	80	4.1	17.5	5	38.9

(Note: mean ± SD for patch test data n=15)

Table 4-4. Correlation coefficient (r^2) of *in vivo* human patch test results and *in vitro* test results

	Order parameter	Visual score	TEWL (g $H_2O/m^2/h$)	MTT_{50} (ppm)	NR_{50} (ppm)	SIRC LC_{50} (ppm)
Order parameter		**0.69**	***0.80***	**0.62**	**0.49**	**0.61**
Visual score			***0.87***	0.50	0.33	0.50
TEWL				0.29	0.18	0.28

The correlation coefficient (r^2) between visual score and TEWL is 0.87, which agrees that TEWL represents skin barrier function and disorder / destruction of intercellular lipid layers is the critical process to induce skin irritation. Order parameter correlates to TEWL with r^2=0.80 and it reflects disorder / destruction of intercellular lipid layers.

The correlation of order parameter with *in vitro* testing such as MTT_{50}, NR_{50} and SIRC LC_{50} were lower than that with TEWL. It can be explained as follows; surfactants contact to the cell membrane and should induce structural alteration in it first. Then they migrate into the viable cells and their toxic reaction starts. Accordingly, the obtained results as MTT_{50}, NR_{50} and SIRC LC_{50} represent two-step reaction; the structural alteration/ destruction of cell membrane and the toxic reaction in the cell.

The low correlation between TEWL, representing barrier function loss due to the alteration/ destruction in the intercellular lipid structure, and *in vitro* testing such as MTT_{50}, NR_{50} and SIRC LC_{50} can be also explained in the same manner. In other words, we have to understand that MTT_{50}, NR_{50} and SIRC LC_{50} are not the index of the interaction to the cell membrane but of the cytotoxicity of surfactants.

4.5 Conclusions

Based upon the results of anionic surfactant effect on the EPR spectra of spin-labeled human stratum corneum, following points are to be summarized.

(1) When 5-DSA was used as a lipid spin label with human (cadaver) stratum corneum, it was partitioned between a non-polar (lipophilic) environment and a polar (hydrophilic) environment.

(2) EPR parameters (e.g. order parameter S) afford an accurate description on the structural alterations in the intercellular lipid bilayers focusing on the fluidity.

(3) SLS showed a significant change in the EPR spectra of spin-labeled human stratum corneum, which suggests that SLS induces conformational changes of spin labels located in the lipid-protein matrix of the stratum corneum. The study of dose dependent order parameter of SLS showed monomeric species probably plays a major role in the interactions between the skin and surfactant.

(4) SLG showing a minimal change in the EPR spectra of spin-labeled human stratum, is the mildest anionic surfactant inducing less

fluidization in intercellular lipid structure amongst several kinds of anionic surfactants; (a) Sodium Lauroyl Glutamate: **SLG**, (b) Sodium Lauryl Sulfate: **SLS**, (c) Sodium Lauryl POE(3) Ether Sulfate: **SLES,** (d) Sodium Laurate: **SL**, and (e) Sodium Lauryl POE(3) Ether Carboxylate: **SLEC**, which are widely used in consumer products on the market.

(5) **SLS** produces disorder (fluidity) of the intercellular lipids at low concentration such as 0.5%wt presumably due to the solubilization of the intercellular lipids by SLS molecules, however, EPR spectral data suggested that the **addition of 1.0%wt SLG** to SLS solutions (0.25 ~ 1.00%wt) inhibits the intercellular lipid fluidization by SLS, which indicates the anti-irritation potential of SLG as discussed in the chapter 2 and chapter 3.

(6) A good correlation, between the order parameters and human clinical data (visual grading and TEWL values), was observed. However, we observed a poor correlation between order parameters and cytotoxicity data (MTT_{50}, NR_{50} and SIRC LC_{50}) because the order parameters are the index only representing the interaction of anionic surfactant to the cellular membrane (lipid bilayer structure) and not representing the cytotoxic reaction of anionic surfactants.

EPR spectroscopic investigation employing nitroxide spin labeling is a robust method in the study of the structure of the intercellular lipid bilayers in human stratum corneum and the order parameters obtained from the EPR spectra can be used as a valuable index predicting the irritation potential of anionic surfactants.

The detection of the perturbation in the intercellular lipid bilayers induced by anionic surfactant may depend on the experimental conditions used for EPR measurement. Many inherent uncertainties of irritation mechanisms might be clearly explained in the future by focusing on the following points;

(1) Water content in the stratum corneum
(2) CMC (critical micelle concentration) of surfactants
(3) Different kinds of surfactants
(4) Different kinds of spin probes,
(5) Different kinds of skin/ stratum corneum systems by developing ex-vivo technique (stripped stratum corneum) or so.

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主論文（5）

Mizushima J, Kawasaki Y, Ino M, Sakamoto K, Kawashima M, Cooke R, and Maibach HI (2001), 電子常磁性共鳴法を用いた界面活性剤の角層に及ぼす影響の評価　水分量の観点から, 香粧会誌, *25, p.130-135*

主論文（7）

Mizushima J, Kawasaki Y, Sakamoto K, Kawashima M, Cooke R, and Maibach HI (2000) Electron Paramagnetic Resonance: A New Technique in Skin Research, Skin Research and Technology, 6, p.100-107

[Synopsis]

An electron paramagnetic resonance (EPR; which is also called as “electron spin resonance (ESR)”) spectroscopy employing a nitroxide spin probe, has been used for characterizing the intercellular lipid bilayer structure in human stratum corneum. Order parameters obtained from EPR spectra are the good index for detecting its fluidity. When stratum corneum was treated with sodium lauryl sulfate (SLS), order parameter S decreased with the increase of SLS concentration. However, the order parameter S increased and became higher value than that of the control followed by drying conditions.

To investigate the observation of high order parameter at a complete dry condition after SLS treatment, we examined the correlation between the order parameters from EPR spectra and the water content of the human stratum corneum treated with different concentration of SLS under the both wet and dry conditions.

Under the wet condition with SLS treatment, the order parameter decreased and the weight of the stratum corneum increased with the increase of SLS concentration. Contrarily, the order parameter S increased and the weight of the stratum corneum decreased with the increase of SLS concentration when dried.

