RSC Advances

Layered silicate clay functionalized with amino acids: Wound healing application

Journal:	*RSC Advances*
Manuscript ID:	RA-ART-06-2014-005216.R1
Article Type:	Paper
Date Submitted by the Author:	28-Jul-2014
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RSC Advances

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Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

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Natural materials such as "healing clays" in mud spas have been used for healing of skin ailments and wounds since prehistoric times. In this study, laponite, a synthetic clay mineral with similar properties to natural healing clays has been functionalized with different amino acids for the purpose of wound dressing application. Physico-chemical properties of prepared laponite/amino acid gels were characterised using various analytical methods. X-ray diffraction and thermogravimetric analysis confirmed the presence of these amino acids in the prepared materials. The release profile of amino acid in simulated wound exudate fluid demonstrated the highest release from laponite/lysine group with up to 50% of the intercalated lysine was released. The effect of extracts (obtained from the prepared laponite/amino acid gels) was tested on human skin fibroblast. Cell proliferation, oxidative stress, anti-oxidant property, and *in vitro* wound healing (scratch assay model) were assessed. Extracts obtained from laponite alone and laponite combined with arginine, lysine and leucine promoted fibroblast proliferation whereas extract from laponite/glutamic acid significantly inhibited cell proliferation. Based on the *in vitro* results presented in this work, laponite/arginine, laponite/leucine and laponite/lysine can be potentially applied as a wound dressing to promote healing process.

Introduction

Natural clay minerals, with many benefits for human health, have been used in traditional medicine and different industrial applications[1]. Use of clays and clay minerals for protection and disease treatment is as old as mankind. Carretero *et al.* [2] reviewed in detail the applications and benefits of the clay minerals upon human health. In recent time, clay minerals have been used more widely in formulation of pharmaceutical and cosmetic products. These clay minerals possess specific properties such as large surface area and adsorption capacity, optimal rheological properties as well as chemical inertness and low toxicity[3, 4], which make them a good candidate for the pharmaceutical applications, particularly for topical administration. Clay minerals can create skin feel upon their application on skin due to their specific rheological property. Clay minerals are also characterised as materials with high absorbency capacity. Therefore, they have also been applied on skin to absorb excess exudates and toxins.[5] Clay minerals in mud bathes can be applied upon the skin to treat wounds and skin ailments. For example, they have been used to treat boils, acne, ulcers, abscess, seborrhoea, and wound.[6]

The need for safe, therapeutically effective, and patient compliant drug delivery systems drives researchers to explore various biomaterials as suitable drug carrier. Local drug delivery systems are designed to administer drugs directly to the site of action and maintain local therapeutic concentration of drugs that is required for an effective treatment. It has been shown that the use of clay in drug formulations is rapidly increasing due to the superior characteristics of clay minerals compared to other drug carriers. These characteristics include controlled release formulations, by intercalating drug molecules between clay nano-discs, improved dispersibility of drugs with low water solubility, better skin absorption and target delivery .[7-9] The main specific characteristics of clay minerals that make them promising in pharmaceutical applications is their high sorptive capacity, optimal rheological behaviour, and ion exchange capacity, which later allows the release of the intercalated drug in contact with body fluid through ionic exchange process.

Skin wounds are damages to skin caused by abrasions, incisions, and burns or in general any kind of trauma.[10] Although the applications of clay minerals for the treatment of skin ailments and wounds have been established in traditional therapies for thousands of years,[11] their potential use for wound healing has not yet been fully assessed scientifically. The clay minerals that have been used in traditional medicine for treatment of skin wounds and diseases are called "healing clays".[12] They mainly contain disordered or highly crystalline kaolinite, smectite (montmorillonite) or ilitite.[13] In these healing clays, bentonite, laponite and montmorillonite have been subject to many studies for various biomedical applications, mostly as a drug delivery system.[4] Despite the occurrence of clay minerals in nature, recently many types of synthetic clays have been produced and used in different industries.[14, 15] Laponite is a synthetic clay mineral with an expandable 2:1 type of alumino-silicate layered structure, which composed of nano-size disk-like particles with approximately 25 nm in diameter and 1 nm in thickness.[16] Laponite is a gel forming material and has “skin feel” when applied on skin, making it suitable for skin applications. In addition, the lamellar space in laponite structure can expand to intercalate different molecules. This expandable structure makes laponite an ideal carrier for biomolecules[14] and drugs[17] to be delivered at the wound site, thereby accelerating and

improving wound healing. Using laponite as a carrier for amino acids that enhance wound healing process is extremely attracting due to laponite low cost, biocompatibility, high surface area (layered structure), eco-friendly characteristics and high cation exchange capacity. The later property facilitates the intercalation of positively charged amino acid molecules into laponite inter-layers and at the same time eases amino acid release from laponite once the gel comes into contact with biological fluids containing cations such as Ca^{2+}, K^{+}, and Mg^{2+}[18]. Clay minerals have been also used in combination with other substrates in form of nano-composites for the application of skin drug delivery and wound healing.[19, 20, 21] For example chitosan/laponite[22] or PVA/ montmorillonite (polyvinyl alcohol)[21] nano-composites have been tested for wound dressing applications.

Previous studies indicated that amino acids like arginine, lysine, proline, glycine, and leucine can ameliorate the healing process of wound through various mechanisms such as enhanced angiogenesis, epithelialization, and promotion of collagen synthesis.[19, 23] It has been shown that there are several mechanisms involved in the loading of amino acids into clay structure. These mechanisms include cation exchange (exchange of Na^{+} ions of laponite, resident between laponite inter-layers, with amino acids of surrounding solution); amino acid dipole interaction with the interlayer cations (Na^{+}), interaction with other intercalated amino acids, hydrogen bonding and van der Waals forces between amino acids and clay particles[24]. In general, positively charged basic amino acids such as lysine, arginine and histidine are more strongly adsorbed than neutral or acidic counterparts largely due to the cationic exchange process.[25]

The present study developed a new class of clay-based wound dressing gel combined with amino acids: arginine, leucine, lysine and glutamic acid. These amino acids were chosen based on their different side chain group: acidic, basic and neutral side chain. Furthermore, these amino acids are all suggested to be essential in wound healing process.[26] For example, arginine is well known for generating nitric oxide (NO), which promotes wound healing process.[27] Moreover, leucine, lysine and glutamic acid have shown to be beneficial in wound healing by increasing deposition of collagen at the wound site.[19] For the first time this study investigated the intercalation of these amino acids into laponite, a synthetic clay mineral.

Material characterisation on prepared laponite/amino acid (Lap/AA) samples was conducted using attenuated total reflectance - Fourier transform infrared (ATR-FTIR), X-ray diffraction analysis (XRD), thermogravimetric analysis (TGA), scanning electron microscopy (SEM) and nitrogen sorption analysis. The release profile of amino acids from Lap/AA samples in the simulated wound exudate fluid was also investigated. Laponite degradation in simulated wound exudate was determined by measuring the amount of released magnesium ion into the medium using atomic absorption spectroscopy. The *in vitro* wound healing characteristics of the prepared materials containing amino acids were assessed on skin fibroblast cells via proliferation and scratch assays.

The effect of oxidative stress on wound healing process is well established and it is known that a decrease in the generation or the effect of ROS (Reactive oxygen species) could be beneficial in healing process.[28] However, low levels of ROS are required for cellular signalling, in particular for angiogenesis.[29] The effect of extracts of materials on generation of oxidative stress and antioxidant capacity were also studied in this work.

