

Development and characterization of hyaluronic acid-lysine nanoparticles with potential as innovative dermal filling

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Skin aging causes changes such as wrinkles and flaccidity leading to a large demand for aesthetic procedures, including dermal filling. A key agent in dermal filling is hyaluronic acid (HA), which is a naturally occurring glycosaminoglycan. However, it is a hydrophilic macromolecule that experiences great difficulty in crossing the skin barrier causing most commercial formulations containing it to be injectable, which in turn brings risks since they involve an invasive technique. In that sense, the aim of this study was to develop and characterize nanoparticles obtained from ionic interaction between HA and lysine (Lys) for use as a potential agent of dermal filling for topical application, increasing and improving its applicability and safety. To this end, nanoparticles were obtained by dripping of Lys over HA under magnetic stirring. A nanometric size was confirmed and a suitable surface charge was obtained by zeta potential. Nanoparticles were almost spherical in shape with a smooth surface. Interaction between raw materials for preparing nanoparticles was studied by FTIR and NMR spectroscopy and an ionic interaction was confirmed. These physicochemical features suggest that obtained nanoparticles can be further used as a topical dermal filling.

Uniterms: Nanotechnology. Skin aging/non-invasive method. Skin aging/treatment/study.

INTRODUCTION

Skin aging is a sign of progressive loss of skin tissue which can be attributed to factors such as epidermal or dermal cell loss and decrease of extracellular matrix, particularly glycosaminoglycans (GAG) (Robert, Labat-Robert, Robert, 2012).

Different types of filler agents are used as cosmetic strategy in order to prevent or reverse loss of extracellular matrix (Sánchez-Carpintero, Candelas, Ruiz-Rodríguez, 2010). The most common fillers reported in literature and clinically used are as follows: collagen (bovine, porcine or human), synthetic or animal hyaluronic acid (HA), poly(L-lactic acid), calcium hydroxyapatite, poly(methyl methacrylate), silicon, and hydroxyethyl methacrylate (Sánchez-Carpintero, Candelas, Ruiz-Rodríguez, 2010). In that context, HA has become more popular and widely used as soft tissue filler due to its major advantages

(Edwards, Fantasia, Iovino, 2006; Requena *et al.*, 2011).

Hyaluronic acid is a GAG, a naturally occurring biopolymer, composed of alternating links between residues of D-glucuronic acid and N-acetylglucosamine (Nelson, Cox, 2011). Considering its high hydrophilicity, HA can provide an improved moisture to skin and an enhanced viscoelasticity to dermal matrix (Brandt, Cassaniga, 2007; Born, 2006). In addition, HA is used as a transitory bioresorbable filler for treating wrinkles and expression lines and for increasing lip volume (Edwards, Fantasia, Iovino, 2006; Requena *et al.*, 2011). However, formulations containing HA are injectable due to its high molar mass and can bring discomfort to patients (Beer, 2009). In that sense, nanostructured systems can be used in order to improve percutaneous absorption of HA and to avoid pain and discomfort that occur during invasive procedure.

Polymeric nanoparticles are widely reported as nanocarriers that can be successfully used to change physicochemical properties of an active agent leading to the improvement of its release into and/or its effect on skin (Guterres, Alvez, Pohlmann, 2007). For this purpose,

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lysine (Lys) was chosen for preparing nanoparticles by ionic complexation with HA since, being a natural polypeptide, both stability and biocompatibility would be ensured. A previous study reported the successful interaction between polypeptides and amino acids (Oyarzun-Ampuero *et al.*, 2011).

Taking into account all these data, the aim of this study was to develop innovative polymeric nanoparticles of HA-Lys, obtained from ionic interaction, for use as a safe dermal filling, and characterize physicochemically ensuring its stability. Our hypothesis was that it is possible to obtain a stable material based on nanotechnology that can be further used as a non-invasive strategy for topical application of HA in order to avoid pain and discomfort to patients.

MATERIAL AND METHODS

Material

Hyaluronic acid *Streptococcus equi* (10,000-30,000 g mol⁻¹) was purchased from Sigma Aldrich (USA). L-Lysine hydrochloride (146.18 g.mol⁻¹) was obtained from Fagron (Brazil). The other reagents and solvents were of analytical grade.

