EFFECTS OF MOLECULAR WEIGHT OF HYALURONIC ACID ON BIOLOGICAL ACTIVITY AND SKIN PENETRATION

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ABSTRACT

Hyaluronic Acid (HA) is a natural glycosaminoglycan with a chemical structure composed of D-glucuronic acid and N-acetyl glucosamine linked by β (1, 4) and β (1, 3) glycoside bonds. HA has been used as a biomaterial for cosmetic formulation, and for pharmaceutical, biotechnical and medical usage. HA commonly has a high molecular weight and hydrophilicity making it difficult to deliver into the skin. Our interest was in reducing the molecules of HA to improve transdermal drug delivery. HA molecular chain was cut which can be done with many methods. The liquid phase plasma process is a new process for HA molecular reduction. This process does not require chemicals or high temperatures. In this study, therefore, we investigated the bioactivities and *in vitro* skin penetration of HA at different molecular weights: 9.5, 19.0, 200 and 1,100 kDa. The antioxidant activities were measured using a DPPH radical scavenging assay and an ABTS assay. We found that HA Mw 9.5 kDa demonstrated the highest antioxidant activity in both assays whereas the lowest antioxidant activity was with HA Mw 1100 kDa. In the DPPH assay, the antioxidant activity of the HA Mw 9.5, 19, 200 and 1100 kDa were 92.05%, 82.36%, 16.89% and 11.76% respectively at the concentration of 5000 µg/ml. In the similar, the ABTS radical scavenging activity of HA MW 9.5, 19, 200 and 1100 kDa were 88.33% , 82.55% , 31.90% and 18.82% respectively at the concentration of 1600 µg/ml. The results of type-1 pro-collagen synthesis showed that tested HA of various molecular

weights was unable to stimulate type-1 procollagen synthesis on human skin fibroblast cells. However, we found that all tested HA were not toxic to human dermal fibroblasts cells at a concentration up to 2,000 μg/ml. The *in vitro* skin permeation study showed that the low molecular weight HA permeated the skin better than the high molecular weight approximately 2%. Therefore, the low molecular weight HA is a better choice for cosmetic formulation than the high molecular weight one.

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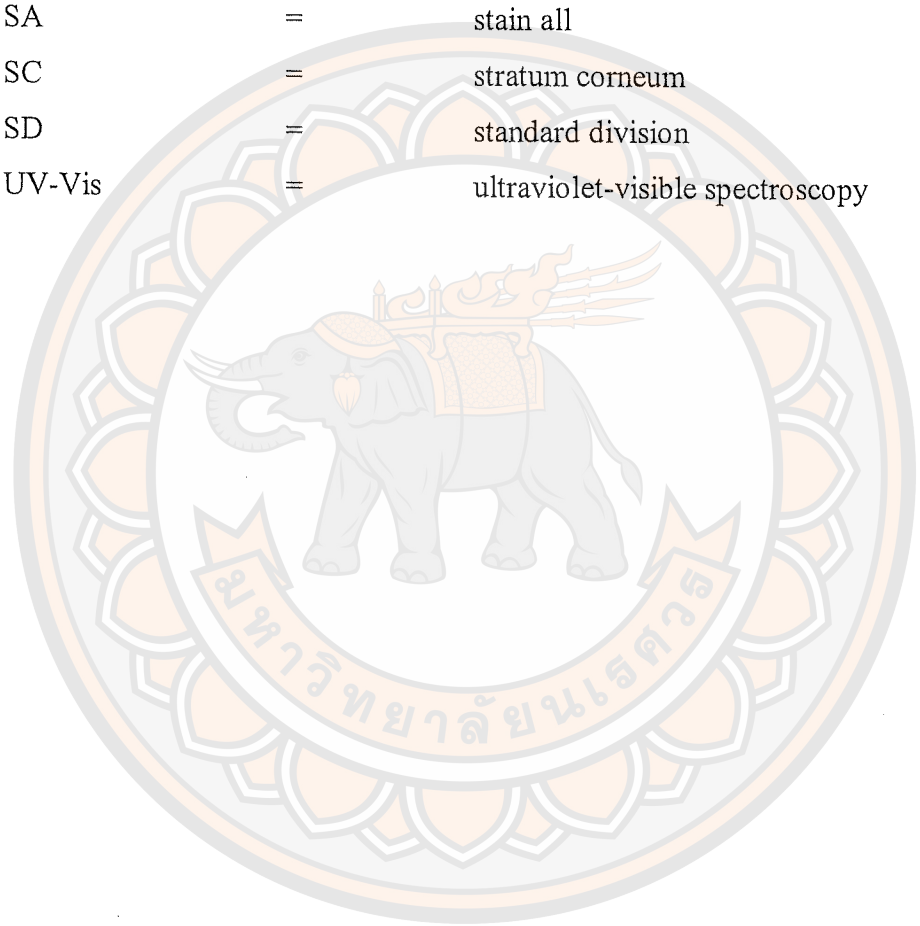
ABBREVIATIONS

β	=	beta
°C	=	degree celsius
µg	=	microgram
µg/ml	=	microgram per milliliter
µl	=	microliter
µM	=	micro molar
ABTS	=	2,2'-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid)
ANOVA	=	analysis of variance
cm^2	=	centimeter square
CO_2	=	carbon dioxide
DI water	=	de-ionized
DMEM	=	dulbecco's modified eagel medium
DMSO	=	dimethylsulphoxide
DPPH	=	1, 1-diphenyl-2-picrylhydrazyl
FBS	=	Fetal bovine serum
g	=	gram
h	=	hour
HA	=	hyaluronic acid
HG	=	high glucose
IC_{50}	=	inhibition concentration at 50%
kDa	=	kilo Dalton
R^2	=	linear correlation
mg	=	milligram
mg/ml	=	milligram per milliliter
ml	=	milliliter
mM	=	millimolar
Mw	=	molecular weights
nm	=	nanometer

ABBREVIATIONS (CONT.)

p	=	probability values
PBS	=	phosphate buffer saline
pg	=	pictogram
rpm	=	revolutions per minute
SA	=	stain all
SC	=	stratum corneum
SD	=	standard division
UV-Vis	=	ultraviolet-visible spectroscopy



CHAPTER I

INTRODUCTION

This chapter contains four parts including the rationale, the objective, the expected outputs and the expected outcomes of the study. The details of each part are described as follows:

The rationale of the study

HA is a natural glycosaminoglycan. Its chemical structure composes of D-glucuronic acid and N-acetyl glucosamine linked by β (1, 4) and β (1, 3) glycoside bonds. HA is an anionic molecule and can be found in the form of sodium salt. It is ubiquitous in many tissues in human body, especially in dermis, soft connective tissue of the skin and joint fluid (1-2). HA in the market is extracted from bovine vitreous, rooster combs, or produced by bacteria (*Streptococcus equi* and *Streptococcus zooepidemicus*) (3-5). It has water retention properties to conserve skin hydration and healing. HA is biocompatibility, biodegradability, viscoelasticity, and non-allergenic (6). Therefore, HA has been used as a biomaterial for cosmetic formulation as a moisturizer, an antioxidant and for increasing skin elasticity. As well, HA is an ideal biomaterial for pharmaceutical, biotechnical and medical usages. For medical usages, it has been applied for tissue engineering, as dermatological filler, and as visco-supplementation for osteoarthritis treatment (7-10). Hyaluronic acids are commonly high molecular weight biopolymers with molecular weights ranging from 2×10^5 to 10^7Da (11). These acids have high viscosity and difficulty in dissolving in water which limits its biological activity and its ability to deliver HA into the skin.

The human skin structure is well known for being a very strong barrier to drug delivery, allowing only very small and appropriate hydrophobic molecules to successfully pass into and through the skin (12). Nowadays, there are many methods that improve dermal and transdermal for topical delivery of bioactive molecules such as the use of delivery vesicles (e.g. liposomes, elastic vesicles), chemical enhancers (e.g. surfactant), physical enhancement (e.g. ultrasound, iontophoresis) and by

reducing the molecular structures of the drug being administered. Each method or combination of each method was successfully improved dermal and transdermal for topical delivery of bioactive molecules (13-14). In our research, the interest was by the properties of the reducing molecules of HA in term of biological activity and ability to transport into the skin.

Preparation of HA small molecules can be done in many ways including acid hydrolysis, ultrasonic degradation, thermal degradation by microwave, or the use of free radical. However, those processes have various disadvantages when applied to HA molecules. Chemical contamination and loss of function due to overheating are often experienced (15-16) . The liquid phase plasma process is a new process discovered by Takai O (17). The principle of this process is to produce the plasma that is surrounded by the liquid phase using electrical potential to stimulate free radicals such as the hydroxyl group ($OH^{\cdot}$) where these free radicals subsequently induce organic degradation. Importantly, the liquid phase plasma process does not require chemicals or high temperature to operate. Therefore, chemical contamination and thermal degradation are not occurred in this process (18).

Therefore, we investigated the effects of various molecular weights of HA fragments generated by the liquid phase plasma process that had been developed by the Faculty of Engineering, Naresuan University, on skin penetration and biological activity.

The objectives of the study

1. To determine the effects of various molecular weights of HA fragments prepared by liquid phase plasma process on antioxidant activities, cell cytotoxicity and Pro-collagen type 1 synthesis.

2. To investigate *in vitro* permeation of various molecular weights of HA fragments.

The expected output of the study

The expected output was to obtain information on the biological activity and skin penetration of hyaluronic acid, with different molecular weights, generated by the liquid phase plasma process which will be of benefit to medical science and cosmetics development.

The expected outcomes of the study

1. The obtained information will be of benefit to medical science and cosmetics development.

2. This research will provide alternative choices of using HA in medical applications and cosmetics formulation.

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

This chapter provides literature reviews related to this study. The reviews consist of two parts: hyaluronic acid, skin structure. Each issue is described individually.

Hyaluronic acid History

In 1934, Karl Meyer and John Palmer first discovered HA at Columbia University, New York. They isolated the substance from the vitreous body of cows' eyes and derived the name from "hyalos", which is the Greek word for glass. They also isolated "uronic acid", which are sugar also found in the vitreous body of cows' eyes. HA was first used commercially in 1942 by Endre Balazs who used it in bakery products as a substitute for egg white and in 1950, was used in a medical application as a substitute for the vitreous humor during eye surgery. Subsequently, HA was isolated from the human umbilical cord, and also, in a highly purified and high molecular weight form, from rooster combs. In 1990, HA found its way into the cosmetics field which is now the most widely known and popular application of this molecule (2, 19).

Physicochemical and structural properties

Hyaluronic acid (HA), as hyaluronan or hyaluronate, is a polysaccharide with a linear, non-branched structure, naturally present in living organisms from bacteria to higher animals including humans. Its chemical structure consists of N-acetylglucosamine acid and D-glucuronic acid, linked via alternating β-1,4 and β-1,3 glycosidic bonds (Fig 1) (1). A completed HA molecule mass can reach approximately 4 million Da. Various authors have reported on the water retention properties of HA which has a greater capacity to hold water than any other natural or synthetic polymer (20) . HA slowly but fully dissolves in water to give a viscous, clear to slightly opalescent and colorless solution, which must be preserved in cosmetic usage (21).

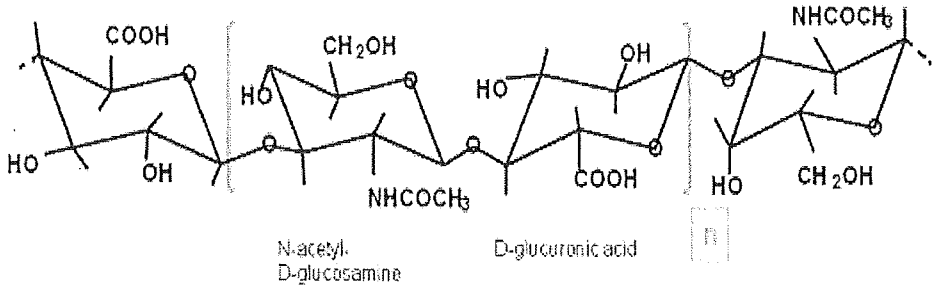


Figure 1 Chemical structure of Hyaluronic acid (1)

Manufacture/Sources

Industrial manufacturing of HA can be extracted from two main processes. The first process, the extraction of HA from animal tissues as fish eye, rooster combs, pig skin, and umbilical cord. The process is still an important technology for commercial products because of available raw material have low costs, but still suffer from low yields, due to the intrinsic low concentration of HA in the tissue, there is a uncontrolled polymer degradation and rick of contamination with proteins and viruses, but this can be minimized by using tissues from healthy animals and extensive purification. Product from the first process is very high molecular weight up to 20 MDa (22). Another method of extraction is by bacterial fermentation using Group A and C *Streptococci*, HA was produced by bacterial fermentation has high molecular weight 1-4 MDa, high yields and purity, but risk of contamination with bacterial endotoxins, proteins, nucleic acid and heavy metals. In the 90s opened the way towards HA production using safe, nonpathogenic recombinant strains (3).

In the past decade a new technology emerged using isolated HA synthase to catalyze the polymerization of the UDP-sugar monomer from *Pasteurella multocida*. The molecular weights of synthetic HA were 5.5×10^5 Da to 2.5 MDa and synthetic HA was no risks of contamination, but large scale production was not achieved yet (4).