Materials and Methods

Preparing laponite/amino acid (Lap/AA) gel

Samples of laponite/arginine (Lap/Arg), laponite/lysine (Lap/Lys), laponite/leucine (Lap/Leu) and laponite/glutamic acid (Lap/Glu) were prepared using laponite XLG (Donated by Amtrade Company, Australia). Amino acid powders were purchased from Sigma-Aldrich, Australia. To prepare samples, 1% (w/v) laponite was first dispersed in de-ionized water for 10 minutes while the pH adjusted to 3 using 0.1 M HCl. When the laponite dispersion was fully hydrated and turned to a clear solution, 0.2 M of amino acid was dissolved in the laponite dispersion. After 24 hours stirring, the dispersion was centrifuged at 5000 rpm for 5 minutes to remove the excess water. Sediment gel was washed once to remove the loosely deposited excess amino acid and centrifuged to recover the gel. The supernatant was decanted and the sediment gel was recovered and freeze-dried.

Extracts of Lap/AA samples were prepared according to the ISO10993.5 (tests for indirect cytotoxicity). In brief, 300 mg of freeze-dried sample was incubated in 10 ml Dulbecco's modified eagle medium (DMEM) cell culture medium at 37 ºC for 24 hours under aseptic condition while it was shaking. The obtained extracts were then subject to filter sterilization. The amount of released amino acid was measured using ninhydrin method as described before.[30] The concentration of released amino acid in DMEM was adjusted to 20 nM by adding fresh DMEM. As control, the extract of freeze-dried laponite powder (without amino acid) was also obtained under the same condition. The obtained extracts were used for fibroblast proliferation assay, scratch test, and oxidative stress analysis.

Physico-chemical characterisation

X-ray diffraction - The powder diffraction patterns of prepared samples were obtained from 2° to 70° 2θ by X-ray diffractometer (D-6000, Shimadzu) using CuKα radiation, with 0.02° step size, 30 mA current, and 40 kV voltage.

The angular 2θ position, d-spacing and the full-width at half-maximum (FW/HM) of the (001) basal reflection of laponite were determined using the obtained XRD patterns from samples. The d-spacing was calculated from the angular 2θ position of the (001) reflection using the Bragg's equation 1.

$$\lambda = 2d\sin\theta \quad \text{(Eq. 1)}$$

Where λ is the wavelength and θ is the scattering angle.

Attenuated total reflectance - Fourier transform infrared (ATR-FTIR) - The ATR-FTIR spectra of samples in form of powder were obtained using Varian 660-IR (ATR-FTIR) spectrometer. All spectra were obtained in the wavelength range from 600 to 4000 cm^{-1} with 2 cm^{-1} spectral resolution.

Thermal properties - To determine the presence of amino acid in laponite structure and its effect on thermal behaviour of laponite, transition temperature of samples was measured using thermogravimetric analysis (TGA), Mettler-Toledo. Total mass of each sample was 5-10 mg. Samples were loaded into alumina pan and heated at the rate of 10 °C/min under a dynamic nitrogen flow (150 cm^3/min) from room temperature to 900 °C. Temperature, sample weight and differential weight were recorded. Differential weight was used for derivative thermogravimetric analysis (DTA).

Specific surface area measurement - Specific surface area and pore volume of samples were determined by nitrogen adsorption/desorption isotherms at 77 K using a micro-meritics Tristar II sorptometer. Samples were degassed with micro-meritics VacPac 0.61 at 50 °C over night under vacuum. Specific surface area was calculated according to the Brunauer-

Emmett-Teller (BET) model. The BET method was used to determine the specific surface area (S_{BET}) using the adsorption data in the relative pressure range of 0.05 – 1.00 p/p° and the pore volume was calculated using desorption of nitrogen isotherm using the Barrett-Joyner-Halenda (BJH) method.

Release study

Release of amino acids in simulated wound exudate fluid- The release of each amino acid from Lap/AA samples was conducted in simulated wound exudate fluid (SWEF) using shaker incubator (50 rpm) at 37 °C over 24 hours. SWEF prepared according to the NSAI standard 13726-1 (National standards authority of Ireland). For this purpose, 100 ml of SWEF was added to the flasks containing 500 mg freeze-dried Lap/AA powder. Aliquots of 5 ml were taken at pre-determined intervals and replaced with the same volume of pre-warmed fresh SWEF. The amino acid released in the SWEF was quantified according to ninhydrin photometric analysis method.[30] Briefly, 1 ml of freshly made ninhydrin-hydrindantin solution (Ninhydrin and Hydrindantin dehydrate, Sigma-Aldrich) was added to 1 ml of amino acid aliquot from the release study. The resulting solution was placed in a test tube and heated for 10 minutes in boiling water bath. Then 5 ml of 50% ethanol was added to each test tube and the tubes were placed in ice bath to terminate the reaction. The absorbance of the resulting solutions was determined using UV spectrometer (UV-1800 Shimadzu) at 570 nm.

Magnesium release from laponite structure in simulated wound exudate fluid - The degradation of materials in SWEF was measured from the amount of Mg^{2+} released in solution. Aliquots taken from release study were used to quantify the concentration of Mg^{2+} using Shimadzu AA-7000 atomic absorption spectrometer (AAS). Although the release of amino acids reported for 24 hrs, the release of magnesium in same medium continued for over 4 days. AAS was performed using 0.7 nm slit width, 8 mA lamp current and 1.8 L/min flow rate of air-acetylene flame gases with a cathode lamp.

Wound healing properties

Cell culture - Primary dermal fibroblasts from male human neonatal foreskin were cultured in DMEM supplemented with 10% (v/v) of fetal bovine serum (FBS), and 1% (v/v) of penicillin/streptomycin/amphotericin B antibiotic solution. Fibroblasts were cultured at 37ºC in a humidified 5% CO_2 atmosphere. The medium was refreshed every 3 days. DMEM and FBS were purchased from PAA (Germany). Antibiotic solution was obtained from Sigma Aldrich.

Proliferation assay- Proliferation of fibroblasts in the presence of released substances from the prepared Lap/AA gels, called "extracts" from now on, was assessed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, CellTiter 96® Aqueous One Solution Cell Proliferation Assay from Promega.

Fibroblasts were seeded in 96 well-plates with the density of 2 × 10^4 cell per well and incubated overnight at 37 ºC. Cells in wells were then treated with 100 µl of extracts for 3 days. After 3-day treatment, MTS reagent was added to the cells according to the manufacturer's protocol and incubated for 2 hours. Finally the absorbance was recorded using microplate reader (Polarstar) at 490 nm.

Scratch assay - To study the effect of extracts obtained from the prepared Lap/AA gels on wound healing process, an *in vitro* wound healing model called "scratch assay" was used. [31] In this assay, 1 × 10^6 fibroblasts per well were seeded into 6-well plate and incubated over night to reach a confluent monolayer. Then a scratch through the central axis of well plate was gently made

using a p10 pipette tip. Debris and dead cells washed twice with Dulbecco's phosphate buffered saline (DPBS). Wounded cells were stimulated with the extracts (2 ml per well) for the experiment time. Cell migration and gap filling were imaged using bright field microscope (Leica) at 0, 24 and 48 hours post-treatment. In order to determine the percentage of cell coverage in the scratch area, the area of cell-free space was measured using image analysis software. Three wells were counted for each condition, one scratch was done per well, and three measurements were carried on each scratch. The cell coverage in the scratch area was expressed as a percentage of that in the control group (without treatment) for each time point.