Preparation of HA-Lys nanoparticles

Hyaluronic acid-Lys (HA-Lys) complex was prepared as reported by Oyarzun-Ampuero *et al.* (2011), with some changes. Nanoparticles were obtained using 1 mol of HA and 16,400 mol of Lys in order to provide a molar equivalency. A Lys solution (4.0 x 10⁻² g.mL⁻¹) was dropped into a HA solution (3.5 x 10⁻⁴ g.mL⁻¹) using a burette (inner diameter of 1 cm) under magnetic stirring (1,700 rpm) at a drip rate of 2.5 mL.min⁻¹. Final colloidal suspension was then dried at 40 °C. This procedure was carried out in triplicate and samples were stored into a desiccator under vacuum at room temperature.

Mean diameter and zeta potential

Mean diameter and zeta potential were measured in triplicate using a Zetasizer Nano ZS90 (Malvern) at 25 °C at a final concentration of 1 mg.mL⁻¹. Samples were diluted in Milli-Q water and NaCl 10 mmol for size and zeta potential analysis, respectively.

Morphological analysis

A morphological evaluation was conducted by

scanning electron microscopy (SEM) in a SSX-550 microscope (Shimadzu). Samples were dried in a vacuum oven (Tecnal), mounted on aluminum stubs, and sputtered with gold (IC-50 Ion Coater, Shimadzu). The micrographs were obtained at accelerating voltage of 10 or 15 kV with different magnifications.

Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was carried out with an IR-Prestige-21 spectrophotometer (Shimadzu) using KBr pellets (4 mg of sample and 196 mg of spectroscopic grade KBr). Spectra were obtained at 64 scans.min⁻¹, resolution of 4 cm⁻¹ and wavenumber range from 4000 cm⁻¹ to 400 cm⁻¹.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra was obtained in an Avance III NMR 400 (Bruker) operating at 400.13 MHz for ¹H and 100.61 MHz for ¹³C. Samples were previously prepared in deuterated water (D₂O).

RESULTS AND DISCUSSION

Preparation of HA-Lys nanoparticles

Nanoparticles were obtained by interaction between HA and Lys solutions without any further purification. This nanoscale material is the result of a spontaneous formation possibly by ionic interaction between carboxylic acid of HA and primary amine of Lys, as demonstrated in Figure 1. Previous studies have already reported this kind of self-assembled nanoparticles using another polysaccharide, chitosan, for various purposes in the pharmaceutical field (Sonvico *et al.*, 2006).

After dripping, HA-Lys nanoparticles in solution showed a macroscopic homogeneous aspect with opalescent bluish white color resulting from the Brownian motion of colloidal structures. After drying, this product presented a powdery consistency and white color.

Mean diameter and zeta potential

Table I summarizes results of size and zeta potential as mean ± standard deviation (SD). The mean size of HA-Lys nanoparticles was 134.1 nm. According to Bos and Meinardi (2000), dermal penetration can occur for chemical compounds lower than 500 Da. Nevertheless, it has been shown that diffusion of high-MW corpuscles is drastically impeded in the