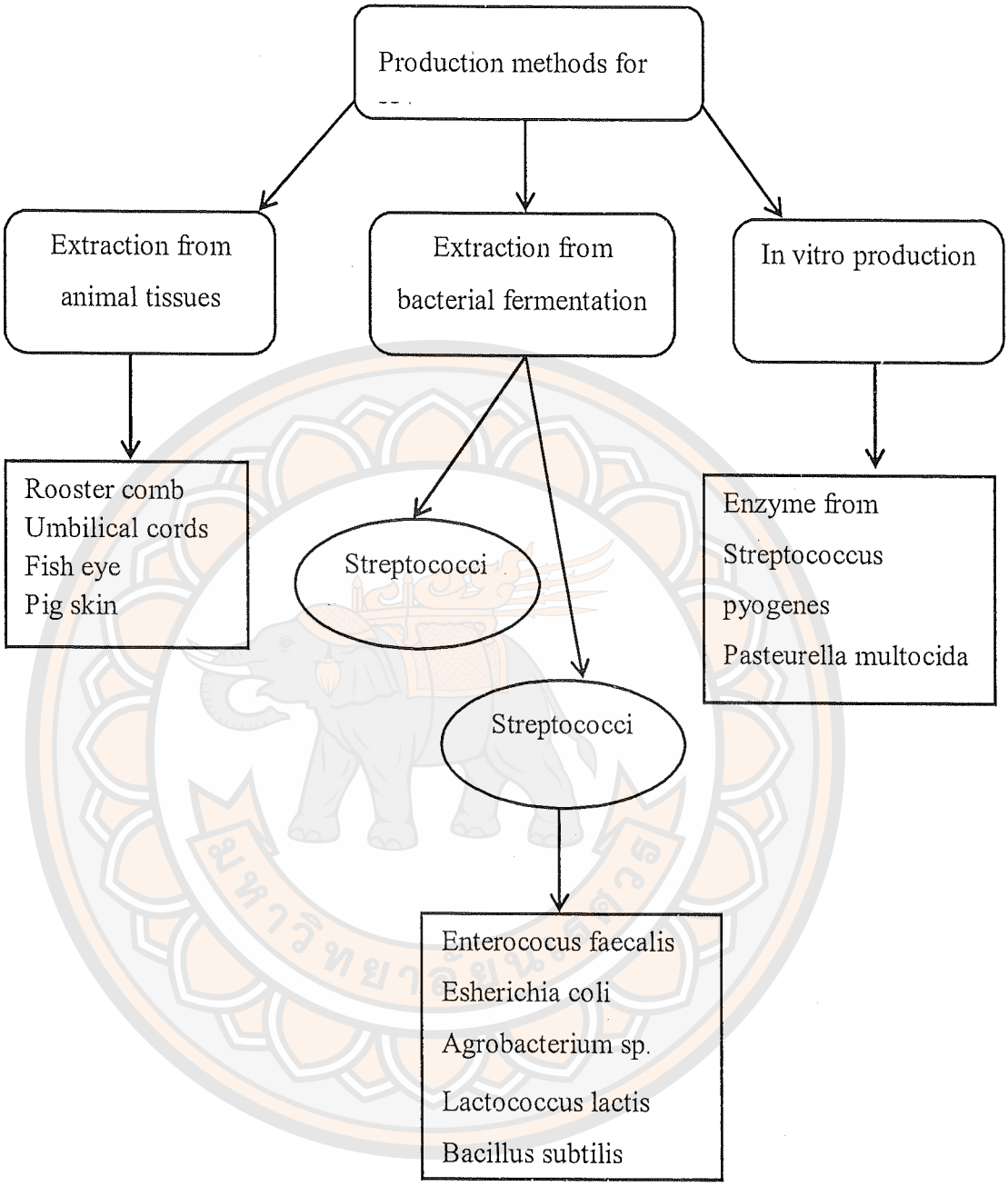


Figure 2 Production methods for HA (27)

Biological activity and application of Hyaluronic acid

In recent years, HA has been reported as having many applications. As given in the International Cosmetic Ingredient Dictionary and Handbook (23), HA functions as a skin-conditioning agent and/ or as a viscosity increasing agent in cosmetic formulations. Voluntary industry reports to the US Food and Drug Administration (FDA) showed that HA is used in 223 products and in concentrations up to 1%. HA is a common ingredient in skin-care products as a moisturizing agent and for restoration of skin elasticity thus achieving anti-wrinkle effects (24). High molecular weight HA solutions, when applied to the skin, can form a hydration viscoelastic film on the skin but low molecular weight HA has been shown to increase the moisture level of damaged skin (25).

As well, previous studies have reported on other biological activities and applications of HA.

1. Antioxidant activity

Nermeen et al. (26) investigated the antioxidant effects of unsaturated HA oligomers by lipid oxidation inhibition assay and DPPH radical scavenging assay, using gallic acid as a polyphenol antioxidant standard. Their results showed that anti-lipid oxidation activities at different concentrations of 50μg/ ml, 10μg/ ml, 15μg/ ml, and 250μg/ ml of unsaturated HA oligomer showed strong anti-lipid oxidation activities than naturally occurring HA, with HA lyase showing the weakest activity. Anti-DPPH Radical properties of naturally occurring HA, native HA lyase protein, and unsaturated HA oligomers, showed that unsaturated HA oligomers provided a stronger radical scavenging activity than naturally occurring HA at different concentrations as 50μg/ml, 10μg/ml, 15μg/ml, and 250μg/ml, with HA lyase protein showing the weakest radical scavenging activity. They suggested that the biological activities of HA depended on its molecular weight and studies on HA oligomers of exact sizes may have more practical significance for future medicinal applications and biological strategies.

In 2013, HA was isolated from the *Amussium pleuronectus* bivalve mollusk by Shankar et al. (27). They examined the antioxidant activity of the isolated HA at various concentrations of 0.2 to 1 mg/ml by using ABTS assay, DPPH radical scavenging activity, and hydroxyl radical scavenging activity. The results of the

scavenging ability of isolated HA towards the ABTS showed the minimum activity was 32.56% and maximum activity was 71.35% when compared with the standard ascorbic acid was 98.04% . The DPPH radical scavenging activity of isolated HA showed the minimum activity was 19.77% and maximum activity was 54.42% . The isolated HA exhibited hydroxyl radical showed the minimum activity was 23.77% and maximum activity was 63.42% . The significantly higher minimum and maximum scavenging activities of the HA from the *Amussium pleuronectus* mollusk indicates that it can be considered as a natural antioxidant.

In the study of Braga et al. (28), the antioxidant effects of HA (MW 900 kDa) were shown to interfere with ROS/RNS during equine PMN respiratory bursts, investigated by ABTS assay. The result showed the lowest concentration that was still active was 0.62 mg/ml and the highest concentration was 2.5 mg/ml, and there was a significant concentration-dependent inhibition of ABTS•+ radicals from 47.22±2.91% to 14.04±0.94%.

2. Anti-inflammatory and wound healing

In the study by Chen and Abatangelo (29), the functions of HA in wound repair were reviewed. They found that HA can act as a promoter of early inflammation that is important in the wound healing process. In a murine air pouch model of carrageenan/ IL-1-induced inflammation, HA was shown to enhance cellular infiltration and to increase pro-inflammatory cytokines TNF-α, IL-1β and IL-8 production by human uterine fibroblasts at HA concentrations of 10 mg/ml to 1 mg/ml via a CD44 mediated mechanism

Law et al. (30) studied the effects of low molecular weight HA (LMW 100kDa) and high molecular weight HA (HMW 1000kDa), in various doses, on wound closure in H_2O_2-damaged H_9C_2 cells. The results show that only HMW-HA significantly reduces the H_2O_2-induced cytotoxic effects in cardiomyocytes and increases wound healing ability in H_9C_2 cells (30). The effective concentration of HMW-HA was 0.3%.

3. Anti-aging and reduce winkle

HA has become popular in anti-aging cosmetics products and in dermal fillers for reducing wrinkles. To derive topical formulation, Farwick et al. (31) studied low molecular weight HA effects on skin ageing, using *in vivo* tests of o/w cream

containing 0.1% the HA derivatives different molecules (MW 50, 130 and 300 kDa), were compared with a placebo control. Their test used 12 female volunteers between the ages of 30 and 60, who applied the cream twice daily for 8 weeks. This study demonstrated the effects of 3 different low molecular weights HA on skin roughness. The 50kDa HA showed significantly reduced skin roughness and also demonstrated strong anti-wrinkle properties.

In 2011, Pavicic et al. (32) were investigated the efficacy of cream-based novel formulations of HA of different molecular weights in an anti-wrinkle treatment. They used 0.1% HA cream formulations of different molecular weights (MW 50, 130, 300, 800 and 2000 kDa) in the periocular area as anti-wrinkle treatment on 76 female volunteers between 30 and 60 years of age who applied one of the formulations twice daily for 60 days. They found that 0.1% HA formulations of MW 50 kDa and 130 kDa significantly improved wrinkle depth using mean roughness (Ra) and maximum roughness (Rz) values after 60 days of treatment compared to the placebo-treated area. However, formulations of 300, 800 and 2000 kDa showed no improvements.

The 20 volunteers in a study by Poetschke et al. (33) were separated into four groups and each group used a different cream with different prices (Balea®, Nivea®, Lancôme®, Chanel®). These creams all contained HA. At the end of the 3-month trial, wrinkle-depth of all volunteers was evaluated using the PRIMOS (pico). Skin-tightness and skin elasticity were evaluated using the Cutometer MP580. The results showed clear and positive effects on wrinkle-depth and skin-tightness in all cases with no reported differences between the cream brands.

With HA fillers for anti-aging treatments, the main differentiators are the concentration of HA injected in a treatment, the source of the HA, and the type of cross-linking agent in the HA. A study of HA fillers for the treatment of the aging face (34) was compared Restylane® (containing HA fillers) with Zyplast® collagen. Restylane® is produced from the fermentation of *equine streptococci* with an HA concentration of 20 mg/ml. The FDA approved Restylane® for use in December 2003 for mid-dermal applications such as deep wrinkle correction, nasolabial fold correction, lip augmentation and glabellar creases. This study used 138 Caucasian females received treatment with both materials, one in each nasolabial fold. The result showed that the wrinkle severity rating scale score for Restylane® was superior at all-

time points; 2, 4 and 6 months after baseline, and was rated superior by 56.9% of the participants, whereas Zyplast® collagen was rated superior by only 9.5% of patients.

In 2008, Allemann et al. (35) studied JUVÉDERM® HA fillers and compared that with Zyplast® collagen as a treatment of nasolabial folds. JUVÉDERM® was approved by the FDA in June 2006. It was used for deep wrinkles and deeper furrows such as nasolabial folds. The HA in JUVÉDERM® is produced from the fermentation of *equine streptococci* and concentrations in each formulation ranging from 18 mg/ g to 30 mg/ g. In this study, they study in 423 patients for 24 weeks for evaluation. The result showed that the JUVÉDERM® efficacy was significantly greater than the Zyplast® collagen products, with efficacy increasing over time and being greatest at 24 weeks after the last treatment (35).

4. Application in osteoarthritis

Osteoarthritis (OA) is a common joint disease characterized by cartilage degeneration and reactive proliferation of bone and cartilage around the joint. The symptoms of OA is pain and stiffness. HA is a major component of the synovial fluid in joints as it supplies nutrients to the articular cartilage and lubricates the joints. The effect of HA on OA has been described in a previous study. In 1996, Kikuchi et al. (36) investigated the effects of high molecular weight HA on cartilage degeneration of osteoarthritis. The knee joints of 72 male New Zealand white rabbits with body weight of 2-3 kg were used. This study compared HA 80 (0.8×10^6 Da, 1%) with HA 190 (1.9×10^6 Da, 0.1-1%) and a control (saline). The injection dose was 0.1 ml/kg of body weight and injected intra-articularly twice a week, immediately after surgery until sacrifice at 2 or 4 weeks. The degenerative change in femoral and tibial cartilages was determined by histopathological score at 2 and 4 weeks after surgery, and scanned the femoral cartilages by electron micrographs. At two weeks after surgery, when HA 190 at 1% concentration was used, dramatic inhibition of cartilage degeneration in both the femoral and tibial was found but H80 had no inhibitory effect. At four weeks after surgery, H80 continued to have no inhibitory effect, but HA 190 showed further significant protection against cartilage degeneration, but only in the femoral cartilage; the tibial cartilage degeneration was not further inhibited in the second 2-week period. This study demonstrated that the higher molecular weight HA is more effective than lower molecular weight HA in inhibiting cartilage degeneration in OA (36).

The long-term efficacy and safety of Hyalgan® containing 20 mg of sodium hyaluronate, administered in 5 weekly intra-articular injections, was studied by Neustadt (37). The study included 76 patients, male and female, ranging in age from 40-80 years, having 92 knees between them, had suffered OA of the knees for more than 3 years. Clinical evaluation was carried out at baseline, weekly, at the time of injections, at the end of the series, and at 6 months, 12 months, and 24 months after termination of the series of injections. The evaluation method was by categorical assessment and visual analog scale (VAS) of 1 to 10 for physical examinations and outcome measures of pain, night pain and pain on walking. The result showed a total of 72% of patients achieved a > 50% improvement for 12 months or longer and only 3.8% did not demonstrate any clinical benefit. The mean duration of response was 9±0.5 months and some patient response exceeded 2 years for no pain or slight pain. In conclusion, intra-articular injections of sodium hyaluronate (Hyalgan®) were effective for pain in difficult-to-treat patients with moderate to severe OA of the knee.

5. Other

In 2017, Groß et al. (38) studied HA for use for Dry Eye Disease (DED), and compared the efficacy and safety of 0.2% and 0.18% HA eye drops in two parallel groups; 35 patients with moderate dry eye disease, and 35 with severe dry eye disease. Eye drops were administered three times a day. At day 35, primary efficacy outcomes were evaluated by ocular staining, with secondary efficacy outcomes similarly evaluated at day 84. The results were compared against the control treatment of ocular staining at day 0, and evaluated the patients' ocular comfort index indicated by the patients and further by the doctors. Statistical analysis of the results showed that at Day 35 neither the 0.2% HA (47.7% reduction in staining score) nor the 0.18% HA (41.2% reduction in staining score) showed a non-significant efficacy. The same outcome was observed at Day 84 although the staining score had further increased to 64.5% for 0.2% HA and 56.4% for 0.18% HA. Both eye drop formulations improved tear film breakup time and ocular comfort index values. Both the investigators and the patients assessed both treatments according to a Likert Scale (1-10), with patient assessment having a median score of 5 and the doctors having a median score of 7.