Oxidative stress - To investigate the potential role of materials extracts in oxidative stress generation, total level of reactive oxygen species (ROS) in fibroblasts were measured after treating the cells with the extracts. The ROS production was measured using H_2-DCF-DA reagent (2′, 7′-dichlorofluorescein diacetate, Invitogen). Fibroblasts were seeded in 96-well plate with the density of 2×10^4 cells per well. After overnight incubation of cells, 10 µM H_2-DCF-DA was added to each well and incubated for 30 minutes. Cells were washed with DPBS and then stimulated with 200 µl extract containing 20 nM concentration of released amino acid. As control, ROS level in cells stained with H_2-DCF-DA without any further treatment was also measured. After 3 hours incubation, the total ROS formation was measured using a fluorescence micro-plate reader (Polarstar) with the excitation of 485 nm and emission of 520 nm.

The total ROS scavenging capacity of Lap/AA extracts were also analysed. Pre-stained cells with H_2-DCF-DA (as mentioned above) were treated with hydrogen peroxide (10 µM H_2O_2 for 30 min) to generate ROS in the cells. After washing cells with DPBS, 200 µl extract was added to each well. ROS was measured as mentioned above using a fluorescence micro-plate reader. The level of fluorescence in H_2-DCF-DA-stained cells treated with hydrogen peroxide without adding extract was used as control.

It should be noted that the reason for treating cells with the material extracts and not directly with the gel was that Laponite inside the gel has high adsorptive capacity and would have easily absorbed the MTS and ROS reagents, resulting in false reading in these assays. Moreover, this study followed the ISO standard for in-direct cytotoxic assay, which is a standard and universal method to test materials for wound healing purpose.

Statistical analysis

All data are expressed as means ± standard deviations of a representative of three similar samples; otherwise it has been mentioned in the experiment. Comparisons between means were conducted using statistical analysis of variance (ANOVA) in conjunction with Tukey's post-hoc test at a significance level of $p < 0.05$ using IBM SPSS statistics version 19.

Results and Discussion

Physico-chemical characterisation

XRD diffraction is a well-established method to investigate clay minerals structure and intercalation between clay nano-layers. The basal reflection corresponding to the (001) plane at $2\theta = 6.2°$ shifted toward lower angles in all Lap/AA samples compared to the laponite sample (Fig. 1). This can be due to the intercalation of amino acids between the laponite inter-layers, resulting in an increase in d-spacing between these layers. It has been reported that the interaction between amino acids and clay is through the hydrogen bond between the carboxyl group of amino acids and the oxygen of the silicate groups $(SiO_4)^{-4}$ on

the surface and edges of laponite layers.[32] Upon the intercalation of amino acids, the interlayer spacing (d-spacing) expanded from 13.28 to 21.43 Å in Lap/Leu group (Fig. 1). The expansion in d-spacing of laponite layers varied between 4 to 8 Å, depending on the intercalated amino acid. The observed expansion in the interlayer spacing of clay due to the amino acid intercalation was also reported in other studies.[33] Amino acids are mainly cationic species at pH 3, and can easily be exchanged with cations of laponite. In the XRD pattern of Lap/Glu, the (001) basal reflection was not present, which can be as a result of the delamination of silicate layers of laponite during the intercalation of glutamic acid between these layers. It should be also noted that glutamic acid might have gradually converted to pyroglutamic acid during the intercalation process. It has been mentioned in other studies that glutamic acid has a strong tendency to cyclise, even under neutral or acidic aqueous media (Scheme 1).[34] Macklin et al.[35] has also reported that silica and alumina based minerals catalyse the attached amino acids to form peptides. For example glycine and glutamic acid can undergo cyclisation or dehydrogenisation and react with the hydroxyl group of clay.[34] The adsorption of glutamic acid on the surface of clay nanoparticles is limited due to the repulsion between negatively charged molecules of clay and glutamic acid. However, some glutamic acid molecules can be converted to pyroglutamic acid (cyclisation of glutamic acid) and the presence of these new formed pyroglutamic acids can attract the non-converted glutamic acids to be adsorbed on the surface of clay mineral.[36] This can be the reason for the difference between XRD pattern of Lap/Glu group and those of other groups.

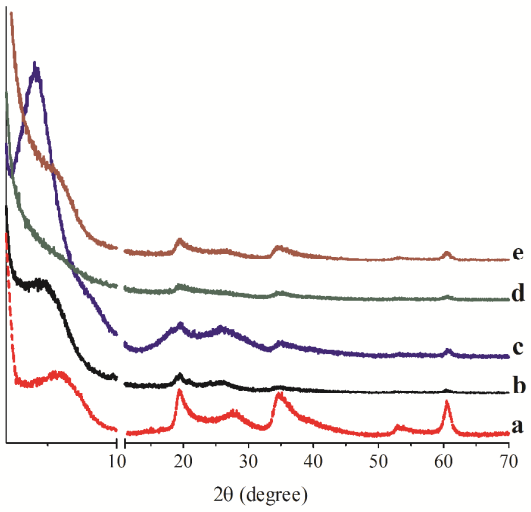
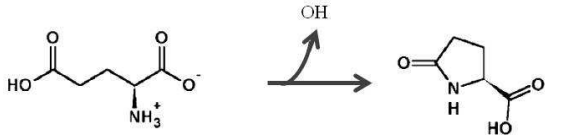


Fig. 1: XRD patterns of (a) laponite XLG; (b) Lap/Lys; (c) Lap/Arg; (d) Lap/Glu; (e) Lap/Leu. The presence of the basal reflection corresponding to the (001) plane at 2θ = 6.2° was observed in all samples except in Lap/Glu. This reflection shifted to lower angles in Lap/AA groups compared to laponite alone (pattern a).



Scheme 1: Cyclisation of glutamic acid

As listed in table 1, in (001) basal reflection, Lap/Leu group showed lower full-width at half-maximum (FW/HM) compared to the other groups. This means that intercalated leucine between the laponite inter-layers caused the least disarrangement in the layers and did not disorder arrays of silicate. This is probably due to a lower extent of leucine intercalation compared to the other tested amino acids, as was shown in results below. It has been shown that, when the layers of laponite are randomly arranged or have low degree of

periodicity, (001) basal reflection of laponite became broader.[37] In contrary, when the layers are well arranged, this XRD reflection is sharper. This is due to the fact that the X-ray beam is diffracted to the same angle in well-arranged and parallel layers.