The order parameter of the stratum corneum treated with higher concentration of SLS was smaller than those of the treated with lower concentration of SLS for the same water content in the stratum corneum.

The dose response profile on the treated SLS concentration clearly showed a bending point at around 0.25% at 37^{o}C, which corresponds to the CMC (critical micelle concentration) of SLS 8.6mM at 40^{o}C. For SLG inducing less fluidization in intercellular lipid structure, the dose response was much smaller that that of SLS and the bending point was observed at around 0.5% at 37^{o}C, which also corresponds to the CMC of SLG 10mM at 40^{o}C.

These results suggest that concentration of surfactant monomer is the key factor inducing fluidization of intercellular lipid bilayers. As for the methodology for measuring EPR spectra on the stratum corneum, wet conditions are desirable, in other words, control of water content in the stratum corneum is required.

5.1 Introduction

We (Kawasaki, et. al., 1997; Kawasaki, et. al., 1999, Lee, et. al., 1994) have reported the effect of anionic surfactants (SLS; sodium lauryl sulfate, SLG; sodium lauroyl glutamate) on the intercellular lipid bilayers in human stratum corneum using EPR spin labeling technique. It was shown that SLS induced “fluidization” of the lipid bilayers, while contrarily SLG did not. TEWL (trans-epidermal water loss) measurement in clinical studies is well known method evaluating skin barrier function and it was confirmed that the order parameters obtained from EPR spectra on cadaver stratum corneum correlated well to the visual scores and TEWL after human patch testing.

Other than using cadaver skin, guinea pig epidermis (Kitagawa, et. al., 1998) and dipalmitoyl phophatidyl choline liposome (Gay, et. al., 1989) were used as a model for the study in order to discuss the lipid structure (both inter- and intra-molecular) and action of chemicals with these lipids.

However, Mizushima, et. al. (2000a) established a stripping method to obtain the stratum corneum for EPR spectral measurement and also validated (2000b) the stripping method for EPR measurement discussing the correlation between the data (visual score and TEWL, stratum corneum hydration and chromametry) based on cadaver stratum corneum and stripped stratum corneum on patch tested sites. The order parameters obtained from the spectra of the stripped stratum corneum correlated to those of cadaver stratum corneum and TEWL values, which means that the stripping method is capable of evaluating the fluidity of lipid bilayers in stratum corneum and correlates with the above bioengineering parameters.

Accordingly, we thought that order parameters from EPR spectra are the valuable index representing the fluidity of intercellular lipid bilayer structure, which correlate well to the skin barrier function.

However, the order parameters on the SLS treated stratum corneum were low just after treatment, although those values increased when order parameters were followed over time under dry condition and finally became higher than that of the control after 24 hours in the report by Kawasaki, et. al. (1997). And a question if order parameters represent fluidity of the lipid bilayers was raised.

Spin probes are specifically incorporated into the lipid. (Alonso, et. al., 2000; Kitagawa, et. al, 2001) and reflect the order parameters of EPR spectra as the properties of a different lipid bilayers region are changed by SLS. Following factors may affect onto the order parameters in their respective EPR spectra; (1) loss of intercellular lipid components, (2) migration of SLS into the lipid layers (surfactant

monomers or micelles), (3) loss of substances responsible for the moisture-holding capacity (water content in the stratum corneum), (4) experimental conditions such as temperature. (Alonso, et. al., 1995; Alonso, et. al., 1996)

In this study, we examined the correlation between the order parameters from EPR spectra and the water content of the human stratum corneum treated with different concentration of SLS under the both wet and dry conditions to investigating observing the high order parameters at a complete dry condition after SLS treatment. Also EPR measurement was conducted to discuss the concentration effect of SLS & SLG onto the order parameters.

5.2 Material and Methods

5.2.1 Materials

5-doxyl stearic acid (5-DSA; FW=384.6) purchased from Aldrich was used as a spin-labeling reagent without further purification. Sodium lauryl sulfate (SLS; Sigma Chemical Co., USA ; purity 99%up), sodium lauroyl glutamate (SLG; Ajinomoto Co., Inc., Japan ; purity >98%) were used as anionic surfactants without further purification. All chemical structures are described in Fig 5-1 and Fig 5-2.

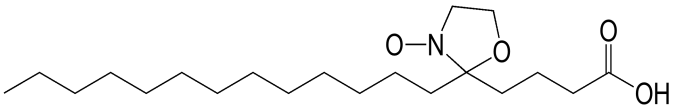


Fig 5-1. Chemical structure of spin label 5-DSA

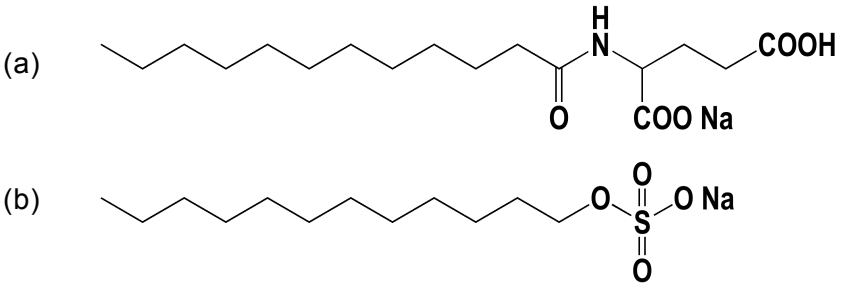


Fig 5-2. Chemical structure of anionic surfactants; (a) Sodium Lauroyl Glutamate: **SLG**, (b) Sodium Lauryl Sulfate: **SLS**

5.2.2 Preparation of human stratum corneum from cadaver skin

Human abdominal skin was obtained from fresh cadaver skin with a dermatome (Northern California Transplant Bank; San Rafael, CA, USA). The epidermis was separated from the dermis by immersing the dermatomed skin in 60^{o}C water bath set for 2 minutes followed by mechanical removal. Then, the epidermis was placed stratum corneum side up, on filter paper and floated on 0.5% trypsin (type II; Sigma), in a Tris-HCl buffer solution pH 7.4, for 2 hours at 37^{o}C. After incubation any softened epidermis was removed by mild agitation of the stratum corneum sheets. Thereafter, the stratum corneum was dried and stored in a

desiccator at -70^{o}C for 3~4 days. Details of the method are described in Quan and Maibach. (1994)