The use of HA as the targeting moiety in developing drug delivery systems for cancer treatment was discussed in terms of there being various tumor cells that are known to over-express the HA receptor, CD44. As a drug delivery system, HA is encapsulated in different types of nano assemblies as a ligand to prepare nano-platforms for actively targeting drugs, genes, and diagnostic agents. In 2009, Ki Young Choi et al. (39) develop self-assembled HA nanoparticles as a drug delivery system for cancer therapy. From a cellular experiment using Cy5. 5-labeled HA nanoparticles in squamous cell carcinoma (SCC7) cell, they found that HA -NPs could be rapidly internalized at the cytoplasm of SCC7 cells via the receptor-mediated endocytosis in the cell culture system. Non-invasive and live animal imaging data demonstrated that HA -NPs were successfully accumulated in the tumor tissue of tumor-bearing mice, owing to the nanoparticle's EPR effect.

Molecular weight reduction of hyaluronic acid

Preparation of HA reduction molecules can be done in many ways including acid hydrolysis (40), ultrasonic degradation (41), thermal degradation by microwave (42), or the use of free radical (43-44). Acid hydrolysis such as ascorbic acid is easy to use with low cost but has the disadvantage of producing chemical waste and chemical contamination (15) . The ultrasonic and microwave techniques, combined with a chemical for preparing low molecular weight HA, are effective methods because they take a shorter time than when using the chemical alone. However, the processes often generate heat that is likely to cause a loss of acid function (45-46). For the preparation of low molecular weight HA, free radicals group are decomposed with a hydroxyl group produced from hydrogen peroxide, which is difficult to eliminate (47) . The liquid phase plasma process is a new process discovered by Takai O. (17) . Importantly, the liquid phase plasma process does not require chemicals or high temperature to operate (18) so chemical contamination and thermal degradation are avoided in this process.

Plasma

Plasma is one of the four fundamental states of matter and was first described by chemist Irving Langmuir in the 1920s (48). Plasma is a result of the breakdown of neutral gas into charged particles or ions and which occurs when the gas is stimulated by electrical energy, heat energy, or microwaves or UV radiation. This process is called "Ionization" in which the stimulation causes the atom to lose its neutral state and release electrons, resulting in the final charge of the plasma being zero, meaning that the plasma then represents the behavior of neutral particles (Quasi-Neutral) and Collective Behavior (49).

Plasma can be divided into 2 types. First, high temperature plasma is plasma produced by providing high energy to the gas which becomes ionized and almost completely disintegrates. The net charge system is a balanced state such as occurs in a nuclear reaction and in jet engines – due to the very high temperatures generated in jet engine fuel combustion. The second type, low temperature plasma, is plasma produced by providing low energy to the gas which becomes ionized but incompletely disintegrated, such as in DC electrical systems with low pressure gas, radio frequency discharge (50-52).

Liquid phase plasma process

The liquid phase plasma (LPP) process, proposed by Takai O. (17), is the process of producing plasma that is surrounded by a liquid phase, and using the potential of an electrical current to stimulate the highly active species (53). The advantages of liquid phase plasma are that it can be used in a variety of ways such as in nanomaterial synthesis, surface modification, water treatment, object sterilization, and decomposition of organic compounds. Previous studies have also focused on acceleration of polysaccharides degradation using LPP the type of solvent and solution used to produce the plasma can be chosen, and the process produces no chemical contaminants, with no thermal degradation (54-56).

Hyaluronic acid fragment generated by the Liquid phase plasma process

The liquid phase plasma patterns are shown in Figures 2 and 3. Plasma occurs at the center point between the electrodes which are surrounded by gas and liquid, resulting in the contact surface between the two areas being stimulated by the high voltage. When the substance is broken into the plasma state, gas bubbles are produced around the plasma. While the plasma is stimulated by the continuous voltage, an increase in free radicals occurs between the electrodes that move to an area of the liquid that has low numbers of free radicals, moving in accordance with the principle of mass transfer. Therefore, the substances in the liquid phase will be replaced and stimulated to become a free radical group (18,55,57).

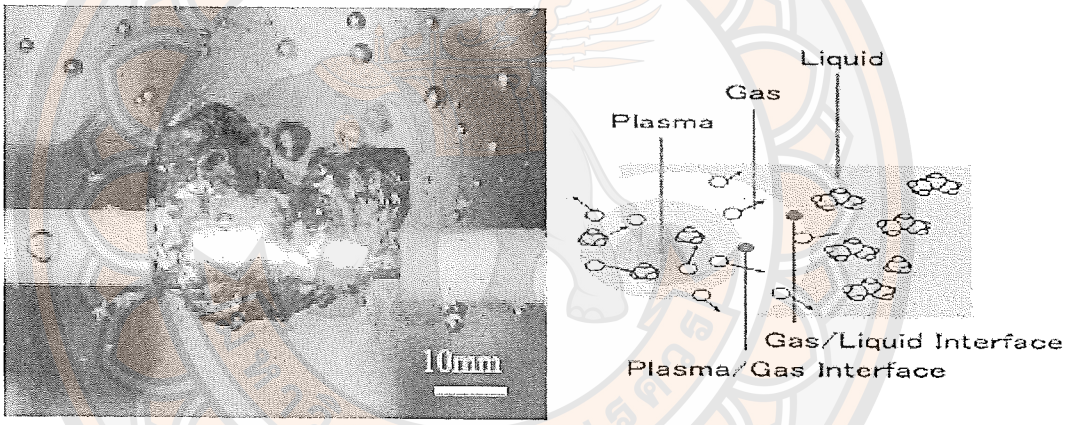


Figure 3 Photos and models of a liquid phase plasma process (17)

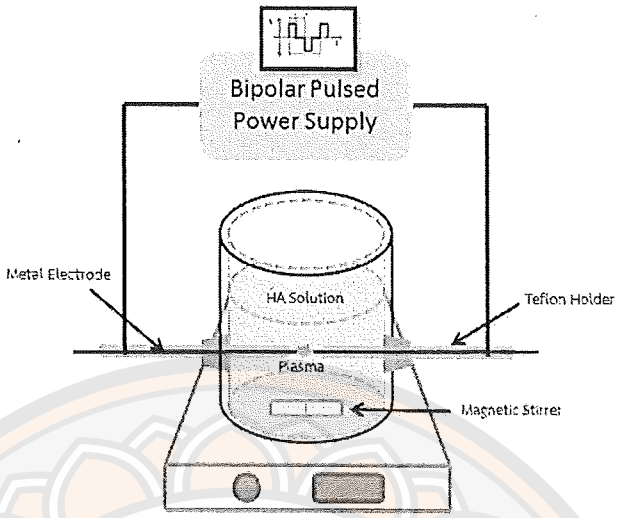


Figure 4 Diagram of a liquid phase plasma generator (55)

Skin structure

As discussed in various places (58-60), the human skin is the outer covering of the body and the largest organ of the body, averaging 1.8 m^2, with a thickness of 1.5-4 mm. The skin structure and morphology varies depending on the location on the body, and according to age, gender, physical environment and exposure. The human skin is composed of three layers; the epidermis, dermis and hypodermis, as illustrated in Figure 4. All layers have both an extracellular protein matrix (ECM) and different cells.

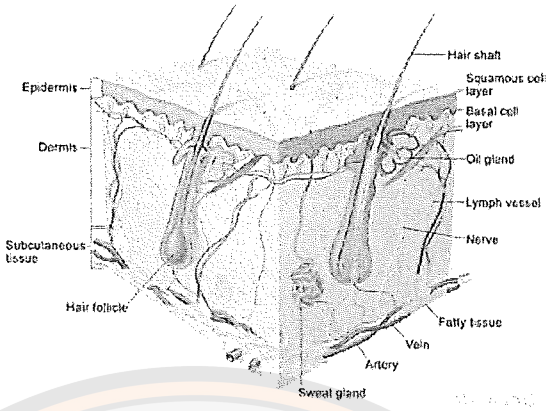


Figure 5 Anatomy of skin structure (60)

1. Epidermis

The epidermis is the outer layer of the skin and is the primary protective structure of the body that prevents loss of water and body fluids, resists mechanical and chemical injury and protects against bacteria, viruses and parasitic infections. The thickness of the epidermis is usually 0.5-1mm but that is dependent upon the site location of the skin, such as the eyelids (0.05mm), the palms and soles of the feet (0.8-1.5mm), or in other protected or unproteced areas of the body. Keratinocyte cells comprise 95% of the cells in the epidermis, with the other 5% being melanocytes, Merkel cells and Langerhans. The pigment in the epidermis plays an important role in protecting the skin from ultraviolet radiation. The epidermis may be divided into five layers, as shown in Figure 5.

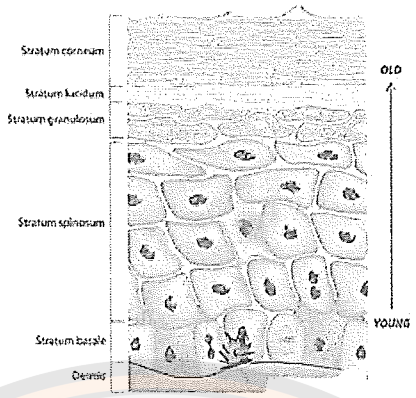


Figure 6 Structure of epidermis (62)

1.1 Stratum corneum

The stratum corneum or horny layer is the top layer of the epidermis containing corneocyte cells which are dead keratinocytes that have lost their nuclei and cytoplasmic organelles. The structure of the stratum corneum has been likened to a brick wall where the corneocyte cells are the bricks and the continuous matrix of specialized lipids provides the mortar. The major lipid species of the stratum corneum are ceramide fatty acids and cholesterol which provide the essential elements of the water barrier, and with corneocytes that protect against the continuous abrasion by chemical and physical injury. The stratum corneum is made of 25-30 layers of flattened epithelial cells and a build-up of the protein keratin. The stratum corneum is thickest on the palms and soles.

1.2 Stratum lucidum

The stratum lucidum is a thin, clear layer of dead skin cells located between the stratum corneum and stratum granulosum. It is readily visible by light microscopy only in areas of thick skin, such as is found on the palms of the hands and the soles of the feet.

1.3 Stratum granulosum

The stratum granulosum or granular layer, are composed of cells that have become flattened and have lost their nuclei. The name 'granulosum' comes from keratinocytes that contain intracellular granules of keratohyalin and cytoplasm, and also contain lamellar bodies of lipids and proteins. The stratum granulosum plays an important role as a barrier function and provides intercellular cohesion within the stratum corneum.

1.4 Stratum spinosum

The stratum spinosum or prickle layer is the thickest layer of the epidermis. Keratinocytes cells in this layer are polyhedral-shaped keratinocytes and known as spinous cells or prickle cells. They have large pale-staining nuclei as they are active in synthesizing fibrilar proteins, known as cytokeratin, which build up within the cells, aggregating together to form tonofibrils. The tonofibrils terminate in desmosomes which allow for strong connections to form between the spinous cytoplasmic protrusions of the keratinocytes. Also, Langerhans cells, which are mainly present to alert the immune system in this stratum, attach to the antigens in damaged skin.

1.5 Stratum basale

The stratum basale is a single layer of cuboidal-shaped keratinocytes with large nuclei. They are firmly attached to the basement membrane with hemidesmosomes and lateral and upper adjacent cells with desmosomes. The stratum basale can act like stem cells with cell division / reproduction (mitosis) in the skin that pushes older cells upwards. Other cell types found within the stratum basale are melanocytes which give color to the skin, and Merkel cells which act as soft touch receptors.

2. Dermis

The dermis is a layer of skin between the epidermis and the subcutaneous tissues. The dermis is composed of interstitial (collagen fiber, elastic tissue, ground substance) and contains mechanoreceptors that provide a sense of touch and thermoreceptors that provide the sense of heat. In addition, hair follicles, sweat glands, sebaceous glands (oil glands), apocrine glands, lymphatic vessels, nerves and blood

vessels are present in the dermis. Those blood vessels provide nourishment and waste removal for both dermal and epidermal cells, as shown in Figure 6.

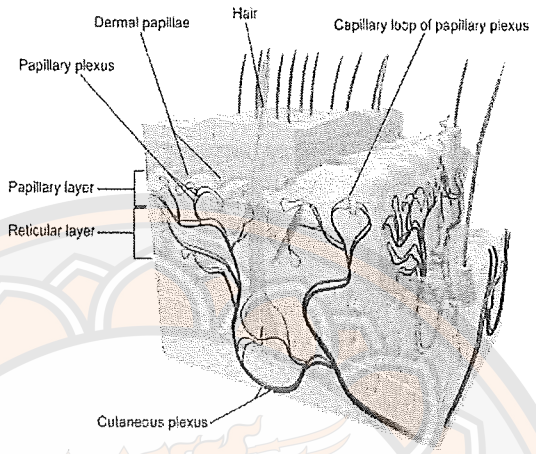


Figure 7 Structure of dermis (72)

The dermis is subdivided into two main layers:

2.1 Papillary dermis

The papillary dermis is the uppermost layer of the dermis with thin fiber components, and is richly supplied with capillaries, sensory nerve endings, and cytoplasm.

2.2 Reticular dermis

The reticular dermis is the lower layer of the dermis, composed of dense collagen fibers, elastic fibers and reticular fibers that weave throughout it.

3. Subcutanous tissue

Below the dermis is a layer of fat that helps to keep the body insulated against temperature extremes and also gives shape to the body. Muscle fibers, nerves and blood vessels also run through this subcutaneous fat, as do the roots of the oil and sweat glands.

The function of the skin

The discussion of the functions of the skin comes from various sources (58, 61-63)

1. Protection

The skin acts as a barrier preventing damage to the internal organs from harmful chemicals, toxins, germs and radiation.

2. Secretion

Waste, in the form of urea, is excreted from the body in the form of sweat. Urea is a mineral that the body does not want.

3. Maintain body moisture

The sebaceous glands produce sebum to nourish the skin in the epidermis layer, moisturizing the skin, making it firm and not dry. In addition, the skin is waterproof and prevents water from entering the body while not allowing moisture in the body to evaporate easily.