Table 1. The 2θ of (001) basal reflection, its corresponding d-spacing and FW/HM

	2θ (Degree)	d-spacing (Å)	FW/HM (Å)
Lap/Arg	3.98	20.54	2.04
Lap/Lys	4.91	17.11	2.36
Lap/Glu	N/A	N/A	N/A
Lap/Leu	5.93	21.43	0.54
laponite	6.65	13.28	1.40

ATR-FTIR was performed to assess interaction between amino acids and laponite. The FTIR spectra (Fig. 2) evidenced the presence of amino acids within the laponite structure. Peaks that correspond to both laponite and amino acids were detected. laponite has a characteristic peak at 966 cm^{-1} corresponding to the stretching vibration of Si-O and Si-O-Si bands,[38] which was present in all spectra of Lap/AA groups (Fig. 2). These peaks shifted towards higher wavelength in all Lap/AA samples (inset of Fig. 2), indicating the molecular interaction between amino acids and laponite, probably attributed to the hydrogen bond between carboxyl group of amino acids and the oxygen of the silicate groups. One multi-component peak was observed at 3000-3700 cm^{-1}, representing hydroxyl groups of physio-adsorbed water. At this region, two main peaks also overlapped: the stretching vibration of Si-OH (3615 cm^{-1}) and Mg-OH (3670 cm^{-1}). Lap/AA samples demonstrated peaks assigned to the amino acid structure: the multi component peak at 1300-1700 cm^{-1} that represents the stretching vibration of NH_3. Moreover, peak at 763 cm^{-1} is due to the stretching of C-N-H group. Bands related to the carboxyl group of amino acids in the region of 600-700 cm^{-1} are overlapped with bending vibration of Si-O and Al-O of laponite. Absorption bands of the intercalated amino acids can be observed among the clay peaks but at lower intensities.[39]

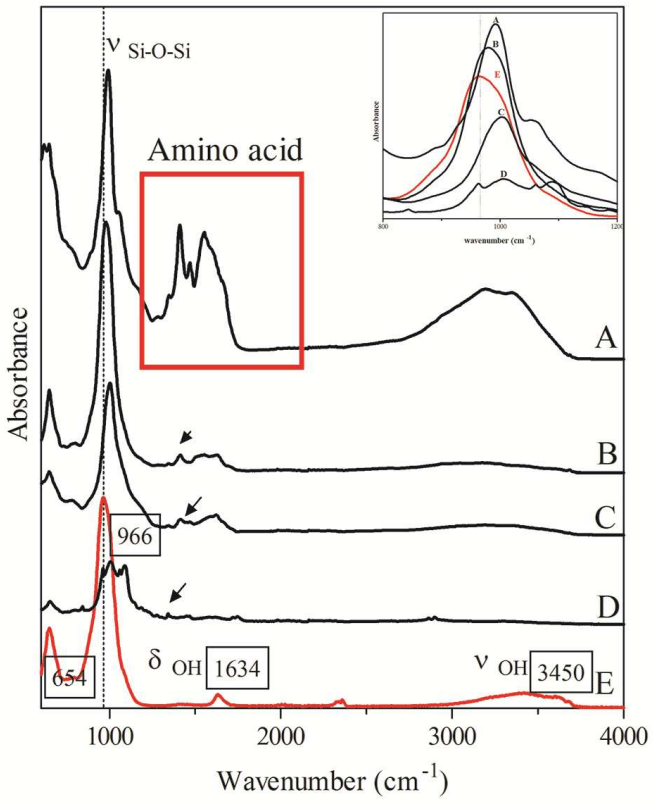


Fig. 2: FTIR absorbance spectra of freeze-dried samples of (A) Lap/Arg; (B) Lap/Lys; (C) Lap/Glu; (D) Lap/Leu; (E) laponite XLG. Arrows show peaks corresponding to vibrations attributed to amino acids molecules. Inset: peaks corresponding to amino acids shifted to higher wavelength in all Lap/AA samples.

Thermogravimetric analysis (TGA) was carried out to confirm the intercalation of amino acids in laponite structure. The derivative thermogravimetric analysis (DTA) and TGA profiles of Lap/AA and pristine laponite (Fig. 3A & B) showed two major

weight losses: an endothermic peak at around 100 ºC, corresponding to the evaporation of absorbed water; the loss of hydroxyl group which occurs at 800-900 ºC. In spite of loosing physio-adsorbed water, laponite structure is stable in the temperature up to 750 ºC, so the weight loss below this temperature should be due to the decomposition of amino acids present in Lap/AA samples. Moreover, there is a peak cantered at 245 ºC, which can be attributed to the decomposition of surface adsorbed amino acids (adsorbed onto inter-particle pores), while the peak present at the temperature range between 360-520 ºC can represent the decomposition of the intercalated amino acids. Intercalation of amino acid molecules between the layers of laponite structure can indeed protect amino acid against decomposition, shifting the decomposition temperature to higher degrees. From the DTA graph of Lap/Glu sample, the decomposition temperature of glutamic acid has moved further up to the higher temperatures. This can be due to the fact that glutamic acid undergoes cyclisation reaction catalysed by laponite and forms pyroglutamic acid. The TGA results demonstrated greater weight loss of Lap/Leu, Lap/Lys and Lap/Arg compared to Lap/Glu and laponite (alone) samples. This indicates that less amount of glutamic acid intercalated into laponite structure compared to the other Lap/AA groups. Glutamic acid has lower pKa than the other used amino acids, which means negatively charged molecules results in lower intercalation into laponite inter-layers.

Looking at the temperature range of 70-100 ºC, which mainly attributed to the escape of physio-adsorbed water; water evaporation occurred at higher temperatures in laponite (100 ºC) and Lap/Lys (120 ºC), while it evaporated in lower temperatures in the Lap/Leu, Lap/Arg and Lap/Glu samples (70-80 ºC). It can be suggested that the presence of amino acids in laponite structure leads to a looser affinity between water and laponite structure.

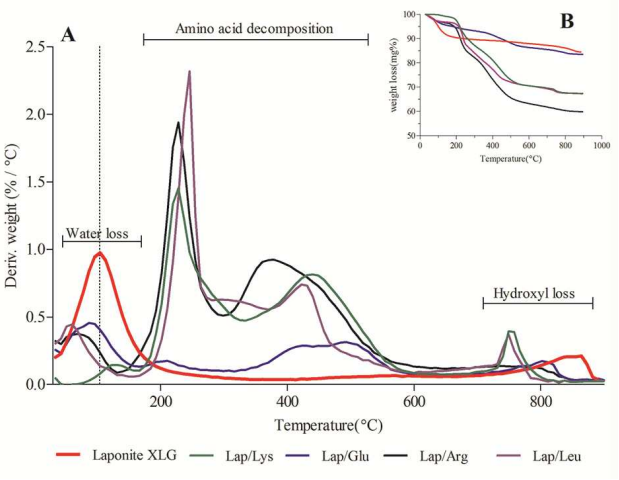


Fig. 3: (A) Differential thermal analysis (DTA) of freeze-dried samples of Lap/Lys, Lap/Arg, Lap/Leu, Lap/Glu and laponite XLG; and (B) The image inset shows the thermogravimetric analysis (TGA) of same samples.

The isothermal graphs of laponite and Lap/AA (Fig. 4A, B, C, D and E) shows different shape for each sample according to the International Union of Pure and Applied Chemistry (IUPAC) classification.[40] The adsorption–desorption isotherm of laponite was of Type IV with a H_3 hysteresis loop at P/Po ~0.2–0.9. Type IV isotherm patterns with H3-type hysteresis loop starting at about 0.4-0.8 partial pressure, which are the characteristics of the mesoporous materials with the cylindrical pores formed in gallery regions indicating a mesoporous solid[41]. Lap/Glu (4D) shows similar isothermal graph to the pure laponite (4E) with dramatic drop in adsorbed volume of nitrogen gas (almost 5-fold decrease).