5.2.3 Spin-labeling procedures and treatment with anionic surfactants

5-DSA was used as a stearic acid spin labeling agent. Some slices of dry stratum corneum sheets (approx. 0.5 cm^2, approx.0.5 cm x approx. 1 cm) were incubated in Tris-HCl buffer solution (pH 7.4) dissolving spin label at 10 mg/L ($2.6x10^{-5}$ M) for 0.5 hour at 37^{o}C. After rinsing spin-labeled (5-DSA) stratum corneum with purified water, immersed it in the surfactant solutions prepared at designated concentration, and incubated at 37^{o}C for 1 hour. After rinsing with purified water and removing excess water by filter paper, set the stratum corneum sheet on the quartz cell. Then, immediately measured the weight of the stratum corneum and EPR spectrum under a wet condition. After EPR measurement under a wet condition, the sample sheet was maintained at room temperature for 1 hour to dry, and measured the weight of the dried stratum corneum and its EPR spectrum. The spin-labeled (5-DSA) stratum corneum treated with purified water instead of surfactant solution was used as a control. The weight of stratum corneum was measured before spin labeling and set as “100”.

To discuss the effect of surfactant concentration and incubation time onto order parameter, multiple slices of spin labeled (5-DSA) stratum corneum were immersed in 0.063 ~ 5.0 %wt solutions and incubated at 37^{o}C for designated hours. After rinsing with purified water and removing excess water with filter paper, set the stratum corneum sheet on the quartz cell. Then, immediately measured EPR spectrum under a wet condition.

5.2.4 EPR spectral measurement and calculation of order parameter

ESR spectrum measurements were carried out by an ER200 Series ESR spectrometer (Brucker Inc., MA, USA) with microwave power out of 25mW and spectrum data were collected by an IBM-PC system with PC/FORTH (ver. 3.2 Laboratory Microsystems, CA, USA). The hyperfine splittings of labeled skin samples were determined with 100 gauss scan width, 4 x 10^2 receiver gain, 2 gauss modulation amplitude and 0.1 second time constant. Each sample was scanned

several times and ESR parameters from each spectrum were averaged to give a single estimate for the example.

Triplet signals, which are sharp as shown in Fig. 5-3, can be observed when the spin probe (doxyl group) moves freely as a solution because there is nothing to inhibit probe mobility. However, the probe cannot move freely due to the rigidity of lipid structure and its EPR spectrum represents the broaden profile such as Fig. 5-4 (a) when the spin probe is incorporated in the highly oriented intercellular lipid structure in the normal skin. When the stratum corneum is treated with surfactant solution, the original structure is destroyed to some extent, the EPR spectrum shows intermediate broadening shape like Fig. 5-4 (b). Accordingly, EPR spectral profile represents the rigidity of the circumstance, which the spin probe is in. To express the rigidity quantitatively, order parameters were calculated according to Griffith OH, et al. (1976), Hubbell WL, et al. (1971), and Marsh D (1981):

$$S = (A_{||} - A_{\perp}) / [A_{ZZ} - 1/2 (A_{XX} + A_{YY})](a_0/a_0')$$

where $2A_{||}$ is identified with the outer maximum hyperfine splitting A_{max}, and $A_{\perp}$ is obtained from the inner minimum hyperfine splitting A_{min} (Fig. 5-4).

a_0 is the isotropic hyperfine splitting constant for nitroxide molecules in the crystal state.

$$a_0 = (A_{XX} + A_{YY} + A_{ZZ}) / 3$$

The values used to describe the rapid anisotropic motion of membrane-incorporated probes of the fatty acid type are:

$$(A_{XX}, A_{YY}, A_{ZZ}) = (6.1, 6.1, 32.4) \text{ Gauss}$$

Similarly the isotropic hyperfine coupling constant for the spin label in the membrane (a_0') is given by:

$a_0' = (A_{||} + 2\,A_{\perp}) / 3$

a_0' values are sensitive to the polarity in the environment of the spin labels since increases in a_0' value reflect an increase in the polarity of the medium.

The order parameter provides a measure of the flexibility of the spin labels in the membrane. It follows that S = 1 for highly oriented (rigid) states and S = 0 for completely isotropic motion (liquid). Increases of order parameter reflect decreases in the segmental flexibility of the spin label, and conversely decreases in the order parameter S reflect increases in the flexibility (Curtain CC, et al. (1984)).