4. Sensation

The skin contains a variety of nerve endings that register touch, pressure, vibration, pain, and tissue injury.

5. Control the body temperature

The skin is a body temperature control mechanism ensuring that body temperature normally remains within a certain temperature range through the function of the contraction or expansion of the blood vessels in the skin and the work of the sweat glands. When the body is heated, the blood vessels will expand to allow more blood to flow to the surface and be cooled, while the blood vessels will shrink when cold, retaining warm blood in the core of the body. The sweat glands that present in the skin layers of the body produce sweat which evaporates, causing the body to cool down.

6. Vitamin D production

The skin is a source of vitamin D to the body, using sunlight to help synthesize 7-dehydrocholesterol into vitamin D3.

Percutaneous absorption

Percutaneous absorption is the study of the mechanism or way of absorption of a drug through the skin or of the specific actions of the drug on the skin, or the absorption of the drug into the circulatory system and its actions throughout the body or in the target organ. The percutaneous absorption acts as a drug diffusion agent, so the skin absorption rate can be explained by the Fick's law of diffusion according to this equation (64-65).

$$\frac{dM}{dt} = \frac{D A K}{h}$$

When dM/dt = rate of diffusion

D = diffusion coefficient

A = surface area of membrane

K = partition coefficient

h = thickness of the membrane

According to Fick's Law, drugs or substances that can penetrate the skin well should have the following properties (66)

1. Low molecular weight (< 500 Da) which will cause the diffusion coefficient (D) to be very valuable.
2. Optimum solubility in lipid and water
3. Key drugs with high K values
4. Low melting point
5. No charge

Route of drug penetration to the skin

The main transport routes for drug penetration through the skin layer are the trans-epidermal pathway and the appendageal pathway, as shown in figure 7 (67-68).

1. Transepidermal pathways

The drug passes across the continuous stratum corneum via the transepidermal pathways which are divided into 2 routes.

1.1 Transcellular route

The transcellular route is the route of drug permeability directly through the skin cells, as shown in Figure 5. This path is the main skin route for drug permeability through the stratum corneum. The drug is transferred through the keratin-packed corneocytes by progressing the drug into and out of the cell membrane into the lipid matrix. The drug must have the properties of being dissolvable in both oil and water to be able to pass through this intracellular channel. The log Po/w value of the drug should be about 1 to 3, with the drug being absorbed and spread through both the corneocyte and lipid matrix cells.

1.2 Intercellular route

In this route, the drug is inserted through the gaps between the corneocyte cells of the stratum corneum layer. The gap between the corneocyte cells are lipid matrix such as cholesterol, fatty acid, and ceramide.

2. Appendageal Pathway

The chemical bypasses the corneocytes by entering the shunts provided by the hair follicles, sweat glands, and sebaceous glands. As the relative surface area of these shunts (appendages) is only 0.1–1.0% of the total area, they are not thought to play a decisive role in the absorption of many chemicals in the human body.

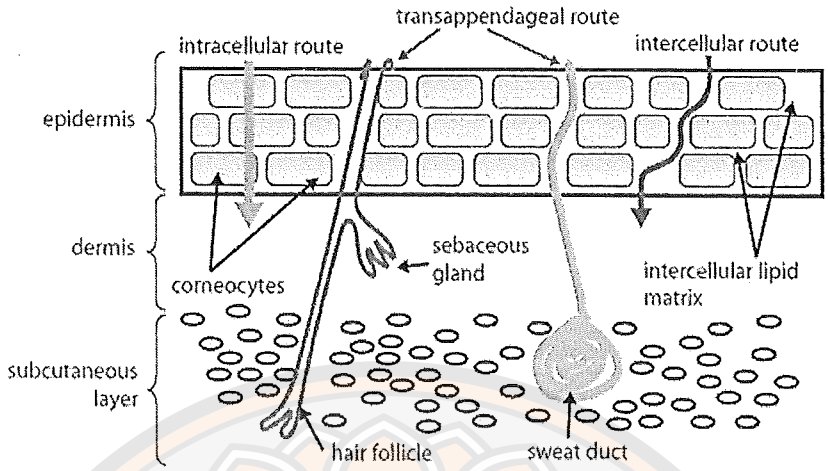


Figure 8 The transport of chemicals through the skin (68)

Factors affecting percutaneous absorption

As discussed in (66, 69-70), the factors affecting percutaneous absorption are:

1. Species

The skin of many species has been evaluated as models for permeation through the human skin. Although rat, mouse, and rabbit skins are more permeable than human skin, they have been used to provide a conservative estimate of skin penetration for safety assessments.

2. Age

Changes that occur in the aging skin include increased stratum corneum dryness, reduction in sebaceous gland activity resulting in a decrease in many skin lipid surfaces, flattening of the dermal-epidermal junction, and atrophy of the skin capillary network resulting in a gradual attenuation of blood supply to the viable epidermis.

3. Anatomical site

Percutaneous absorption varies depending on the location or site of the skin on the body. The skin thickness of the eyelid, for example, is approximately 0.05 cm whereas that of the palm and sole is about 0.4 cm.

4. Skin condition

The skin condition can have a significant impact on the penetration and permeation of chemicals, especially when the barrier function is disrupted by the skin being damaged.

5. Temperature

Skin that has high temperature makes the skin absorption rate faster and more efficient because the heat will help increase the rate of diffusion, increases the molecular weight, and increases the dissolution of the drug. The viscosity of the receptor increases the blood flow rate, allowing the drug to be absorbed more efficiently, resulting in a higher level of concentration of the drug in the bloodstream.

6. Hydration

When the skin is wet, the gap between the proteins in the skin is enlarged, and the skin becomes swollen, reducing the firmness of the skin which increases the rate of diffusion of the drug

7. The properties of drugs

In general, a drug has a log P value in the range 1-3 (similar to this property like lipid), is absorbed through the skin more effectively. Also, a drug with a molecular weight of 500 Da or less has a small particle size that can more easily penetrate the skin well.

8. Concentration of drug

Where the dosage of the drug is high, the concentration of the drug is also high. This results in a high penetration rate of the drug through the skin.

***In vitro* test for skin permeation**

Principle of the Franz Diffusion Cell (71)

In an *in vitro* test, the test substance, which may be radio-labelled, is applied to the surface of a skin sample, separating the two chambers of the diffusion cell. The chemical remains on the skin for a specified time under specified conditions, before removal by an appropriate cleansing procedure. The receptor fluid is sampled at time points throughout the experiment and analysed for the presence of the test chemical.

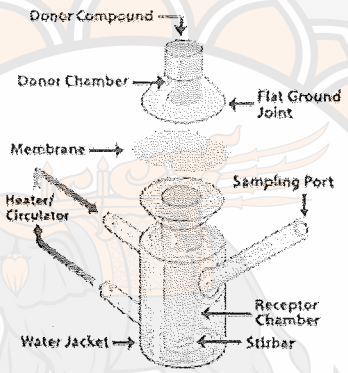


Figure 9 Typical design of a diffusion cell (71)

***In vitro* skin permeation of hyaluronic acid**

In the study of Ming Chen et al. (72), the SPACE peptide technique, to enhance topical delivery of a macromolecule as HA (HA, MW: 200–325kDa), was studied. They used the SPACE-ethosomal system (SES) for enhanced HA penetration into porcine skin and also full thickness abdominal human skin. *In vitro* skin penetration and deposition experiments of different test formulations containing HA were performed in Franz diffusion cells under occlusive conditions at 37 ± 1°C with an effective penetration area of 1.77 cm^2 and receptor cell volume of 12.0 ml. The receptor compartment was filled with PBS at pH 7.4 and then 200µl of the test formulation was applied to the skin surface using a pipette. The incubation time was 24 h at which time a 1 ml sample was withdrawn from the receptor compartment and the concentration of HA was determined using a micro-plate reader. The formulations were removed from the skin by washing five times with PBS (pH 7.4). The skin was then transferred onto a device for tape-stripping the startumcorneum. The results

showed the SPACE-ethosomal system (SES) significantly greater penetration of HA into the skin than the ethosomal control: higher by 9.3% ± 1.2% , $p<0.01$ The SES formulation also increased the penetration depth of HA into the epidermis skin; 3.3% ± 0.6% of the applied dose, $p<0.01$ greater than the ethosomal control.

An *in vitro* permeation study by Chawankorn Ka, et al. (73) investigated the simultaneous treatment of elastic liposomes (ELs) / low frequency ultrasound (LFU) on porcine ear epidermis to improve transdermal delivery of the HA (HA MW 1500 kDa) using franz diffusion cells maintained at 32±0.5°C. The diffusion area was 2.46 cm^2 with 12 ml of DI water (receptor medium). At incubation times 0, 5, 10, 30, 60, 120, 180, 240 and 300 min, 400ml samples were collected, and replaced by an equal volume of fresh receptor medium. The amount of HA permeation was determined by UV-Visible spectrophotometer at 640 nm. The results of this study demonstrated that pure HA solution cannot permeate through the porcine epidermis. However, HA in combination with Els/LFU showed greater HA permeation than both Els alone and the HA /LFU combination.

Martha L et al. (74) investigated the permeation of HA encapsulated in liposomes incorporating two penetration enhancers (PEs): Tween® 80, and Transcutol® P, by vertical Franz-type diffusion cells with a diffusion area of 0.6 cm^2 using cellulose membranes, with 300μL of the test formulation applied on the membranes. The receptor chamber was filled with milliQ water or buffer and maintained at 32.0 ± 0.5°C with continuous shaking with a magnetic stirrer. After an incubation time was 5h, the concentration of released HA was assessed by HPLC. The results showed that there was a significant improvement in HA release when supplemented with PEs, compared with control liposome.

CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this research, the materials and equipments.

Materials

Chemicals and Reagents

1. 2-Diphenyl-1-picrylhydrazyl, DPPH (A.R. grade, Sigma-Aldrich Co. , St.Louis, Missouri, USA)
2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (A.R. grade, Sigma-Aldrich Co., St.Louis, Missouri, USA)
3. Bovine serum albumin (Sigma-Aldrich Co., St.Louis, Missouri, USA)
4. Dimethylsulphoxide, DMSO (A.R grade, LabScan Asia, Co. Ltd, Bangkok, Thailand)
5. Dulbecco's Modified Eagel Medium, DMEM (A.R. grade, High glucose, Gibco™, New york, USA)
6. ELISA kit for type 1 procollagen (Abcam, Cambridge, USA)
7. Ethanol (A.R grade, LabScan Asia Co. Ltd, Bangkok, Thailand)
8. Fetal bovine serum, FBS (A.R. grade, Gibco™, California, USA)
9. Gluta-MAX (A.R. grade, Gibco™, New York, USA)
10. Hyaluronic acid (Faculty of Engineering, Naresuan University.)
11. L-ascorbic acid (A.R. grade. Chem-Supply Pty Ltd, Gillman, Australia)
12. Methanol (A.R grade, LabScan Asia Co. Ltd, Bangkok, Thailand)
13. PBS buffer (A.R. grade, Gibco™, New York, USA)
14. Penicilin-Streptomycin (A.R. grade, Gibco™, New york, USA)
15. Stain-all, 75%, pure (Acros Organic, USA)
16. Trypan blue solution (A.R. grade, Gibco™, New York, USA)
17. Trypsin EDTA (A.R. grade, Gibco™, New York, USA

Cell culture

Human dermal fibroblast cells

Apparatus

1. Centrifuge (Universal 320R, Hettich, Becthai Bangkok Equipment & Chemical Co., Ltd., Bangkok, Thailand)

2. CO_2 incubator (Steri-Cult CO_2 incubators, Thermo Scientific Forma, Becthai Bangkok Equipment & Chemical Co., Ltd., Bangkok, Thailand)

3. Franz diffusion cell (Logan FDC-6, Meditop Co., Ltd., Bangkok, Thailand)

4. Freeze-dryer (VP190D, s/n VP95C03, Trivac, New York, USA)

5. Hot air oven (UN110, Memwert, P.P. Chemical & Holding Co., Ltd., Phitsanulok, Thailand)

6. Inverted microscope (AE2000, MOTIC®, Rushmore Precision Co., Ltd., Bangkok, Thailand)

7. Laminar air flow cabinet (1300 Series A2, Thermo Scientific, Becthai Bangkok Equipment & Chemical Co., Ltd., Bangkok, Thailand)

8. Microplate reader (Eon, BioTek, Science Tech Co., Ltd., Bangkok, Thailand/ M3, Molecular Devices, Lab connection Co., Ltd., Chiang mai, Thailand)

9. Microplate shaker (SLM-MPS-250, Merck Specialties Pvt. Ltd., Bangkok, Thailand)

10. pH meter (Mettler Toledo S2220, P.P. Chemical & Holding Co. Ltd., Phitsanulok, Thailand)

11. Refrigerator (SNL-190C, SNOWLAND, Becthai Bangkok Equipment & Chemical Co., Ltd., Bangkok, Thailand)

12. Shaker (SK-O330-Pro, P.P. Chemical & Holding Co. Ltd., Phitsanulok, Thailand)

13. Sonicator (Bransonic® Ultrasonic Cleaner, Brason Ultrasonic Co. Ltd., *Connecticut, USA)*

14. Vortex (Vortex-2 Genie, Scientific Industries, Merck Specialties Pvt. Ltd., Bangkok, Thailand)

15. Water bath (Memmert GmgH+ Co. KG, Silvia, Germany)

Methodology

Hyaluronic acid molecular weight of 9.5, 19.0, 200 and 1,100 kDa prepared using the liquid phase plasma process by Assistant Professor Dr. Isarawut Prasertsung, Faculty of Engineering, Naresuan University was used in the study.

1. Determination of antioxidant activity

1.1 DPPH radical scavenging assay

DPPH is a common abbreviation for the organic chemical compound 2, 2-diphenyl-1-picrylhydrazyl that is used in a DPPH assay to show free radical scavenging activity. It is a dark-colored which turns yellow when scavenged. The ability of HA to scavenge DPPH free radicals was measured by the method described by Hafsa et al. (75).