Lap/Leu (4A) and Lap/Lys (4B) samples demonstrated different type of isothermal graph (Type III) than laponite and Lap/Glu. Type III isotherm demonstrates the formation of multilayer amino acids on the surface of laponite as there is no

turning point in the curve, indicating no monolayer is formed. The increasing presence of amino acid molecules facilitates the on-going adsorption of nitrogen gas that is demonstrated in the isotherm Type III.

Lap/Arg (4C) shows the Type V isothermal graph that indicates the occurrence of capillary condensation and saturation.[41] In this isotherm the saturation level reaches at a pressure below the saturation pressure. This can be explained on the basis of a possibility of nitrogen gas is condensed in the tiny capillary pores of Lap/Arg.

In table 2 analyses of the isothermal graphs reveals that the capacity of laponite interlayer space, and thus its surface area, reduced extensively when cation exchange process happened. This means that amino acids in fact intercalated between laponite layers and filled the structural spaces, which are formed due to the house-of-cards configuration of laponite discs (Fig. 5).[42] According to the BET results, basic amino acids (arginine and lysine) decreased the specific surface area at higher extent than hydrophobic (leucine) and acidic amino acids (glutamic acid). In the BET graph of the Lap/Glu sample, the isotherm shape is similar to the pure laponite (Type IV), showing that glutamic acid most likely did not cover or block the pores of laponite as the negative charge of glutamic acid molecules hindered the intercalation of glutamic acid into the negatively charged laponite inter-layers.

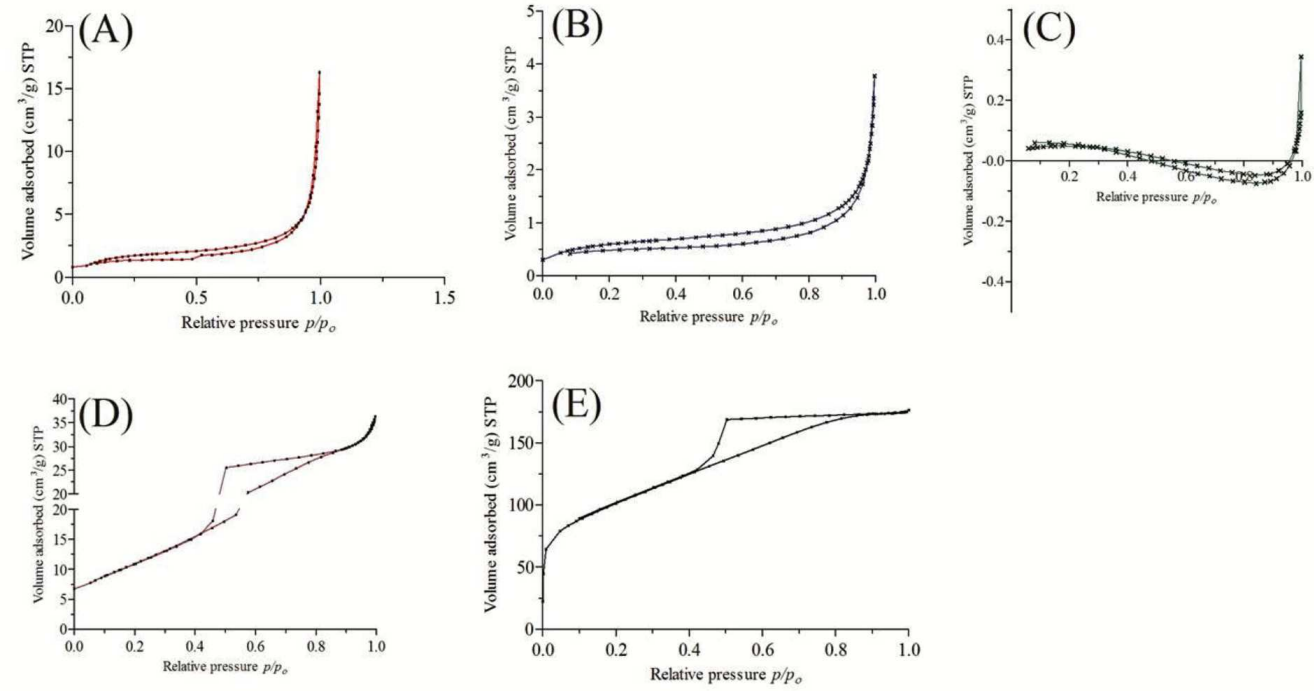


Fig. 4: Nitrogen adsorption/desorption isotherms of (A) Lap/Leu, (B) Lap/Lys, (C) Lap/Arg, (D) Lap/Glu and (E) laponite XLG samples were evaluated with Brunauer–Emmett–Teller (BET) method.

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Furthermore, in Type IV, the desorption isotherms of samples do not retrace the adsorption isotherms but rather form a hysteresis loop before re-joining the adsorption isotherm. Generated hysteresis loops in isothermal graphs indicate that laponite discs are stacking and creating house-of-cards structure. SEM image of laponite XLG and schematic image in figure 5 shows this house of cards structure. According to the other study, montmorillonite (natural type of laponite) intercalated with surfactants (cationic agents), similar isothermal curves have been observed.[43]

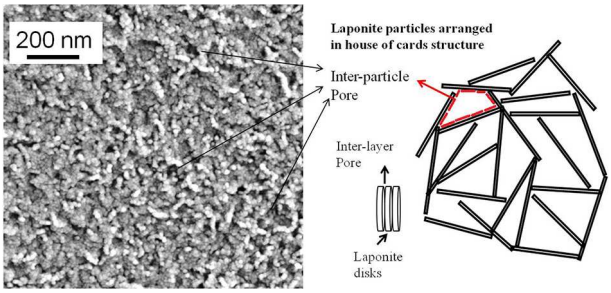

Fig. 5: SEM image of laponite XLG and schematic image that shows pores in relation to the house-of-cards structure of clay.

Table 2. Specific surface area and total pore volume of laponite, Lap/Lys, Lap/Arg, Lap/Leu and Lap/Glu measured using BET

	Total surface area (g/m^2)	Pore volume (cm^3/g)
Lap/Arg	0.08 ± 0.05	0.0005
Lap/Lys	1.69 ± 0.32	0.004
Lap/Leu	7.20 ± 1.24	0.001
Lap/Glu	50.13 ± 8.59	0.005
Laponite XLG	345.58 ± 4.11*	0.03*

*Significantly ($p < 0.05$) higher than other groups

It has been established that smectite clays are able to have inter-lamellar expansion. Thus intercalation of organic materials, for example amino acids, can expand these layers as it has been shown in other studies.[44] Previous studies indicated that surface area reduction of organo-silicates is due to two mechanisms. The first is that surface area can be covered with the organic compound and therefore the inter-layers are not accessible for nitrogen molecules in specific surface area measurement. The second mechanism is that pores are filled with the organic material and inhibits the passage of nitrogen molecules into interconnected pores. Microstructure of laponite and Lap/AA, including specific surface area and pore volume are listed in table 2. A dramatic decrease in surface area and pore volume was observed in Lap/AA samples, which could be attributed to three reasons; first covering the surface of laponite with amino acids, second, blockage of pores and third, intercalation of amino acids between laponite inter-layers. Previous studies showed that cations not only enter into the clay inter-layers but also occupy the pores available between the clay particles (house-of-cards structure).[45] Lap/Arg, Lap/Lys and Lap/Leu showed a massive decrease in total surface area compared to laponite alone (Table 2), while Lap/Glu sample showed higher total surface area compared to the others Lap/AA. This may be explained by two speculations; first, due to negative charge of glutamic acid, the repulsion between laponite and glutamic molecules hinders the intercalation though, the surface area of laponite was not decreased. Second, delamination of laponite discs happened in contact with glutamic acid which XRD results (Fig. 1) for Lap/Glu supporting that delamination

happened. Pore volume of samples was measured using nitrogen adsorption/desorption analysis, in Lap/AA samples, pore volumes decreased as low as 0.004 - 0.0005 cm^3/g compared to laponite (0.03 cm^3/g).