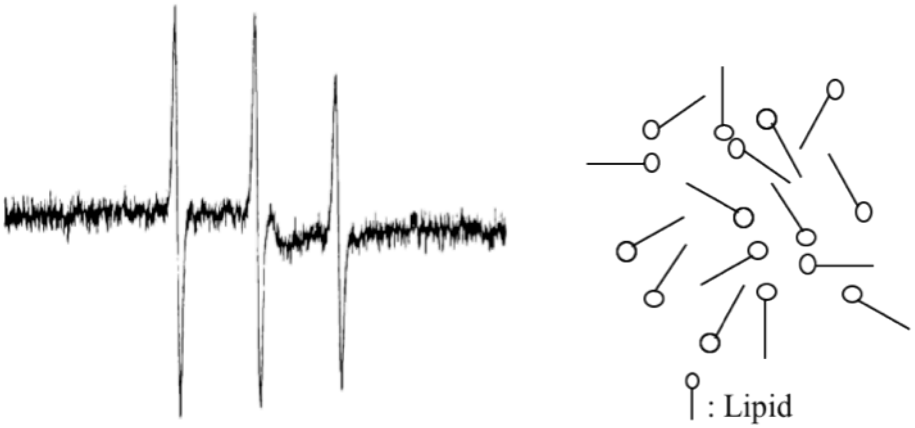


Fig. 5-3. EPR spectrum of 5-DSA in aqueous solution

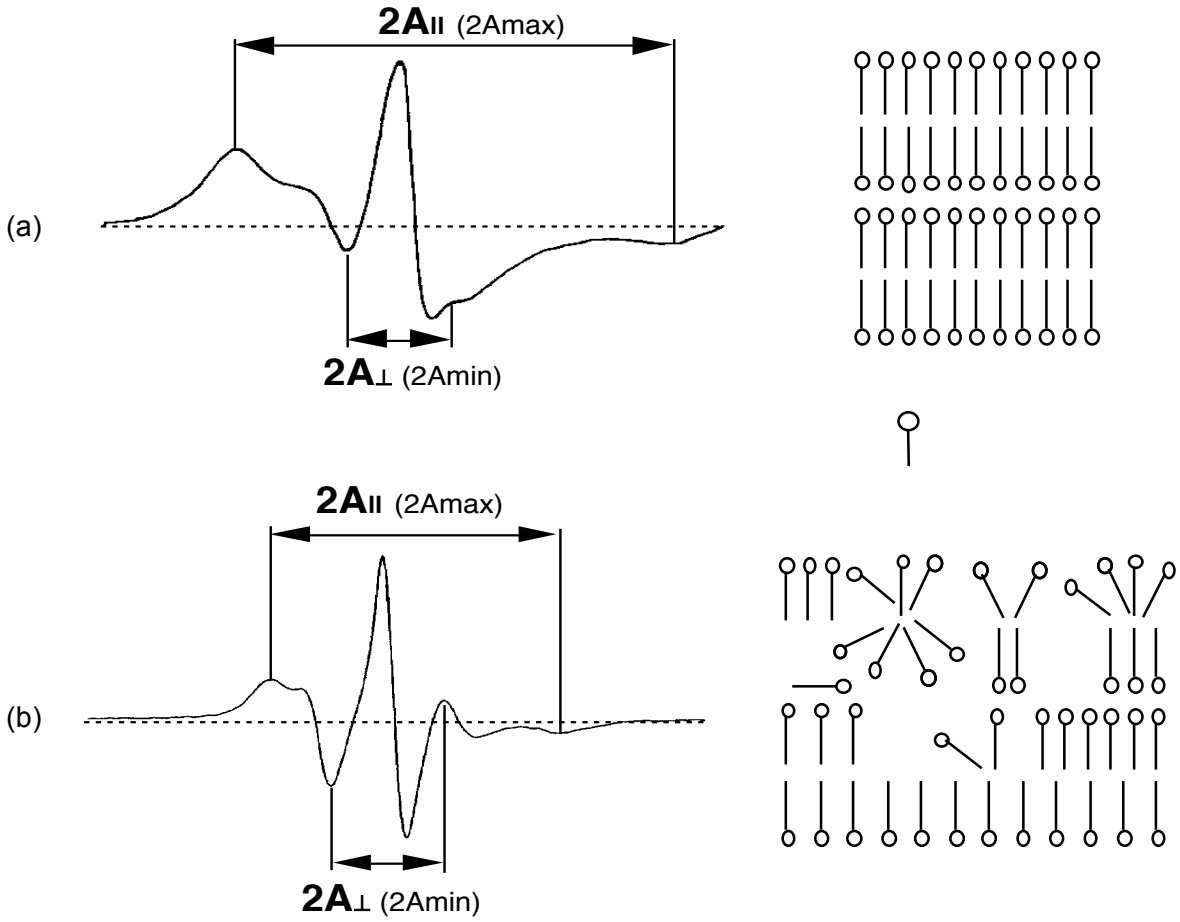


Fig. 5-4. EPR spectrum of 5-DSA labeled stratum corneum from cadaver,
(a) non treatment (control), (b) treated with surfactant solution

5.3 Results & Discussion

5.3.1 Dependence of SLS and SLG concentration and exposing time to surfactant solution onto order parameter

1 wt% SLS induced fluidization as shown in Fig. 5-5. We also examined the following point; how much alteration is induced depending upon SLS concentration and exposing time to SLS.

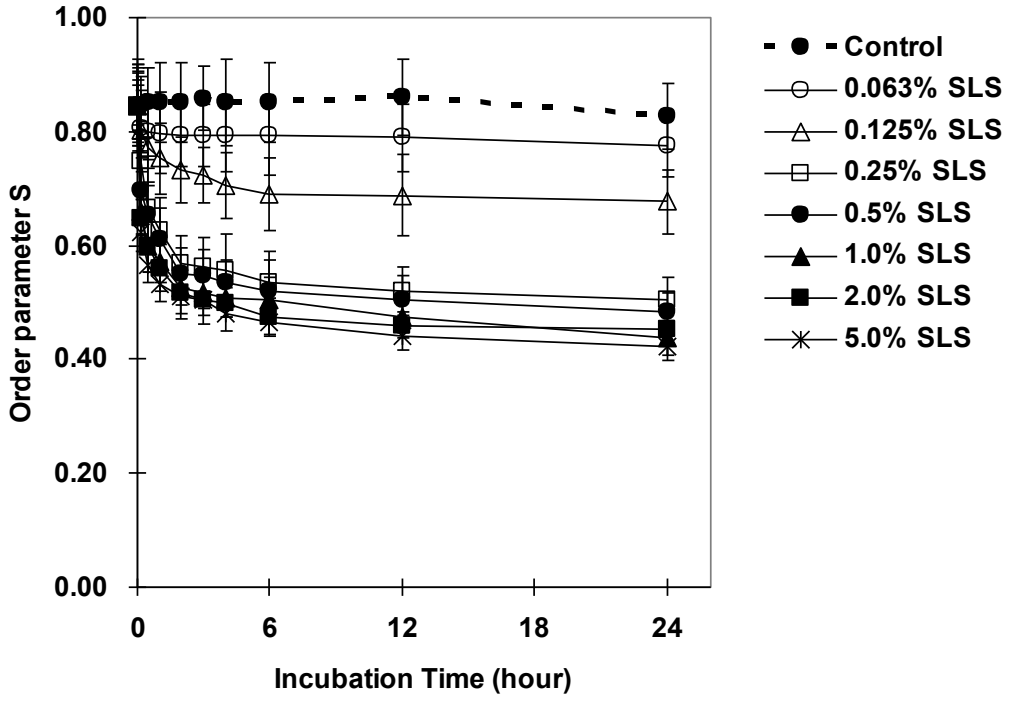


Fig 5-5. Time dependence of order parameters of 5-DSA labeled stratum corneum in SLS solution at various concentrations (Error bars: Mean ± SD, n=5)

Fig. 5-5 shows the incubation time dependence of an EPR spectrum with different SLS concentrations. With increasing incubation time, the order parameter was decreased. However, each profile of incubation time dependence had a plateau at the region of 6 hours and thereafter. The skin lipid alteration induced by SLS was typically completed within 6 hours at a given concentration. However, each alteration level in intercellular lipids depended on its SLS concentration.