Hyaluronic acid solutions were prepared at various concentrations in methanol. L-ascorbic acid, used as the positive control, was also prepared at various concentrations in methanol. Fifty µl samples of both the L-ascorbic acid solutions ad HA solutions were added to a 96-well plate. DPPH solution (50 µl of 0. 2 mM solution) were added to each well and left standing in the dark for 30 min at room temperature. The absorbance of DPPH was measured by the remaining volume of DPPH by spectrophotometer at a wavelength of 517 nm. The % Inhibition was calculated by the following equation.

$$\% \text{ Inhibition of DPPH radical} = \frac{(A517 \text{ of control} - A517 \text{ of sample}) \times 100}{A517 \text{ of control}}$$

1.2 Scavenging of ABTS•+

ABTS•+ (2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) is a stable radical not found in the human body. This method uses a diode-array spectrophotometer to measure the loss of color when an antioxidant is added to the blue-green chromophore ABTS•+. The ability of HA to scavenge ABTS•+ radical was measured by the method described by RE et al. (76).

ABTS radical cation (ABTS• +) was produced by mixing 7. 4 mM ABTS and 2.6 mM potassium persulfate in dark conditions at room temperature for 12 h. The reagent solution, ABTS•+, was diluted with ethanol to obtain the absorbance of

1. 170±0. 02 units at 734 nm. An aliquot of 120 µl of the previously prepared HA solution was mixed with 80 µl ABTS solution and the mixed solution was kept in the dark condition for 5 min and the absorbance of the mixed solution was measured at 734 nm by using a UV-VIS spectrophotometer.

The positive control, Trolox was prepared in the same method. The % Inhibition was calculated by the following equation.

$$\% \text{ Inhibition of ABTS radical} = \frac{(A734 \text{ of control} - A734 \text{ of sample}) \times 100}{A734 \text{ of control}}$$

2. Determination of the Type-1 procollagen synthesis

2.1 Cytotoxicity test

The cytotoxicity of the HA was determined by using 3-(4, 5-Dimethylthiazol-2-yl) -2, 5-Dipenyltetrazolium Bromide in an MTT assay. The MTT assay is a colorimetric assay to assess cell cytotoxicity and cell viability. The reduction occurs by dehydrogenase and reducing agents present in metabolically active cells that can be converted to MTT as formazan crystals that are then dissolved in dimethylsulfoxide (blue-violet) and have the highest absorbance at 550-600 nm (77).

Human dermal fibroblasts cells were incubated in a 96-well plate at a density of 10^4cells/well for 24 h at 37°C in 5% CO_2 and a humidified atmosphere. The hyaluronic acids were prepared in various concentrations and PBS solutions were used for sample dilution. Cells were exposed to various concentrations (0 to 2000 µg/ml) of HA for 24 h at 37°C in 5% CO_2 and a humidified atmosphere. After 24 h, cells were grown in 1 mg/ml MTT (prepared in PBS, filtered with a 0.2 mm membrane) for 3 h at 37°C in 5% CO_2 and humidified atmosphere. After 3 h, 100 µl of dimethylsulfoxide (DMSO) was added to each well. The absorption value was read at 595 nm using a microplate reader. The percentage of cell viability was calculated by the following equation.

$$\% \text{ Cell viability} = (A595 \text{ of sample} / A595 \text{ of control}) \times 100$$

2.2 Determination of the Type-1 procollagen synthesis by sandwich ELISA

Human dermal fibroblasts cells were incubated in 96-well plate at a density of 10^4cells/ well for 24 h at 37°C in 5% CO_2 and a humidified atmosphere. After 24 h, the cells were exposed to 200 µl of hyaluronic acids or ascorbic acid (positive control) for 48 h following which the cell-free supernatant was collected. The amount of type-1 procollagen was determined by using human procollagen alpha 1 matched antibody pair kit (ab216064, Abcam).

Caption antibody 50 µl of 0.5µg/ml was incubated in 96-well plate for 2 h and washed 3 times with 300 µl of washing buffer (1x) . 300 µl of blocking solution was added and kept at room temperature for 2 h. The plate was washed before adding 50 µl of standard pro-collagen type 1 and 50 µl of samples per well, then kept at room temperature for 2 h with agitation at 400 rpm. The plate was then washed and 50 µl of detection antibody added. The plate was then incubated for 45 min with agitation at 400 rpm. After incubation, the plate was washed 50 µl of 0. 005µg/ ml Streptavidin/HRP added. After incubation for 45 min with agitation at 400 rpm, the plate was washed and 50 µl of TMB substrate was added. This was incubated for 3 min with agitation 400 rpm at room temperature. The reactions were stopped by adding 50 µl stop solution. The absorbance was determined by a microplate reader at 450 nm.

3. *In vitro* human skin penetration of hyaluronic acid

3.1 Calibration curve of HA by colorimetric assay

The amount of HA was determined by complexes with stain-all (SA) as reported by Fagnola, et al. (2009) (78). Stain all (SA), a cationic dye, is known to bind glycosaminoglycans and forms complexes. Hyaluronic acid solutions were prepared in various concentrations. The stain all solution (0.1 mg/ml) was prepared by dissolving 1 mg of Stain All in 22 ml of water and 3 ml of methanol. In this study, Hyaluronic acid solution was mixed with stain all solution in the ratio 7: 1 and the absorbance spectrum of the hyaluronic acid/ stain all complex was determined by colorimetric assay using UV-Vis spectrophotometer at 640 nm.

3.2 *In vitro* skin penetration studies

In vitro skin penetration of HA was studied using vertical Franz diffusion cells maintained at 37±0.5°C. The diffusion area was 2.46 cm^2 with 4 ml of the receptor medium. DI water was used as the receptor medium. The artificial membrane (Strat-M™ Membrane) was fitted between the donor and the receptor chambers. After skin equilibration for 30 min, 100 µl of the HA solution samples were evenly spread on the membrane under non-occlusive conditions. Then, 500 µl of the receptor medium was taken at predetermined time intervals of 1, 2, 3, 4, 6, 8 and 24 h and immediately replaced by the same volume of fresh receptor medium. The amount of HA penetration were determined by UV–Vis spectrophotometer at 640 nm as described previously.

Statistical analysis

All values are expressed as mean ± standard error of the mean. Data were analyzed by one-way ANOVA using the SPSS version 17. 0 software package. Significant differences between multiple means were determined by Tukey's Honestly Significant Difference test. The $p < 0.05$ was considered to be statistically significant.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter presents the findings obtained from the study, which includes analysis of hyaluronic acid, determination of antioxidant activity, determination of the Type-1 procollagen synthesis and *in vitro* permeation study

Determination of antioxidant activity

1. DPPH radical scavenging assay

The used of the 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) reacting with antioxidant compounds was discussed just over two decades ago in (79). DPPH• is a stable free radical, due to the delocalization of the spare electron on the molecule. Thus, DPPH• does not dimerize, as happens with most free radicals. The electron delocalization on the DPPH• molecule determines the occurrence of a purple color (80). Hyaluronic acid at the concentration of 5000 µg/ml and at molecular weights 9.5 to 1100 kDa showed that decreasing the molecular weight of HA significantly increased the antioxidant activity ($p < 0.05$) testing by DPPH assay. These data are shown in Tables 1- 4 and Figure 10. HA MW 9.5 kDa, HA MW 19 kDa, HA MW 200 kDa and HA MW 1100 kDa exhibited the DPPH radical scavenging activity at 92.06%, 82.37%, 16.89% and 11.76% respectively. Ascorbic acid was used as an antioxidant standard (shown in Table 5). The inhibitory concentrations (IC_{50}) are shown in Tables 1-4. IC_{50} of the HA MW 9.5 and 19.0 kDa were 1.383 and 5.8 µg /ml, respectively. While the IC_{50} of HA MW 200 and 1,100 kDa were more than 5000 µg/ml. The results showed that the low molecular weight HA possessing pronounced free radical scavenging when comparing to the high molecular weight HA.

Our results is in agreement with another previous study which showed that the DPPH radical scavenging activities of HA MW 14.5 and 45.2 kDa were 59.38% and 50.23% at concentration 1600 µg/ml (81). The smaller HA molecules were superior than bigger HA molecules in their DPPH radical scavenging activity. Molecular weights of polysaccharide are one of many factors that influences the

antioxidant activity of polysaccharide because low molecular weights would have more reductive hydroxyl group terminals to accept and eliminate the free radicals but high molecular weights polysaccharide have a more compact structure, resulting stronger intra-molecular hydrogen bond and thus making the hydrogen and amino groups restricted (82).

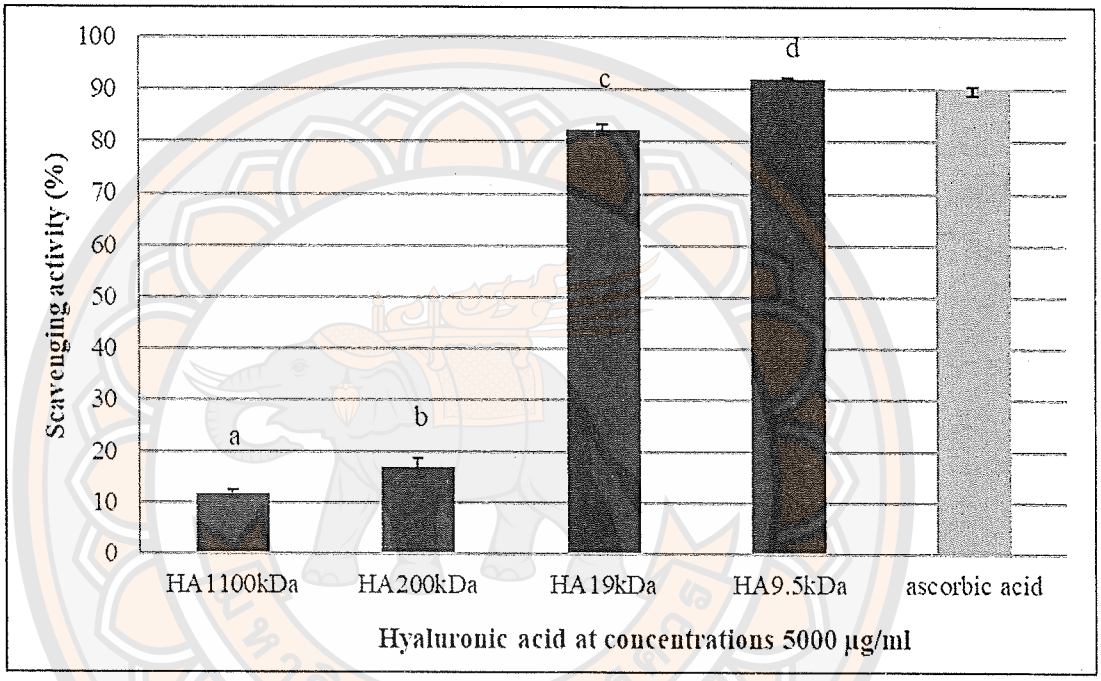


Figure 10 The percentage of free radical scavenging of HA MW 9.5, 19.0, 200 and 1100 kDa at concentrations 5000 µg/ml tested by DPPH assay (Each bar represents the mean value ± SD of three replicated determinations (n = 3). One way ANOVA; Post hoc multiple comparisons, Equal variances assumed by Tukey, considered significantly different at $p < 0.05$)

Table 1 The percentage of free radical scavenging and IC_{50} of HA MW 9.5 kDa by DPPH assay

Concentration of HA MW 9.5 kDa (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
39.10	3.28±0.69	1383.00±1.06
78.10	3.99±0.53	
156.30	7.99±0.42	
312.50	15.19±2.06	
625.00	27.33±1.33	
1250.00	48.41±2.09	
2500.00	87.35±0.31	
5000.00	92.06±0.76	

Table 2 The percentage of free radical scavenging and IC_{50} of HA MW 19.0 kDa by DPPH assay

Concentration of HA MW 19 kDa (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
39.10	2.86±0.51	2931.00±0.56
78.10	2.59±0.70	
156.30	3.76±0.49	
312.50	5.75±0.63	
625.00	11.62±0.93	
1250.00	22.21±1.21	
2500.00	51.58±0.19	
5000.00	82.37±1.85	

Table 3 The percentage of free radical scavenging and IC_{50} of HA MW 200 kDa by DPPH assay

Concentration of HA MW 200 kDa (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
39.10	1.43±0.40	> 5000
78.10	1.31±0.22	
156.30	1.57±0.25	
312.50	2.79±1.27	
625.00	4.32±0.99	
1250.00	7.87±1.63	
2500.00	11.52±0.59	
5000.00	16.90±0.94	

Table 4 The percentage of free radical scavenging and IC_{50} of HA MW 1100 kDa by DPPH assay

Concentration of HA MW 1100 kDa (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
39.10	0.08±1.79	> 5000
78.10	0.83±0.97	
156.30	0.06±0.44	
312.50	0.37±1.52	
625.00	3.15±1.29	
1250.00	5.61±0.75	
2500.00	8.11±1.15	
5000.00	11.77±0.33	

Table 5 The percentage of free radical scavenging and IC_{50} of L-ascorbic acid by DPPH assay

Concentration of L-ascorbic acid (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
0.25	0.19±0.16	7.338±1.15
0.50	0.78±0.50	
1.00	2.93±1.30	
5.00	27.24±1.52	
7.50	48.34±2.17	
10.00	58.65±1.98	
30.00	88.79±0.80	
40.00	88.69±0.72	
50.00	88.84±0.97	
100.00	88.83±0.86	

2. Scavenging of ABTS•+

Generating the ABTS (2, 2' -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances (76). The blue/green radical ABTS•+ is generated by oxidation of ABTS with potassium persulfate and decolorized by antioxidant compound (83). The antioxidant activity profile from an ABTS assay exhibited almost the same characteristics as that from the DPPH assay. The results showed that decreasing the molecular weight of HA significantly increases the antioxidant activity ($p < 0.05$) testing by ABTS assay is shown in Figure 11 and Tables 6-9. The highest antioxidant activity for HA MW 9.5 kDa was 88.04% while HA MW 1,100 kDa showed the lowest antioxidant activity at 18.81%. This result confirmed the effect of the molecular weight of HA on antioxidant activity. The inhibitory concentration (IC_{50}) of HA, needed to inhibit 50% of the ABTS radicals, is shown in Tables 6-9. The HA MW 9.5 showed IC_{50} was 167.70 µg/ml and IC_{50} of 19.00 kDa was 477.30 µg/ml. IC_{50} of HA MW 200 and 1,100 showed more than 1600 µg/ ml. The result shows that low

molecular weight HA is superior in the antioxidant activity than the high molecular weights HA. The ABTS•+ scavenging activity of various molecular weights is in agreement with the DPPH radical scavenging activity as shown in the previous section.