Release of amino acids and Mg^{2+} in simulated wound exudate fluid - The release profile of amino acids from samples was investigated in SWEF under continuous shaking at 37 ºC (Fig. 6A). Lysine demonstrated the highest release rate (60% of loaded amino acid at the end of experiment), followed by arginine, glutamic acid and leucine with 17, 10 and 7% cumulative release, respectively. This was in agreement with other studies that showed lysine was almost completely released from the mineral clay complex, and in general, basic molecules such as lysine have a high release rate from clay minerals.[46] As a function of the isoelectric point (pI) of amino acids, clay mineral can absorb some amino acids at neutral pH, however due to their negative charge, acidic amino acids has very low adsorption into clay mineral at pH 7. Hedges et al.[47] reported that at pH 7, glutamic acid, which has a total negative charge, does not adsorb onto montmorillonite with intrinsic negative charge. It should be noted that the loading percentage of amino acids in different gels varied based on the type of the amino acid. The initial amount of amino acid when formulating the gels was 200 μM and after the washing and centrifugation process the percentage of loading for different amino acid was: 30% of glutamic acid, 50% of leucine, 60% of lysine and 80% of arginine.

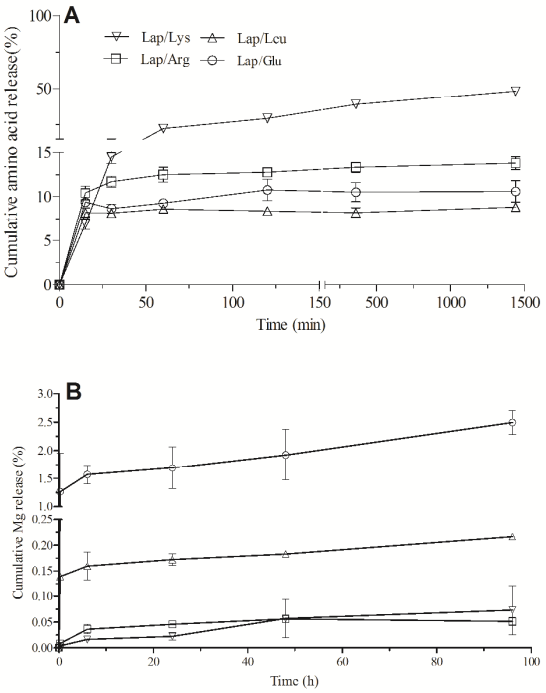

Fig. 6: (A) release of Lap/Lys, Lap/Arg, Lap/Leu and Lap/Glu in SWEF; (B) Mg^{2+} release into SWEF as a result of laponite degradation.

The amount of released Mg^{2+} from laponite structure in the exudate-like fluid is an indication of dissolution rate of laponite in the release medium. Released Mg^{2+} from Lap/Leu, Lap/Lys and Lap/Arg samples were 0.2, 0.05 and 0.04% respectively, whereas Lap/Glu sample released significantly higher Mg^{2+} up to 1.5%, showing its higher degradation rate (Fig. 6B). It is known that, in acidic conditions, laponite starts to degrade and release Mg^{2+} following equation 2:

$$Si_8Mg_{5.45}Li_{0.4}H_4O_{24}Na_{0.7} + 12\ H^+ + 8H_2O \rightarrow$$

$$0.7\ Na^+ + 8\ Si(OH)_4 + 5.45\ Mg^{2+} + 0.4Li^+ \qquad \text{(Eq. 2)}$$

Lap/Glu sample released more Mg^{2+} due to acidic nature of glutamic acid. However the released Mg^{2+} did not exceed the 2% of the total magnesium content of Lap/Glu sample. Arginine and lysine are basic and leucine is aliphatic amino acid, which explains the lower content of Mg^{2+} release from the

clay complexes with these amino acids. The released Mg^{2+} has been suggested to enhance cell growth[48] and further accelerates wound healing process,[49] which are favourable properties for wound dressing application. Laponite dissolves in acidic condition and elutes Al^{3+}, Si^{4+} and Mg^{2+} and other ions. Only the amount of released Mg^{2+} in simulated wound exudates fluid was measured in this study as among all these ions Mg^{2+} has the most significant role in wound healing. Mg^{2+} affects metalloenzmes function, where metaloenzimes take part in repair and regenerative processes in skin wounds.[50]

Wound healing properties of Lap/AA

Fibroblast proliferation in the presence of extracts of Lap/AA and laponite was determined by cells metabolic activity using MTS assay. The metabolic activity of cells was normalized with the fibroblasts cultured in medium without extract and this rate of proliferation considered to be 100%. The proliferation of fibroblasts (Fig. 7) showed that the presence of amino acids in laponite increased cell proliferation, except in glutamic acid group, in which cell proliferation was significantly lower than laponite alone. Extracts from lysine, arginine and leucine groups demonstrated 130, 125 and 110% proliferation respectively, while the proliferation of fibroblasts cultured in the presence of Lap/Glu extract decreased to 55% compared to the control group (Fig. 7). Fujiwara et al. investigated the effect of arginine on fibroblast proliferation. They reported that cell proliferation increased dose-dependently and was significant compared to non-treated cells at L-arginine concentrations of 6 mM.[51]

In another study that investigated the effect of amino acids in combination with hyaluronic acid on fibroblasts, the authors reported that this combination enhanced cell proliferation.[52] Other studies investigating different types of cells such as, osteoblasts, lymphocites and hepatic tumour cells claimed different proliferation patterns depending on the cell type and amino acid type and its concentration.[53] Number of different mechanisms has been suggested in showing beneficial effects of arginine on wound healing process. Dominating mechanism was postulated by Stechmiller and Shi who reported that the supplement of arginine had wound healing property via enhancement of wound strength and collagen deposition in wound site.[54] It has been also demonstrated that arginine plays a key role in signalling pathway of t-cell mediated immune function cells through the production of nitric oxide.[55] Nitric oxide has shown to be beneficial in diabetic wound ulcers.[56]

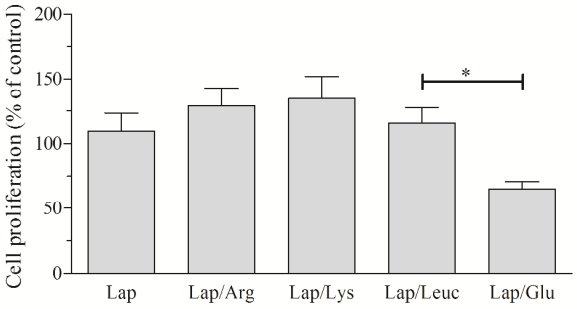

Fig. 7: Effect of extracts from prepared materials on the proliferation of skin fibroblasts. *Significant difference by one-way ANOVA analysis followed by Tukey Post-Hoc test.