As the concentration of SLS increases, the order parameter at 24-hour incubation decreases drastically in the range of 0 to 0.25 %wt of SLS. (Fig. 5-6) However, the order parameters calculated from the stratum corneum treated with SLS at more than 0.25%wt had no significant difference, showing around 0.45 ~0.50. This critical point around 0.25%wt (8.7mM) corresponds to the CMC (Critical Micelle Concentration) for SLS at 37°C. Rosen MJ (1978) published the data that the CMC of SLS is 8.6 mM at 40°C and 8.2 mM at 25°C.

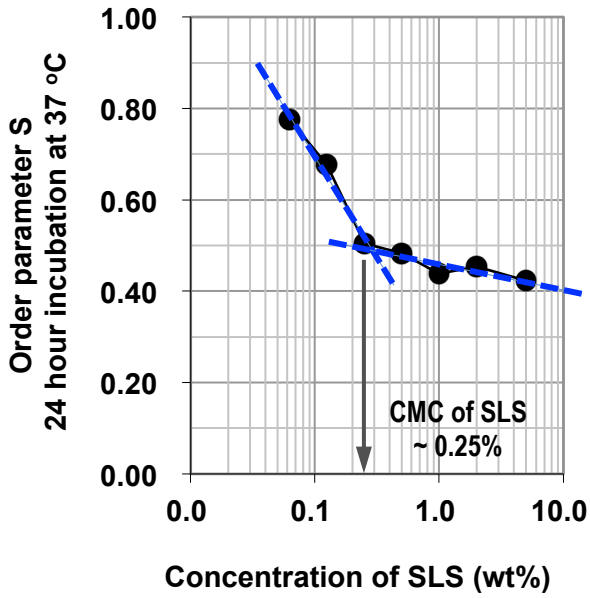


Fig.5-6. Correlation between SLS concentration and order parameters of 5-DSA labeled cadaver stratum corneum incubated at 37 °C for 24 hours

This behavior is consistent with the following general concerns among experts: monomeric surfactants can penetrate the skin. Monomeric molecules are also the species that are initially adsorbed into the various surfaces within the skin; we cannot ignore secondary bonding due to hydrophobic effects. Thus, the concentration of monomeric species probably plays a major role in the interactions between the skin and surfactant. (Rieger MM (1995)).

However, the correlation between SLG concentration and order parameters of 5-DSA labeled stratum corneum incubated at 37°C for 24 hours was different from that of SLS as shown in Fig. 5-7, which shows much gentler slope against the treating

concentration than SLS. Sakamoto (1995) reported that the CMC of SLG (MW=351) is ~ 10mM at 40^{o}C, which corresponds to ~0.35%wt.

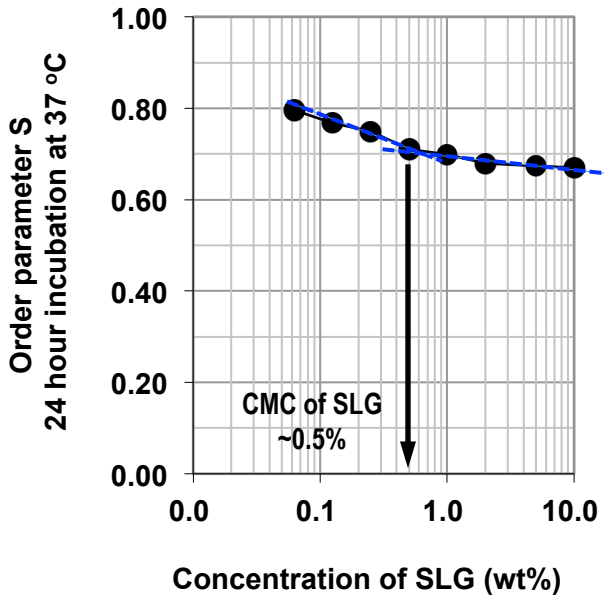


Fig.5-7. Correlation between SLG concentration and order parameters of 5-DSA labeled cadaver stratum corneum incubated at 37 ^{o}C for 24 hours

5.3.2 Correlation between order parameter S and water content in the stratum corneum Treated with SLS

The order parameters on the SLS treated stratum corneum and its weight measured under wet and dry conditions were summarized in Table 5-1 and Fig. 5-8.