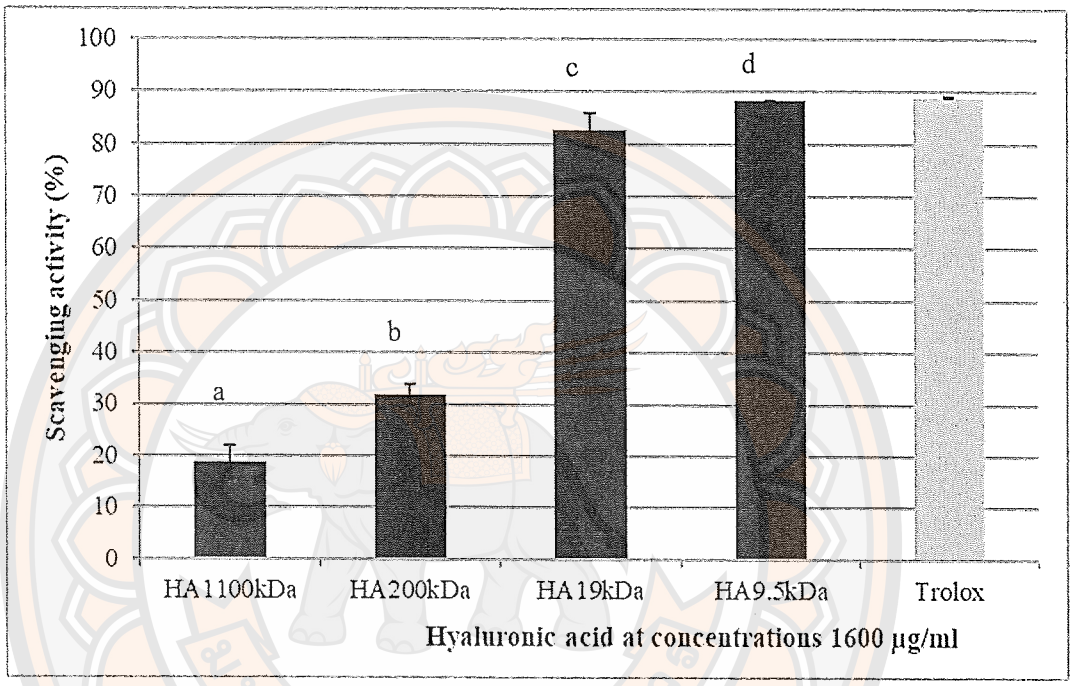


Figure 11 The percentage of free radical scavenging of HA MW 9.5, 19.0, 200 and 1100 kDa at concentrations 1600 µg/ml tested by ABTS assay (Each bar represents the mean value ± SD of three replicated determinations (n = 3). One way ANOVA; Post hoc multiple comparisons, Equal variances assumed by Tukey, considered significantly different at $p < 0.05$)

Table 6 The percentage of free radical scavenging and IC_{50} of HA MW 9.5 kDa by ABTS assay

Concentration of HA MW 9.5 kDa (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
12.50	2.82±1.68	167.70± 0.64
25.00	10.53±0.25	
50.00	17.70±2.31	
100.00	30.09±0.31	
200.00	52.73±3.12	
400.00	78.43±3.82	
800.00	87.67±2.11	
1600.00	88.33±0.05	

Table 7 The percentage of free radical scavenging and IC_{50} of HA MW 19.0 kDa by ABTS assay

Concentration of HA MW 19.0 kDa (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
12.50	1.48±0.19	477.30±1.38
25.00	4.28±1.54	
50.00	5.01±3.12	
100.00	9.13±1.30	
200.00	17.28±1.69	
400.00	37.82±1.57	
800.00	69.36±2.99	
1600.00	82.55±3.21	

Table 8 The percentage of free radical scavenging and IC_{50} of HA MW 200 kDa by ABTS assay

Concentration of HA MW 200 kDa (µg/ml)	Free radical scavenging (%)	IC_{50} (µg/ml)
12.50	1.57±1.26	> 1600
25.00	1.13±1.53	
50.00	3.29±1.47	
100.00	4.90±1.77	
200.00	6.63±3.12	
400.00	12.86±2.72	
800.00	20.88±1.26	
1600.00	31.90±2.04	

Table 9 The percentage of free radical scavenging and IC_{50} of HA MW 1100 kDa by ABTS assay

Concentration of HA MW 1100 kDa (µg/ml)	Free radical scavenging (%)	IC_{50}(µg/ml)
12.50	0.87±0.13	>1600
25.00	2.13±1.72	
50.00	2.17±0.70	
100.00	2.69±0.51	
200.00	4.67±0.78	
400.00	7.23±3.17	
800.00	13.09±2.25	
1600.00	18.82±3.11	

Table 10 The percentage of free radical scavenging of tolox by ABTS assay

Concentration of Tolox (µg/ml)	Free radical scavenging (%)	IC_{50}(µg/ml)
0.50	1.44±0.29	11.01± 1.90
3.90	14.20±3.52	
5.00	25.04±1.71	
7.81	32.60±2.33	
15.62	64.31±0.93	
31.25	82.83±1.61	
50.00	92.34±0.42	
62.50	94.44±0.80	
100.00	94.48±0.73	
125.00	94.57±0.53	

Determination of the Type-1 procollagen synthesis

1. Cytotoxicity test on human dermal fibroblast cells

The percentages cell viability of human dermal fibroblasts cells after HA MW 9.5, 19.0, 200 and 1100 kDa treatment at various concentrations (3.91-2,000 µg/ml) are shown in Figures 12-15. The results showed that HA at all molecular weights were unable to inhibit cell growth at concentrations 3.91-2000 µg/ml (p>0.05) when compared with the control. Therefore, the chosen maximum concentration of HA for pro-collagen type 1 synthesis test was 2000 µg/ml.

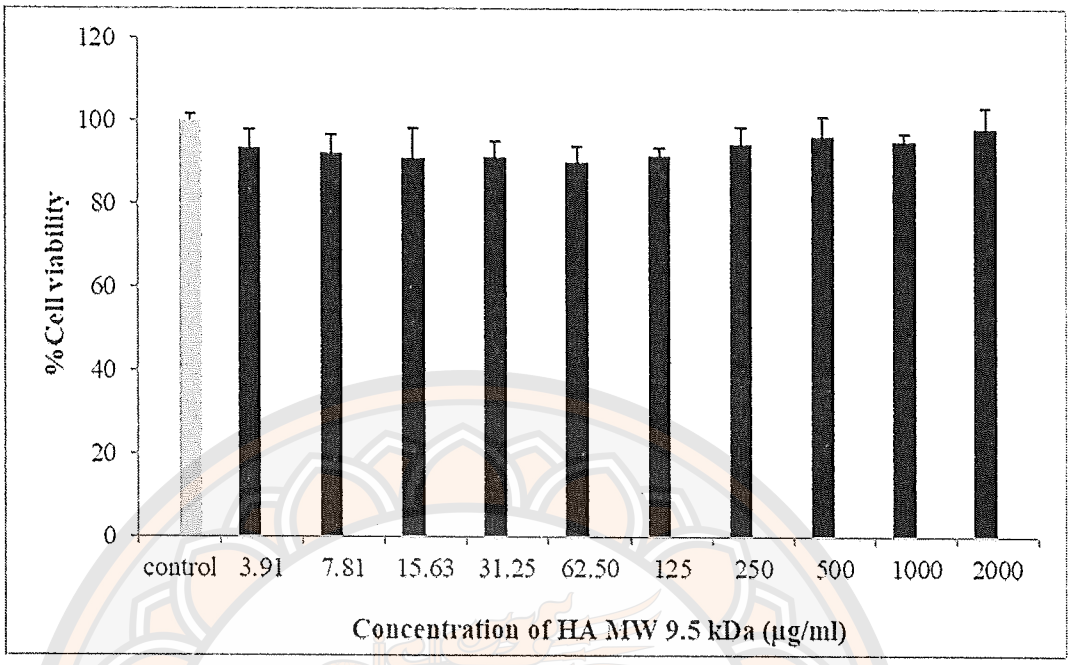


Figure 12 The cell viability of human dermal fibroblast cells treated with various concentrations of HA MW 9.5 kDa. The control group was untreated cells. Each bar represents the mean value ± SD of three replicated determinations (n = 3). Comparisons were subjected to a Student *t*-test, considered significantly different at $p < 0.05$

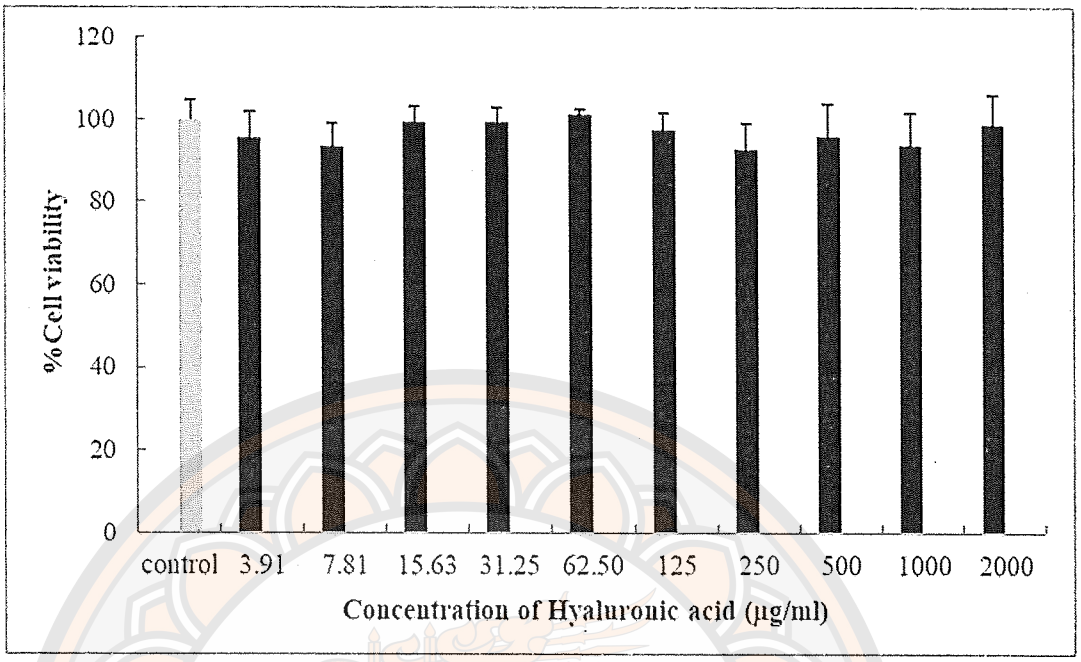


Figure 13 The cell viability of human dermal fibroblast cells treated with various concentrations of HA MW 19.0 kDa. The control group was untreated cells. Each bar represents the mean value ± SD of three replicated determinations (n = 3). Comparisons were subjected to a student *t*-test, considered significantly different at $p < 0.05$

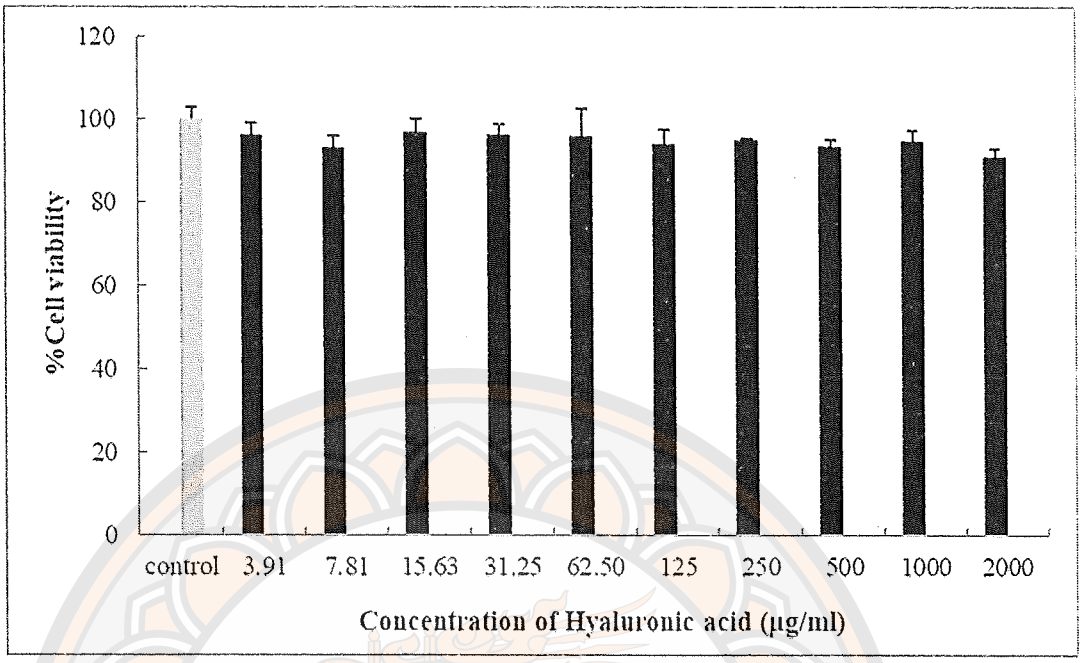


Figure 14 The cell viability of human dermal fibroblast cells treated with various concentrations of HA MW 200 kDa. The control group was untreated cells. Each bar represents the mean value ± SD of three replicated determinations (n = 3). Comparisons were subjected to a student *t*-test, considered significantly different at $p < 0.05$