In this study, the extract from Lap/Leu slightly increased cell proliferation (110 %). Leucine has anabolic effect on protein production hence increased collagen production and deposition.[57] Fibroblast proliferation increased in the presence of extract from Lap/Lys (125%). Lysine directly increases synthesising and depositing collagen in wound area or indirectly through activation of cell proliferation.[58] In clinical studies, lysine deficiency had a negative effect on wound healing process and after lysine supplementing, wound healing improved.[59] Although Suguna and Santhanam demonstrated

that glutamic acid supplementation has positive effects and led to a faster wound healing *in vivo*, in our *in vitro* study extract from glutamic acid group decreased cell proliferation by 55%.[60] Cyclisation of glutamic acid to pyroglutamic acid in the presence of clay mineral might be the reason of this cytotoxic behaviour. Finally, laponite extract did not stimulate any cytotoxicity on fibroblasts and in fact increased slightly the cell growth (110%). Gaharwar et al.[61] reported that laponite does not trigger any cytotoxic reactions at concentrations below 1 mg/mL and it can potentially be used for biomedical applications. Silica and magnesium released from laponite has been suggested to increase osteoblast growth in culture.[62]

Scratch test was applied as an *in vitro* wound model. Figure 8 demonstrates images acquired using light microscope from the scratched areas at certain time points (0, 24 and 48 hours) treated with each Lap/AA extracts. The closure of scratch in fibroblasts cultured in the presence of extracts was normalized to that of control (cultured without extracts in medium and represented 100% closure area) and presented as wound closure area percentage (Fog. 9). All scratched area became confluent after 2 days except wells treated with Lap/Glu extract. There was no significant difference between control and any of treated wells except for glutamic acid group showing a smaller closure area after 2-day period. In wells treated with extract from Lap/Glu, gap did not healed after 48 hours and the rate of wound closure was nearly 40% of that of control. Among the other extracts, laponite extract (from sample without amino acid) was the only extract that closed the gap slightly faster than control but the difference was not significant. Among all laponite/AA samples, Lap/Arg with almost 92% wound closure rate showed the fastest cell migration to close the scratch. Lap/Lys and Lap/Leu extracts closed the *in vitro* wound up to 80 and 82% respectively after 48 hours.

In this study, the results of scratch test did not show any significant difference between control cells and cells treated with Lap/Lys, Lap/Leu and Lap/Arg extracts (Fig. 9). However, the beneficial effects of these amino acids, particularly arginine, on wound healing process have already been demonstrated in many *in vivo*[63] and clinical[64] studies. It should be noted that arginine was traditionally considered to be a non-essential amino acid as it is produced endogenously when is required by tissues. Arginine has been now defined as conditionally essential amino acid due to insufficient endogenous synthesis of arginine during metabolic stress, organ maturation, and wound healing[51]. For this reason, supplementation of arginine during metabolic stress and wound healing is essential. The molecular mechanisms through which arginine stimulates wound repair is still unknown. A number of studies showed that various intra-cellular and inter-cellular pathways are activated and coordinated to facilitate fibroblast migration and proliferation for wound healing. These pathways however are observed and discussed in *in vivo* setups in the presence of other biological pathways, which do not occur in *in vitro* scratch test as a wound model.

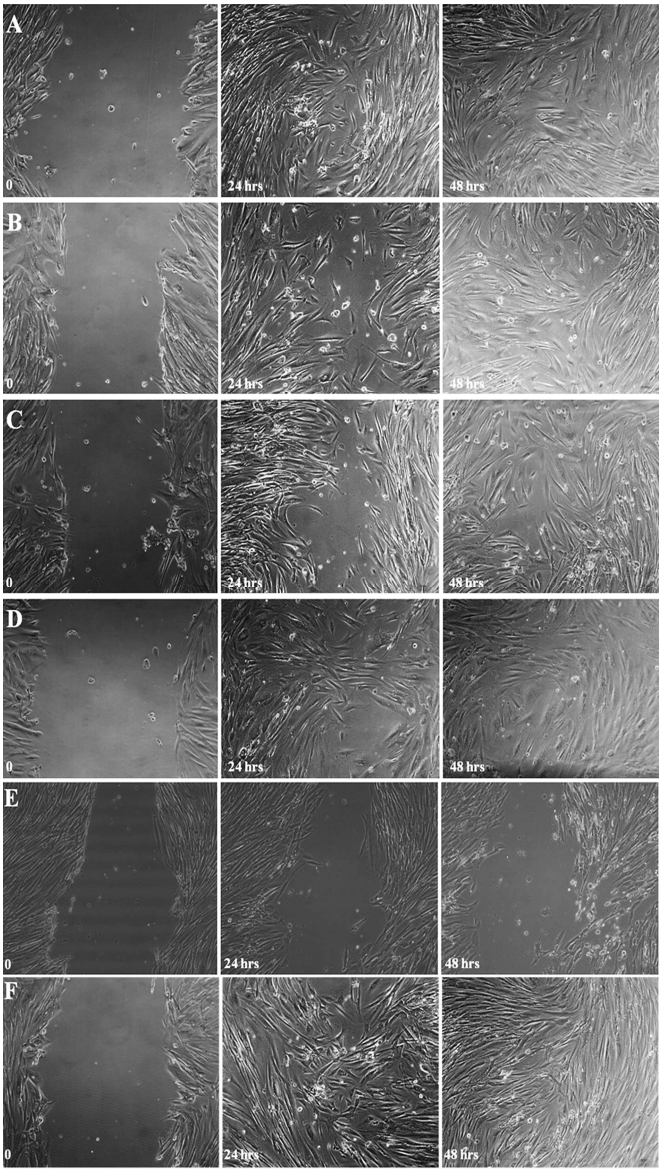

Fig. 8: Representative microscopic pictures of scratched monolayer of fibroblasts were shown at t=0 h, t=24 h and t=48 h treated with (A) laponite, (B) Lap/Arg, (C) Lap/Lys, (D) Lap/Leu, (E) Lap/Glu, extracts and (F) control cells without any treatment. Pictures were made using an inverted microscope (Leica) and the open area was calculated with image analysis software at the indicated time points. 10 × magnification.

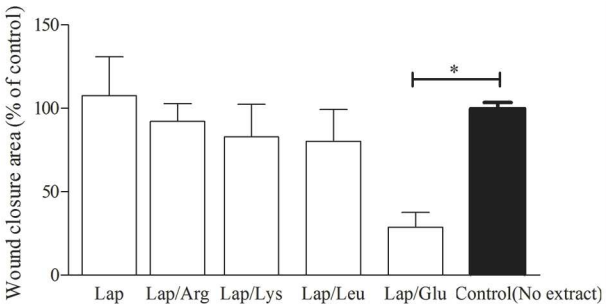

Fig. 9: Effect of extracts from laponite and laponite intercalated with arginine, lysine, leucine and glutamic acid on skin fibroblast migration in an *in vitro* "wound model", scratch assay. Results were compared to the scratched cells without any treatment. Percentage of wound closure area in comparison to control was measured after 48 hr. Cell migration in the presence of Lap/Glu extract was significantly inhibited compared to the other groups (*$p < 0.05$).