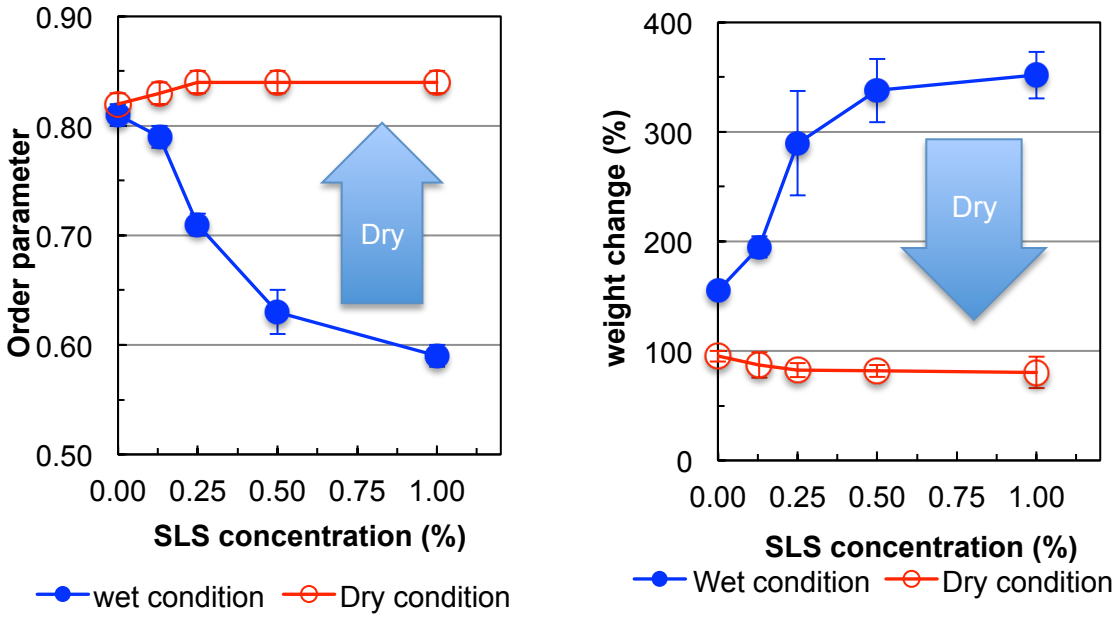
Under wet condition, the order parameters decreased and the weight of the sample stratum corneum increased as the SLS treating concentration increased, which suggested that SLS pulled water into the stratum corneum and induced fluidization of intercellular lipid layers. When the stratum corneum was treated with water (no SLS), water content increased to ~50% from the dried stratum corneum and the order parameter was “0.81”. When the stratum corneum was treated with 0.125% SLS, it hold 100% weight of water and its order parameter slightly decreased to “0.79”, but there was no statistical significance from the control ($p>0.05$). When the stratum corneum was treated with SLS at 0.25% or more, the water content of the samples and their order parameters drastically increased up to ~250% and decreased to ~0.6, respectively.

After 1 hour dry for each sample, the order parameter of the sample treated with water was “0.82” that was slightly higher than that of wet condition and its dry weight was 95.2 ± 4.9%, however, there was no significant difference ($p>0.05$) in the order parameters and the weight between wet and dry conditions, which suggested that the stratum corneum was just swollen by water but the process was reversible, in other words, water does not alter the structure of intercellular lipid and does not remove any components such as lipids, proteins, and the substances responsible for the water holding capacity. As for the stratum corneum treated with 0.125% SLS, the weight was slightly lighter than the control and the order parameter was slightly higher than the control. When the stratum corneum treated with SLS at 0.25% or more, the weight got down to 80 ~ 83% and the order parameters went up to “0.84”, that was constant. The SLS treatment at 0.25% or more, which is CMC and higher concentration of SLS at 40^{o}C, reduced 17 ~ 20% water content in the stratum corneum from the control under the dry condition. It can be concluded that SLS treatment at 0.25% or more induced the depletion or change of the components such as fatty acids, cholesterol derivatives, protein and amino acids as natural moisturizing factors, responsible for the water holding capacity, and irreversibly destroyed the structure of intercellular lipid.

Table 5-1. The order parameters on SLS treated stratum corneum (n=3) and its weight, measured under wet and dry conditions Error bars: Mean ± SD, n=3

[Note] The weight of the stratum corneum sheet is defined as 100 (%) just before labeling with 5-DSA aqueous solutions

SLS concentration (%wt)	Order parameter		% of weight increase	
	Wet condition	Dry condition	Wet condition	Dry condition
0.00	0.81 ± 0.01	0.82 ± 0.01	155.2 ± 4.3	95.2 ± 4.9
0.125	0.79 ± 0.01	0.83 ± 0.01	195.0 ± 9.8	87.1 ± 11.5
0.25	0.71 ± 0.01	0.84 ± 0.01	289.5 ± 47.7	82.5 ± 6.4
0.50	0.63 ± 0.02	0.84 ± 0.01	337.7 ± 28.8	81.6 ± 5.3
1.00	0.59 ± 0.01	0.84 ± 0.01	351.9 ± 21.3	80.3 ± 14.2



(a) (b)

Fig. 5-8. The order parameters on SLS treated stratum corneum (n=3) and its weight, measured under wet and dry conditions Error bars: Mean ± SD, n=3

[Note] The weight of the stratum corneum sheet is defined as 100 (%) just before labeling with 5-DSA aqueous solutions

5.3.3 Effect of water content in the stratum corneum treated with SLS onto the order parameters; using a sealed EPR cell.

In order to define the effect of water content in the stratum corneum treated with SLS onto the order parameters, we designed a sealed EPR cell (Fig.5-9), which covers the stratum corneum with a glass and sealed with vacuum grease, and conducted following experiments.

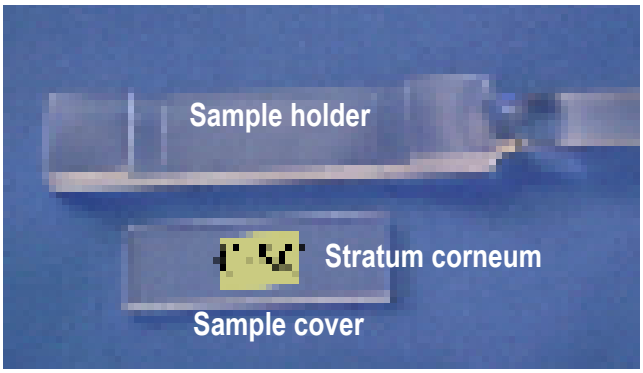


Fig.5-9. Sealed EPR cell

[Note] After setting a piece of stratum corneum on the cover, turn it over onto the holder to make the sample inside the cell

Several pieces of stratum corneum (approx. 0.5 cm^2, approx. 0.5 cm x approx. 1 cm) were spin labeled with 5-DSA in accordance with the procedure described in the previous section. After rinsing spin-labeled (5-DSA) stratum corneum with purified water and removing excess water by filter paper, they were dried in a vacuum drier (ULVAC SINKU KIKO MINIVAC PD-102; Yamato Scientific Co., Ltd., Tokyo, Japan) for 24 hours. Then, they were immersed it in the SLS solutions prepared at 0.1%wt, 0.5%wt and 1.0%wt, and incubated at 37^oC for 1 hour. Rinsed with 10 mL purified water 5 times per sample and measured the weight (Y) before and after EPR measurement. After EPR measurement, the sealed EPR cell was opened and the sample was dried for a while. Then, sealed it with vacuum grease again and measured EPR spectrum and the stratum corneum weight (Y). This procedure was repeated until the weight became constant. When the weight became constant, the sample stratum corneum was dried in the vacuum drier at ambient temperature for 12 hours and measured the weight (X) and EPR

spectrum. Water content at each EPR measurement was calculated by the following formula.