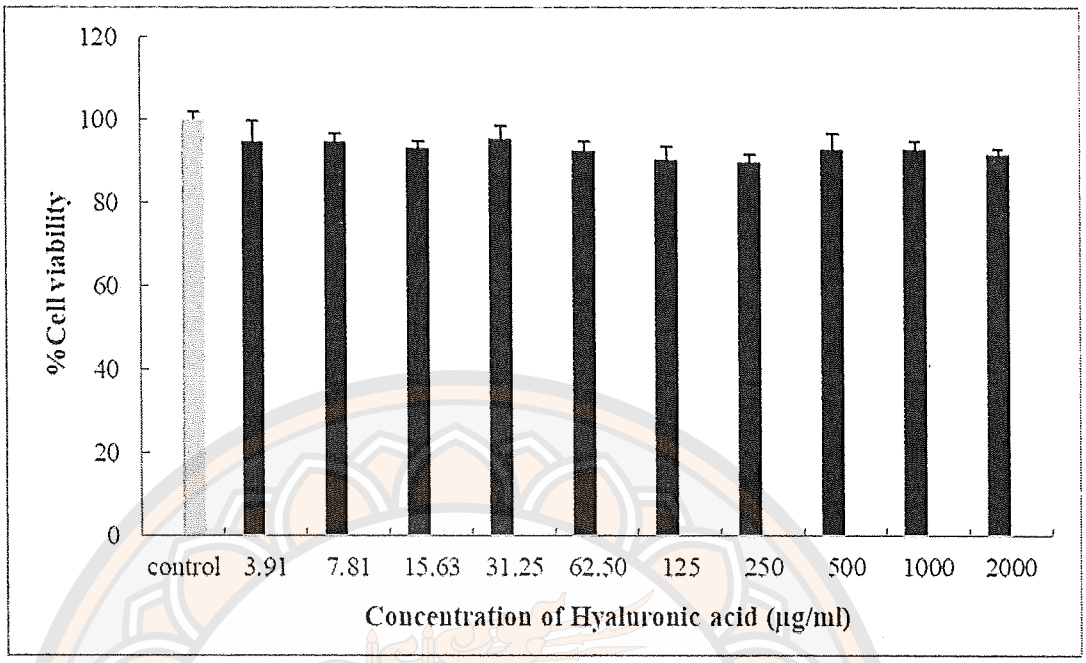


Figure 15 The cell viability of human dermal fibroblast cells treated with various concentrations of HA MW 1100 kDa. The control group was untreated cells. Each bar represents the mean value ± SD of three replicated determinations (n = 3). Comparisons were subjected to a student *t*-test, considered significantly different at $p < 0.05$

2. Determination of the Type-1 procollagen synthesis by sandwich ELISA

The aging of the skin is a natural occurrence caused by two main factors: firstly, intrinsic aging due to deterioration of the body as the time passed and, secondly, exposure to external factors (extrinsic aging) such as UV light (84). The aging of the skin has a noticeable appearance with deep grooves, wrinkles, and lack of elasticity. When comparing older skin to young skin, the skin of older people has reduced collagen proteins, elastin and glycosaminoglycan (85). Collagen distributed in the dermis is type I and III.

In our study, the stimulation in collagen synthesis was intended to qualtitate by pro-collagen type-1 content in the cell-free supernatant of fibroblast cells after treatment with various concentrations of HA MW 9.5, 19.0, 200 and 1100 kDa using PIP EIA Kit. The results showed that the human dermal fibroblast cells treated with HA MW 9.5, 19.0, 200 and 1100 kDa at concentration 2000 µg/ml showed no difference in the amount of type 1 procollagen when compared to that of the control at 48 h ($p > 0.05$) (Figure 17). The procollagen type 1 standard curve was presented in Figure 16. However, There was a report by Frank Wang et al indicated that when HA fillers known as nonanimal stabilized hyaluronic acid (NASHA) injected into the skin at 4 and 13 weeks, respectively, the type 1 procollagen was increased for 10.5 ± 2.4-fold (P = .003) and 17.6 ± 6.9-fold (P = .04), and type 3 procollagen was increased for 7.9 ± 1.8- fold (P = .004) and 11.6 ± 4.6-fold (P = .045). They indicated that NASHA injections induced robust collagen production through several potential mechanisms, including the mechanical stretching of fibroblasts, stimulation of growth factors, and inhibition of collagen breakdown (86-87). In *in vitro* test, we were unable to test on dermal human fibroblasts for a long period of time due to the limitation of the cell culture area to the growth of fibroblasts. Therefore, this could be one possible reason that our *in vitro* results were not in agreement with the mentioned *in vivo* report.

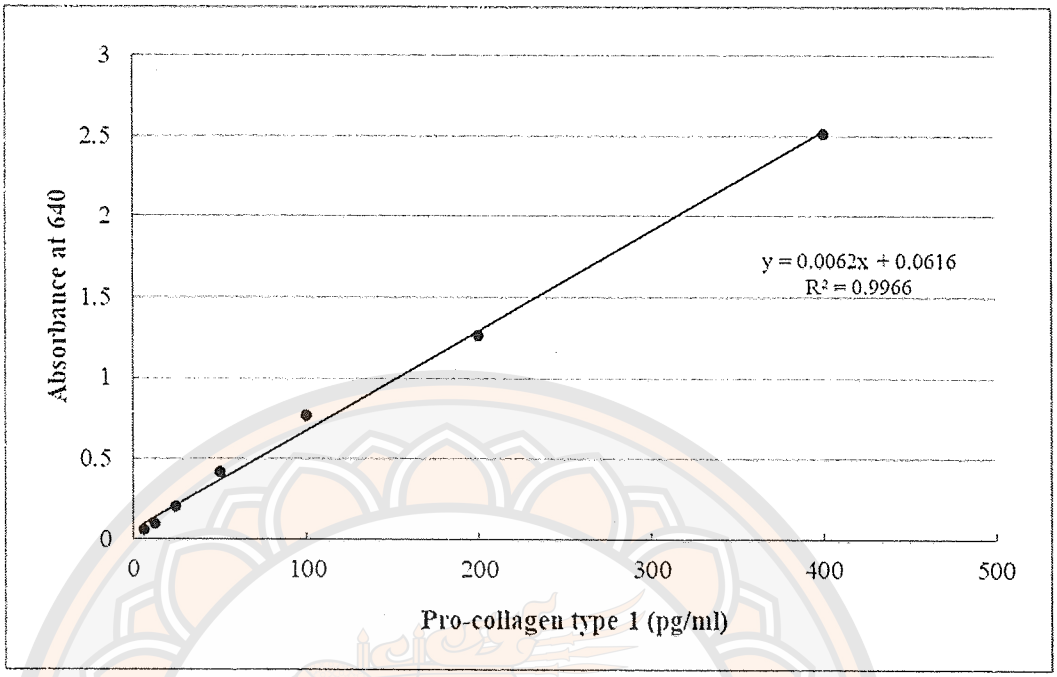


Figure 16 The Pro-collagen type-1 standard curve

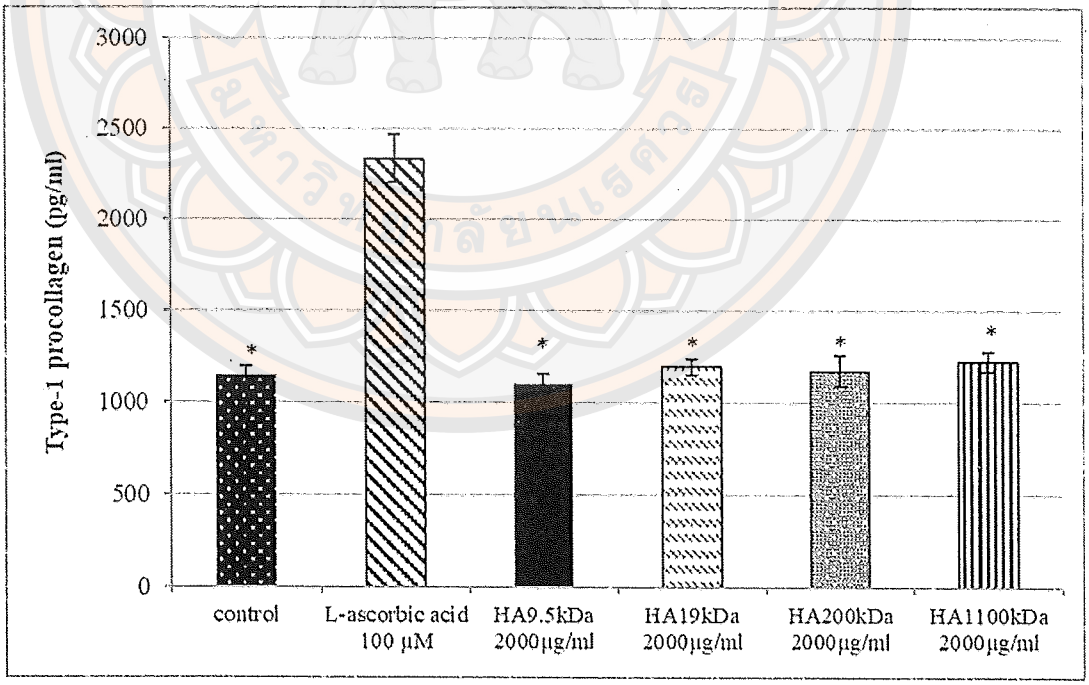


Figure 17 The Pro-collagen type 1 synthesis of human dermal fibroblast cells after the cell medium was changed to a new medium and incubated for 48 h. Each bar represents the mean value ± SE of three replicated determinations (n = 3) considered significantly different at $p < 0.05$

***In vitro* human skin penetration of hyaluronic acid**

1. Calibration curve of HA by colorimetric assay

HA MW 9.5, 19, 200, 1100 kDa were determined by colorimetric assay using UV-Vis spectrophotometer at the wavelength of 640 nm. Stain All (SA) has magenta color and cationic dye which binds with HA (anionic polymer) to form HA/dye complexes by ionic interaction (78). The calibration curves of HA MW 9.5, 19, 200, 1100 kDa in the range 0.039-20.0, 0.029-15.0, 0.078-10.0 and 0.078-10.0 µg/ ml respectively were constructed by their absorbance values versus HA concentrations. The calibration curve equations for all HA were as follow: the standard curves for determination of HA MW 9.5 kDa, which were y = 0.0138x + 0.0431 (R^2= 0.0998) (Figure 18), HA MW 19 kDa was y = 0.0235x+ 0.0399 (R^2=0.0996) (Figure 19), HA MW 200 kDa was y=0.0403x+0.0388 (R^2 = 0.0997) (Figure 20), HA MW 1100 kDa was y = 0.0507x + 0.0466 (R^2 = 0.0998) (Figure 21).

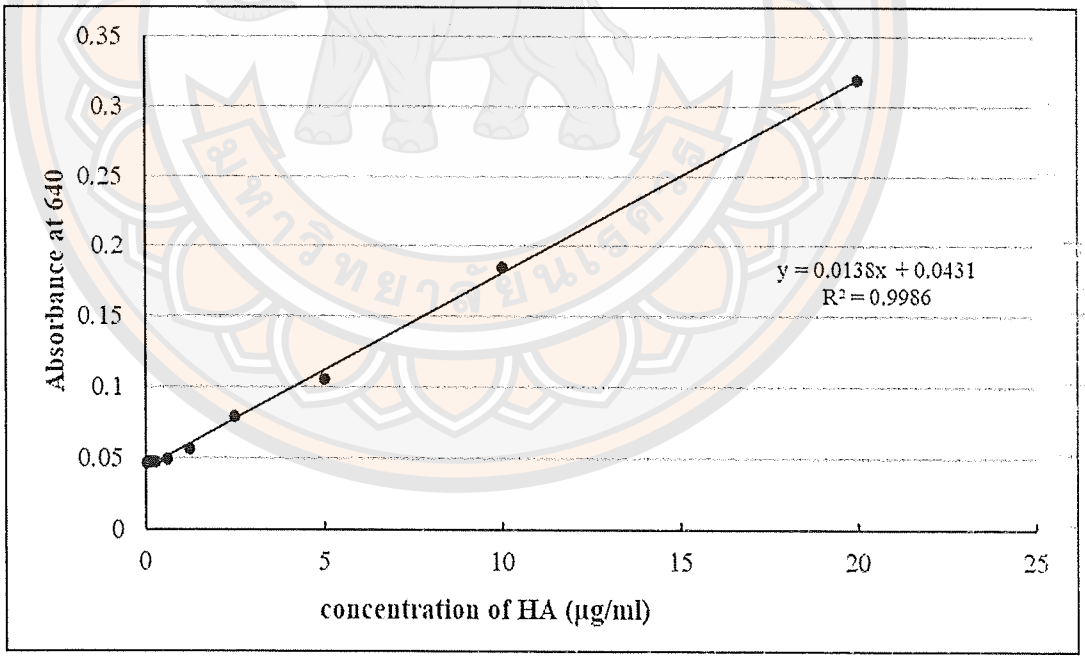


Figure 18 Calibration curve of HA MW 9.5 kDa using colorimetric assay by UV-Vis spectrophotometer at 640 nm