Reactive oxygen species (ROS) are essential for wound healing, through the activation of immune system to defend against infection. However, an excessive amount of ROS can damage cells and thereby hinders the wound healing process. Wound healing process therefore requires a fine balance between the positive and negative effects of ROS. Effect of the extracts obtained from different samples on ROS formation in fibroblast culture is demonstrated in Figure 10. Results indicated that the extracts from glutamic acid and lysine samples both increased ROS formation in fibroblasts, which %ROS was significantly higher in Lap/Lys group compared to the untreated cells and other groups (Fig. 10A). Lysine induced ROS formation up to 125% (normalized to the untreated control cells which considered as 100%), while extract from Lap/Glu increased ROS formation slightly to 105%. Although the level of generated ROS is higher in Lap/Lys group than the untreated cells, it is still tolerable for cells. In contrast, extracts from arginine and leucine samples and laponite alone sample reduced

significantly the fluorescence rate induced by ROS generation, indicating that the level of ROS indeed decreased in the fibroblasts treated with these extracts or in the other words they have ROS scavenging capacity. Previous studies demonstrated the suppressing effect of some amino acids on induced oxidative stress using H_2O_2 treatment. These amino acids include Cys, Ala, Ile, Leu, Trp, Val, Lys, and His.[65] The effects of amino acids on reactive oxygen species has not been fully studied and the few that investigated these effects found to be controversial, depending on the type of amino acid and treated cells.[65] Meucci et al. reported that the proteins in plasma are in fact responsible for the antioxidant capacity of plasma, and further suggested that amino acids with phenolic hydroxyl group (tyrosine) can act as an antioxidant agent.[66] In another study investigating the antioxidant properties of amino acids, it was shown that arginine, lysine and glutamic acid had protective potential on gout eye lens against oxidative stress.[67]

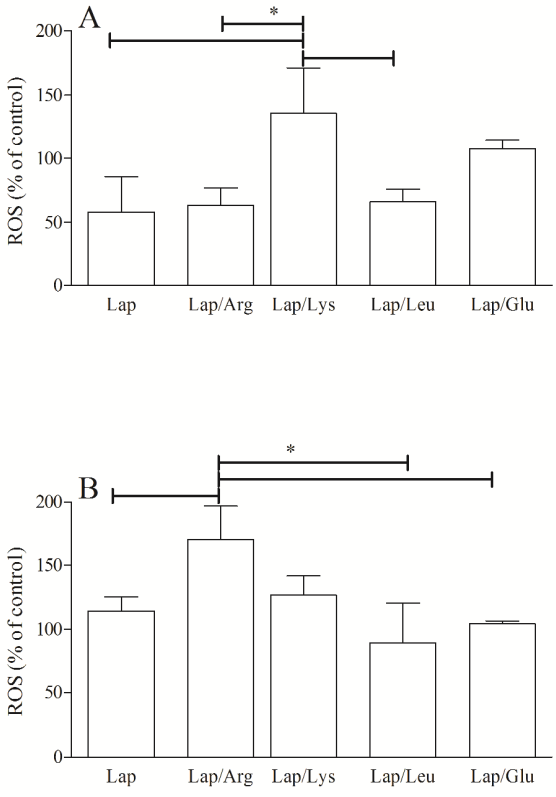

Fig. 10: Percentage of generated ROS (normalized with control) was determined with H_2-DCF-DA fluorescence intensity in skin fibroblasts at (A) the presence of extracts of laponite, Lap/Lys, Lap/Arg, Lap/Leu and Lap/Glu and (B) %ROS was measured after induction of oxidative stress with H_2O_2 treatment and then treated with extracts of laponite, Lap/Lys, Lap/Arg, Lap/Leu and Lap/Glu. Values are the mean and SD of three independent experiments versus control (**p* < 0.05).

The antioxidant property of extracts was also investigated after introducing reactive oxygen species to fibroblasts by treating the cells with 10μM H_2O_2. Figure 10B shows that the extracts from Lap/Arg, Lap/Lys and laponite alone enhanced ROS formation in the presence of OH species induced by H_2O_2 treatment; with ROS% was significantly higher in Lap/Arg (160%) compared to the other groups and control, whereas extracts from Lap/Leu and Lap/Glu showed antioxidant behaviour to some extent by decreasing the level of induced ROS. However these differences were not significant in comparison to untreated control cells.

The reason of high %ROS in arginine samples can be related to the fact that arginine is the physiological substrate for nitric oxide (NO) synthesis. In the presence of hydrogen peroxide, arginine is catalysed by NOS (nitric oxide synthase) to NO. Therefore the increase in fluorescence intensity observed in our study in Lap/Arg group is due to the generation of NO from arginine. It has been shown that H_2-DCF-DA can detect both ROS and NO radicals.[68]

NO substrate has positive or negative effect on wound healing, Witte et al.[69] reported that the main mechanism that correlates arginine's beneficial activity in wound healing is by generation

NO. *In vitro* studies have also shown that exogenous NO in cultured skin fibroblasts increased collagen synthesis.[70] In contrast, it has been previously shown that the amount of NO increases at the wound site followed by tissue damage.[71] Extract from the leucine group did not generate ROS and the ROS level was lower than untreated cells, suggesting that leucine may scavenge intrinsic ROS formed in the cells. Findings from other studies regarding the effects of leucine on oxidative stress are quite contradictory, probably due to the different experimental methods. In a study conducted by Stoppigilia et al.,[72] the results showed that at high concentrations (30.5 mM), leucine acts as an antioxidant in pancreatic islets, due to its role as a metabolic substrate. In contrast, Bridi et al.[73] reported that leucine caused oxidative stress in cerebral cortex due to the inhibition of antioxidant enzymes like catalase and glutathione peroxidase (GSH-PX1) and the oxidation of protein thiol groups. De Obanos et al.[74] also demonstrated that leucine increased ROS formation in cell culture due to the effect of collagen synthesis via phosphorylation mechanism.

ROS level in cells treated with laponite extract was not significantly different from control. However, there was a slight increase in total ROS in cells in contact with laponite extract. It was shown that using low concentration of laponite (< 1 mg/ml) on human mesenchymal stem cells (hMSC) did not generate oxidative stress, while at higher concentrations (IC_{50} = 4 mg/ml), ROS generation damaged the treated cells.[61] The same findings also demonstrated that the treatment with high concentration of montmorillonite (>500 μg/ml) generated ROS in human normal intestinal cells.[75]

Conclusion

Lap/AA gels were prepared and characterised, and their application as a wound healing gel was evaluated using various *in vitro* assays. Amino acids were intercalated between the inter-layers of laponite. It was observed that amino acids react with laponite differently, depending on amino acids charge during intercalation process. The *in vitro* effects of Lap/AA extracts on fibroblasts indicated that the presence of amino acids promoted fibroblast proliferation, except in glutamic acid sample. Based on scratch test and ROS analysis it was found that the Lap/Arg, Lap/Lys and Lap/Leu samples have the potential to be applied as a topical wound healing gel.

Acknowledgment

The authors acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Australian Centre for Microscopy and Microanalysis, The University of Sydney. Authors would like to thank Prof Fariba Dehghani from the School of Chemical and Biomolecular Engineering, University of Sydney for assisting in ATR-FTIR analysis. We appreciate Prof Rebecca Mason's help from The Department of Physiology, University of Sydney, for providing us with fibroblasts.

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Laponite holds amino acid between its interlayer spaces and releases it in contact with wound fluid through ionic exchange process.