Water content = (Y – X) / X x 100 (%)

The obtained results were summarized in the Fig-5-10. As the treating concentration of SLS increased, required amount of water to saturate in the stratum corneum increased the order parameter decreased and the water content in the stratum corneum increased; ~150% for no SLS, ~325% for 0.1%wt SLS, ~410% for 0.5%wt SLS and ~460% for 1.0%wt SLS. The order parameter at each saturated point showed the SLS dose response; “0.8” at ~150% for no SLS, “0.68” at ~325% for 0.1%wt SLS, “0.56” at ~410% for 0.5%wt SLS and “0.51” at ~460% for 1.0%wt SLS.

In the drying process, the order parameter was consistently at around “0.8” for the control (no SLS treatment), although the water content in stratum corneum varied from 150% to zero. As for 0.1%wt SLS treatment, the order parameter maintained at around “0.7” while the water content is between ~300% and 100% and straightly went back to “0.8” as it was dried. However, for 0.5%wt and 1.0%wt SLS treatment, the order parameters did not back to around “0.8” of the control and showed higher values.

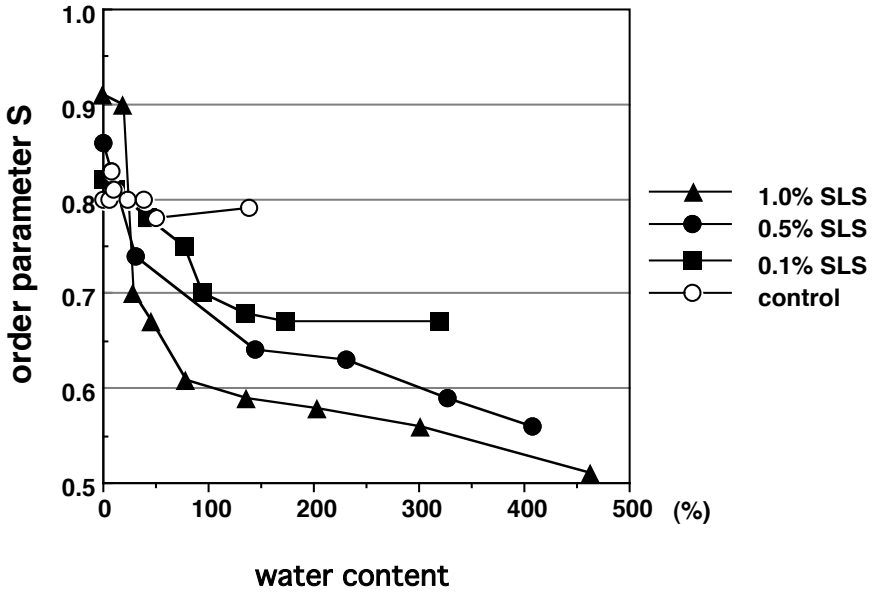


Fig. 5-10. The relationship between the water contents and the order parameter treated with SLS at three concentrations (0.1%wt, 0.5%wt and 1.0%wt)

These results discussed above, suggest the following; as far as the stratum corneum is treated with SLS at the concentration under its CMC (0.25% at 40^{o}C; Rosen MJ (1978)), the induced fluidization of intercellular lipid layers is a reversible alteration. Conversely, irreversible alteration happens in the stratum corneum when treated with SLS at the concentration over the CMC, which is not a simple fluidization of intercellular lipid layers but destruction of the structure by removing and/ or changing the components responsible for water holding capacity.

Two phases can be hypothesized in the increase of fluidity in lipid structures. (1) The first phase is in the effect of surfactants incorporated into the lamellar structures. If the surfactant interferes with or decreases lateral interactions between lipids, mobility increases in a way similar to the phase conversion from gel to liquid crystal in the lamellar layers, which is reversible. (2) The second phase is the destruction of the lamellar structure by means of micellization or solubilization of the lipid layer by the surfactant. In this case, lipids no longer have dimensional restrictions and gain much higher mobility. The surfactant might have changed the water-holding capacity of the stratum corneum, and the water content may change the fluidity of stratum corneum lipids.

5.4 Conclusions

Following points are to be summarized as conclusions.

(1) Three independent experiments suggest that surfactant monomer plays a critical factor inducing fluidization of intercellular lipid layers

   a. The order parameter on the stratum corneum treated with SLS drastically decreased until reaching at the 0.25%wt 37^{o}C, which is nearly equal to the published CMC of SLS 8.2mM at 40^{o}C (Rosen MJ, 1978). Beyond CMC, the order parameter did not change much.

   b. The order parameter on the stratum corneum treated with SLG gently decreased until reaching at the 0.5%wt 37^{o}C, which is close to the published CMC of SLG 10mM at 40^{o}C (Sakamoto K, 1995). Beyond CMC, the order parameter did not change much.

(2) As far as the stratum corneum is treated with SLS at the concentration under its CMC (0.25% at 40^{o}C; Rosen MJ (1978)), the induced fluidization of the intercellular lipid layers is a reversible alteration. Conversely, irreversible alteration happens in the stratum corneum equal to/ treated with SLS at the concentration over the CMC, which are not a simple fluidization of the intercellular lipid layers but destruction of the structure by removing and/ or changing the components responsible for the water holding capacity.