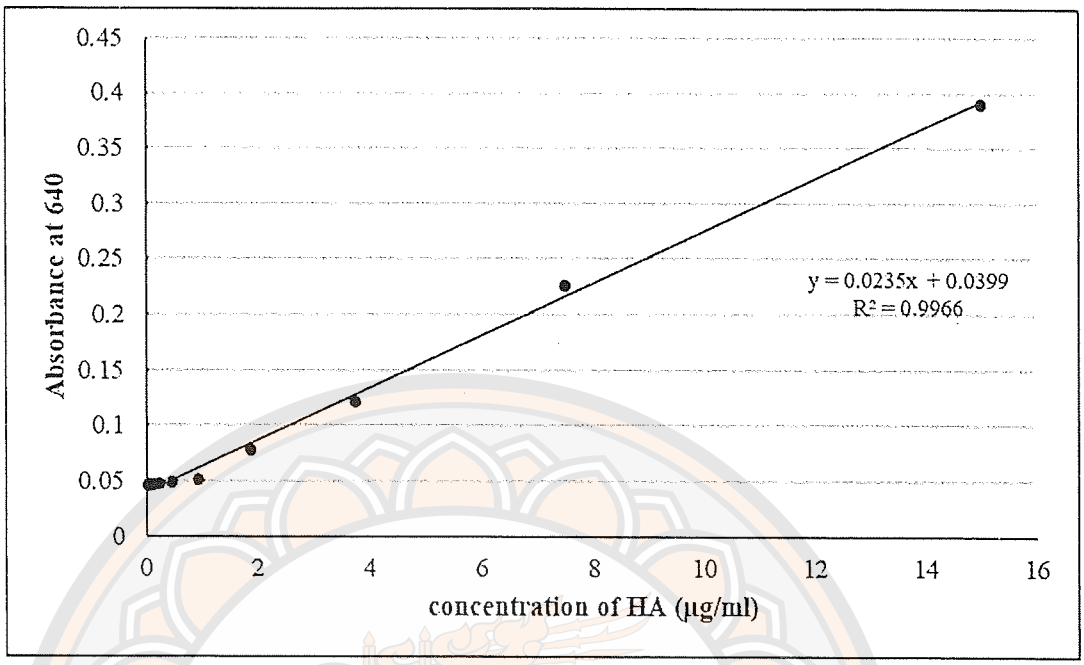


Figure 19 Calibration curve of HA MW 19.0 kDa using colorimetric assay by UV-Vis spectrophotometer at 640 nm

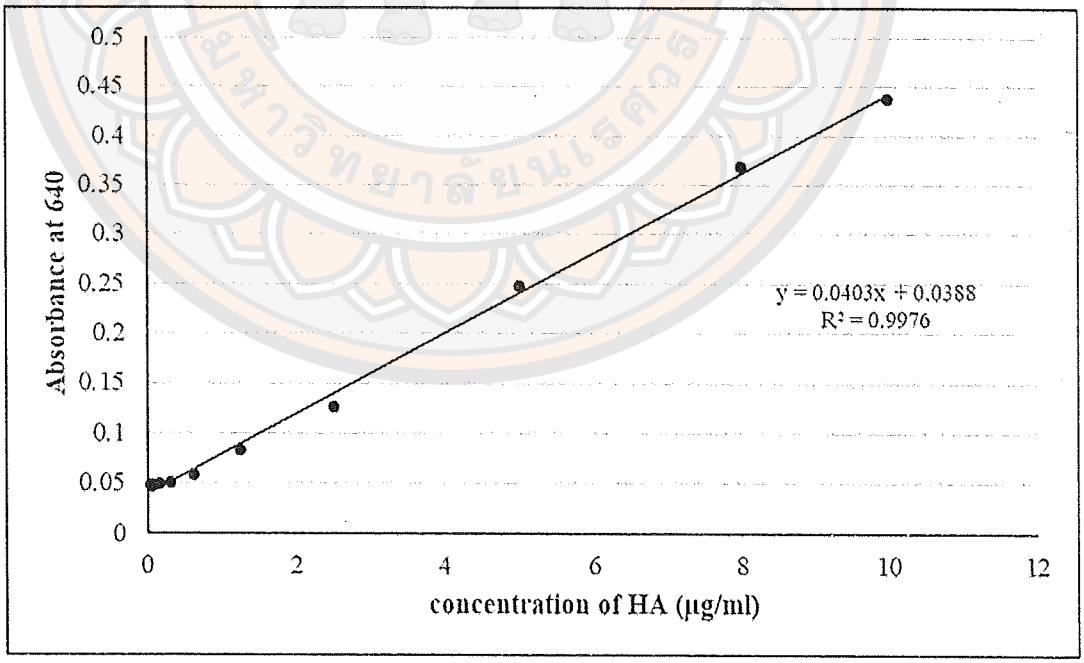


Figure 20 Calibration curve of HA MW 200 kDa using colorimetric assay by UV-Vis spectrophotometer at 640 nm

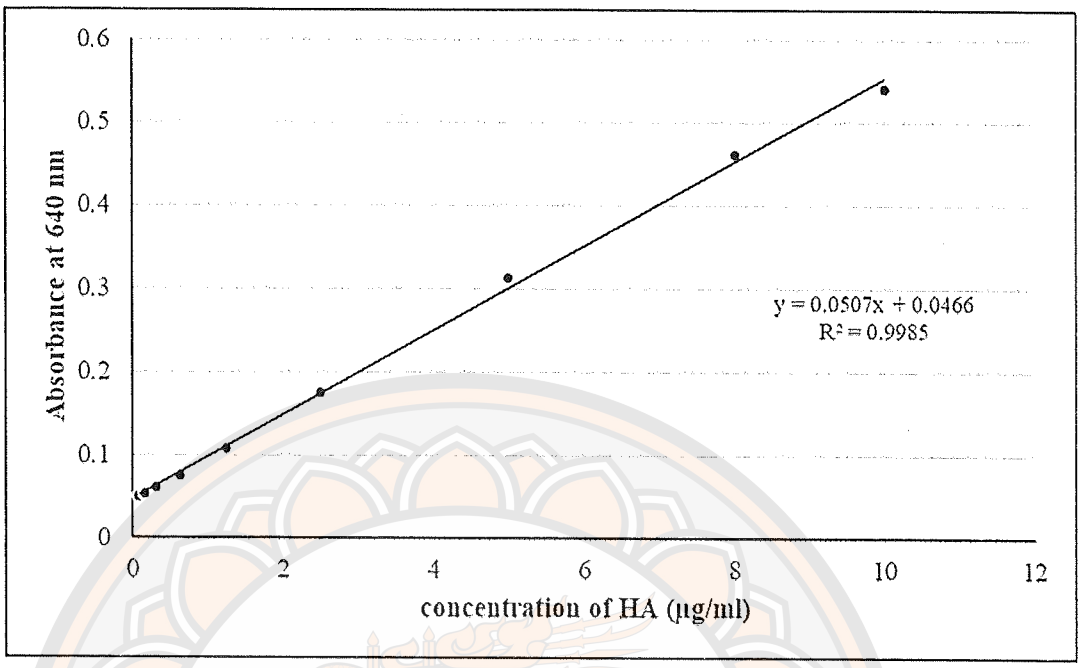


Figure 21 Calibration curve of HA MW 1100 kDa using colorimetric assay by UV-Vis spectrophotometer at 640 nm

2. *In vitro* skin penetration studies

In vitro permeation profiles for solutions of the different HA molecular weights are shown in Table 11 and Figure 22 as cumulative HA over time. The HA Mw 9.5 kDa solution (2.915±0.42%) demonstrated a superior skin penetration over HA Mw 19.0 kDa (2.659±0.06%), HA Mw 200 kDa (1.792±0.01%) and HA Mw 1100 kDa (0.468±0.04%) at 24 h study (p <0.05). In Table 12 showed that HA Mw 9.5 kDa solution demonstrated a higher skin penetration into the membrane of artificial skin 9.44±0.67% when compared with that of the higher Mw HA ($p < 0.05$). This result corresponds with previous reports indicated that the low molecular weight HA (50-300 kDa) demonstrated better skin penetration abilities which resulted in a significant improvement in skin hydration and wrinkle depth. HA with a high molecular weight (more than 1000 kDa) stayed only at the surface of the skin where it created a film that caused a diminution of skin evaporation (88) . Brown et al have shown that HA enhanced significant partitioning of diclofenac into human skin and its retention and localization in the epidermis when compared with an aqueous control (89). Farwick et al did show that HA molecular weights more than 300 kDa penetrated only small

amounts intopig ear skin. In contrast, low molecular weigh HA (50 kDa) revealed at least three times higher skin penetration after only 5 h study (90) . HA has high hygroscopic properties andhigh capacity to blind water, therefore, these properties effect on modulating tissue hydration. The possible explanation of low molecular weight HA penetration into skin is the increased hydration of the surface layer of skin because when skin hydration the compact substance of the stratum corneum was opened by loosening the dense, closely packed cells thus increasing the permeability of HA (91).

Table 11 The percentage of cumulative permeation of HA different molecular weights at various times (n=3)

Time (h)	Cumulative HA permeation (%)			
	HA 9.5 kDa	HA 19.0 kDa	HA 200 kDa	HA 1100 kDa
1	1.369±0.29 [a]	1.265±0.07 [b]	0.935±0.07[c]	0.224±0.04 [d]
2	1.701±0.30 [a]	1.537±0.12 [b]	1.151±0.02 [c]	0.244±0.05 [d]
3	1.892±0.27 [a]	1.747±0.10 [b]	1.314±0.02 [c]	0.297±0.08 [d]
4	2.051±0.41 [a]	1.962±0.05 [b]	1.403±0.07 [c]	0.310±0.07 [d]
6	2.238±0.13 [a]	2.011±0.08 [b]	1.553±0.06 [c]	0.329±0.07 [d]
8	2.522±0.40 [a]	2.139±0.06 [b]	1.596±0.04 [c]	0.338±0.08 [d]
24	2.915±0.42 [a]	2.659±0.06 [b]	1.792±0.01 [c]	0.468±0.04 [d]

Note: One way ANOVA; Post hoc multiple comparisons; Equal variances assumed by Tuckey; $p<0.05$

a, b, c and d: Mean values with different letters in the column are significantly different for all molecular weights hyaluronic acid

Table 12 Disposition of HA (expressed as a percent of applied formulation ± SE) in artificial skin after topical application 24 h (n=3)

Hyaluronic acid (kDa)	Compartments			% Recovery
	Donor	membrane	Receiver	
9.5	85.46±0.47[a]	9.44±0.67[a]	2.92±0.42[a]	97.815±1.44
19.0	91.86±0.70[b]	4.20±0.03[b]	2.66±0.06[b]	98.72±0.54
200.0	93.87±0.87[c]	3.64±0.05[c]	1.79±0.0[c]	99.30±1.10
1100.0	95.93±0.81[d]	1.22±0.06[d]	0.49±0.04[d]	97.64±0.69

Note: One way ANOVA; Post hoc multiple comparisons; Equal variances assumed by Tuckey; $p<0.05$

a, b, c and d: Mean values with different letters in the column are significantly different for all molecular weights hyaluronic acid

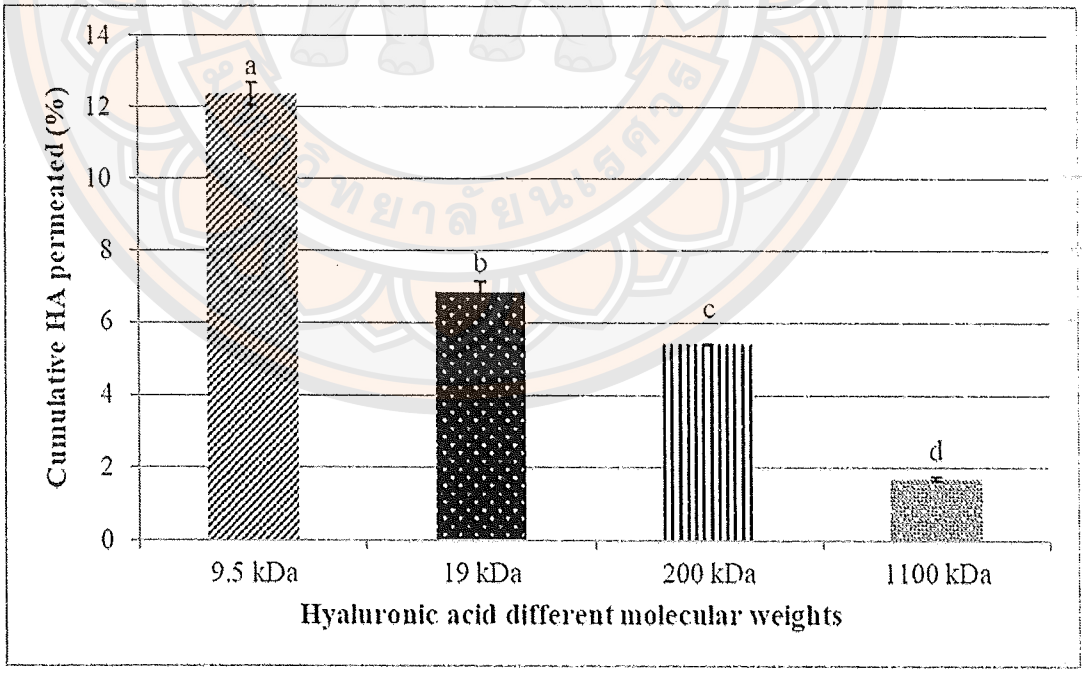


Figure 22 Amount of HA different molecular weights (mean±SE)transported into and across artificial skin after topical application 24 h (n=3)

CHAPTER V

CONCLUSION

In this study, HA at the molecular weight of 9.5, 19.0, 200, 1100 kDa, was prepared by the liquid phase plasma process at the Faculty of Engineering, Naresuan University. These HA solutions were used to investigate the antioxidant activity, pro-collagen synthesis activity, and *in vitro* skin penetration.

We showed that the biological activity of HA MW 9.5 and 19.0 kDa were promissing to be used as an antioxidant agent according to the results from DPPH and ABTS radicals anti-oxidant studies. We also demonstrated that HA MW 9.5, 19.0, 200 and 1100 kDa at a concentration 2,000 µg/ml were non-toxic on human dermal fibroblasts cells. However, the lower molecular weights (9.5 and 19.0 kDa) and the higher molecular weights (200 and 1100 kDa) were unable to stimulate type-1 procollagen synthesis on the human fibroblast cell culture. In the *in vitro* skin permeation study, the low molecular weights of HA showed better permeation into the skin than the high molecular weight HAs. Therefore, the low molecular weight HA generated by the liquid plasma process is of interest as a promising ingredient for cosmetic formulation.

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