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6. Conclusion

We selected several kinds of anionic surfactants and examined their irritation potential on the human skin by occlusive patch testing, Draize tests and several kinds of alternative methods (cytotoxicity tests) using human keratinocyte, which we developed prior to commercially available test kits. Also we conducted the tests employing established techniques in 1990s; SKINTEX® and SIRC colony forming assay. Throughout the various irritation tests conducted, the results demonstrated that sodium lauroyl glutamate (SLG) shows extremely low irritation potential on human amongst anionic surfactants, which are extensively used on the market.

The stratum corneum is the first contact substrate for surfactants to induce irritation reaction and the intercellular lipid bilayers intensively regulate the barrier function of the human skin. Accordingly, we developed the electron paramagnetic resonance (EPR) spectroscopic method on employing nitroxide spin labeling in order to investigate the mechanism of skin irritation focusing on the structural alteration/ fluidization of intercellular lipid bilayers in human stratum corneum.

Sodium lauryl sulfate (SLS) showed a significant change in the EPR spectra of spin-labeled human stratum corneum, which suggests that SLS induces conformational changes of spin labels located in lipid-protein matrix of stratum corneum, in other words, induces “fluidization” of the intercellular lipid bilayers. However, SLG showing a minimal change in the EPR spectra of spin-labeled human stratum corneum is the mildest anionic surfactant inducing less fluidization in intercellular lipid structure amongst several kinds of anionic surfactants tested, which may be due to the “peptide (-CO-NH-)” bond in the SLG structure. When SLG added to SLS solutions, EPR spectral data suggested that SLG inhibits the intercellular lipid fluidization by SLS and works as an anti-irritant to SLS.

We confirmed a good correlation between the order parameters and human clinical data (visual grading and TEWL values). However, a poor correlation between order parameters and cytotoxicity data (MTT_{50}, NR_{50} and SIRC LC_{50}) was observed because order parameters are the index representing the interaction of surfactant to the cellular membrane (lipid bilayer structure) and not primarily representing the cytotoxic reaction of surfactants.

Based on the results of the order parameters of the stratum corneum treated with different concentrations of SLS, it was demonstrated that the SLS monomer plays a critical role in inducing fluidization of intercellular lipid layers. An irreversible alteration happens in the stratum corneum treated with SLS at the concentration equal to/over the CMC (0.25%wt at 37^{o}C), which are not a simple fluidization of the

intercellular lipid layers but destruction of the structure by removing and/or changing the components responsible for water holding capacity. The dose response profile of the order parameter on SLG treated stratum corneum was completely different from that of SLS, which indicates that SLG has a different type of interaction with the intercellular lipid bilayers.

EPR spectroscopic investigation employing nitroxide spin labeling is a robust method in the study of the structure of intercellular lipid bilayers in human stratum corneum, and the order parameters obtained from the EPR spectra can be used as a valuable index predicting the irritation potential of anionic surfactants.

After establishing the EPR spectroscopic method on the cadaver stratum corneum, a lot of advancements have been made by Mizushima J, et al. (2000a & 2000b), Nakagawa N, et al. (2006), Yagi E, et al. (2006), Yagi E, et al. (2008), Yagi E, et al. (2010) until now.

Mizushima J, et al. (2000a) developed a “stripping method” to obtain the stratum corneum for EPR measurement. To assess the “stripping method” comparing to the standard methods (Kawasaki Y, et. al, 1997 & 1999), they conducted human patch testing (visual score and transepidermal water loss (TEWL)), stratum corneum hydration and chromametry, and EPR spectra measurement were conducted with the cadaver stratum corneum respectively treated by three types of surfactants (sodium lauryl sulfate (SLS; anionic), stearyltrimethyl-ammonium chloride (MSAC; cationic) and N-[3-alkyl (12.14) oxy-2-hydroxypropyl]-L-arginine hydrochloride (HEA; amphoteric by Ajinomoto)) and the stripped off stratum corneum from the patch applied sites. 5-Doxyl stearic acid was used as a spin probe. The order parameters obtained from the stripped stratum corneum correlated with those of cadaver stratum corneum and TEWL values. The results suggest that “stripping method” is capable of evaluating the fluidity of the intercellular lipid layers.

Nakagawa N., et. al. (2006) investigated the chain ordering of the lipid bilayers in the stratum corneum using EPR spin probe (5-DSA) method in conjunction with slow-tumbling simulation and demonstrated that EPR slow-tumbling computer simulation can differentiate the subtle spectral changes and provide more detail information on the lipid bilayers structure.

Yagi E, et. al. (2006) reported that structural ordering of the outermost stratum corneum layer is less tight, whereas the structure of the inner layers becomes more rigid by means of EPR slow-tumbling simulation in conjunction with spin probe (5-DSA) studies. In 2008, Yagi E, et. al. optimized the preparation method of *ex vivo* stratum corneum specimen and established an accurate *ex vivo* EPR analysis of stratum

corneum lipid ordering. He also demonstrated that polyoxyethylene/ polyoxypropylene dimethyl ether (EPDME) is effective for skin moisturization by means of EPR studies of ex vivo specimens of the stratum corneum by “stripping method”. (Yagi E, et al., 2010)

The toxic manifestations of topically applied substances may induce immediate phenomena such as corrosion or primary irritation, and delayed phenomena such as sensitization, in addition to the phenomena that require an additional vector such as phototoxicity, and systemic phenomena. Such reactions cannot occur unless the toxic agent reaches a viable part of the skin by penetrating through the stratum corneum with accompanying intercellular lipid structure disruption. If the toxicant can be stored in or absorbed by a skin layer without any alteration in lipid structure, it may not reach the viable tissues at all or may be released relatively slowly, thus effectively prolonging the symptoms.

EPR (Electron paramagnetic resonance) spin probe method has been improved/ advanced for the last decade and now becomes a robust method for monitoring the structural change in intercellular lipids induced by topically applied not only surfactants also any chemicals. We have shown that order parameter is an easy to use and quantifiable index for predicting irritation reactions in the skin. It may also aid in investigating the irritation potential of general chemicals, effects of topical penetration enhancers, drug delivery systems and skin diseases such as xerosis and atopic dermatitis.

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