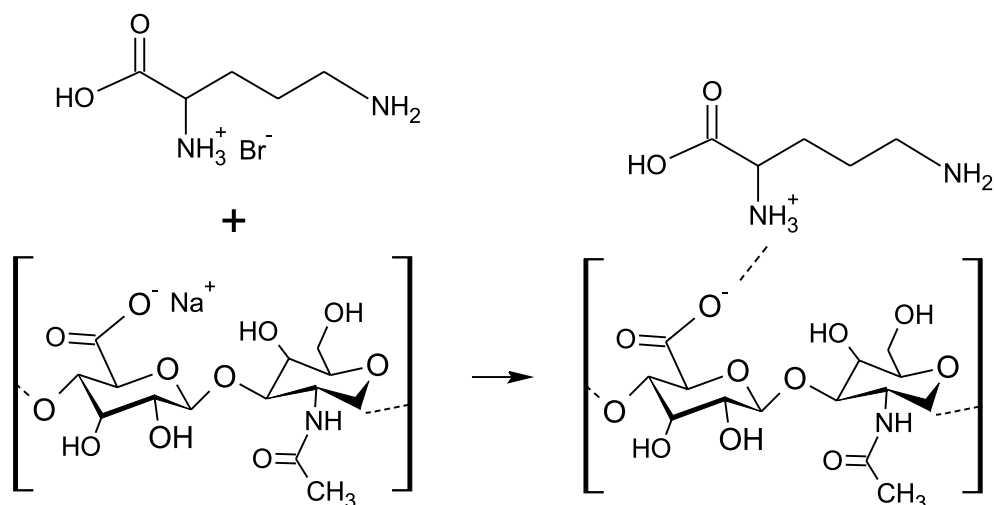


FIGURE 1 - Scheme of ionic interaction between HA and Lys.

stratum corneum. As nanoparticles have molar masses in the order of 10^6 Da and greater, no significant transport over an intact dermal barrier is to be expected for human beings (Schneider *et al.*, 2009). In that sense, nanoparticles lower than 300 nm show penetration most likely along two possible routes: the intercellular route, following lipid channels between corneocytes to deeper skin layers and the appendage route through hair follicles and sweat glands (Schneider *et al.*, 2009; Rancan *et al.*, 2012). Therefore, HA-Lys nanoparticles present a suitable mean size for topical application and penetration may occur through lipid channels between corneocytes, hair follicles or sweat glands.

TABLE I - Size and zeta potential of HA-Lys nanoparticles

Material	Size (nm \pm SD)	Zeta potential (mV \pm SD)
HA-Lys nanoparticles	134.1 \pm 32.0	-59.8 \pm 9.0

Considering zeta potential, HA-Lys nanoparticles demonstrated a negative value, which is consistent with the anionic nature of HA due to the presence of carboxylic groups. Zeta potential is an important parameter to

determine stability of nanoparticles in aqueous systems. In general, higher zeta potential values (>30 mV) provide an electrostatic repulsion among particles that leads to the formation of more stable nanosystems (Frozza *et al.*, 2010). In that sense, HA-Lys nanoparticles revealed suitable stability in colloidal suspension.

Morphological analysis

Images obtained by SEM of HA and Lys (Figures 2a and 2b, respectively) were performed at 2,400 X magnification. Both HA and Lys showed agglomerates of large size. However, HA-Lys complex at 35,000 X magnification (Figure 2c) were almost spherical in shape and showed nanometric size and smooth surface. These data are consistent with the mean size obtained by Zetasizer Nano ZS90 in the literature (Sonvico *et al.*, 2006; Oyarzun-Ampuero *et al.*, 2011).

Fourier-transform infrared spectroscopy

Figure 3 depicts FTIR spectra of HA, Lys and HA-Lys nanoparticles. A broad stretching band of hydroxyl

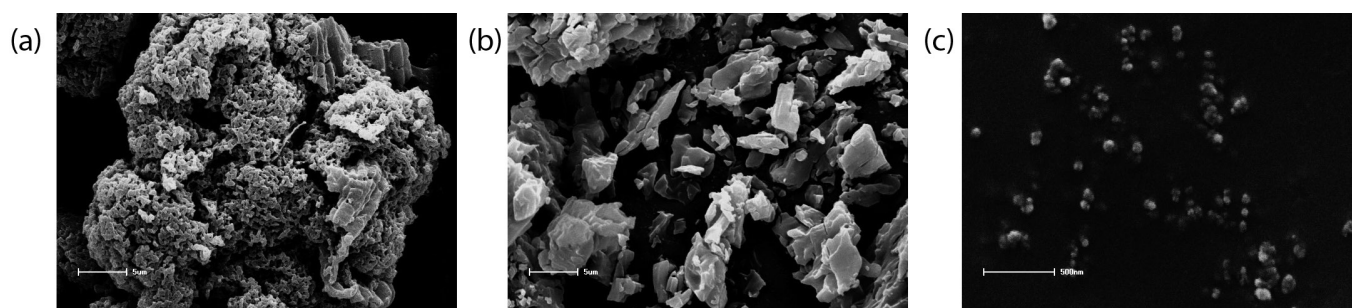


FIGURE 2 - SEM micrographs of HA (a), Lys (b) and HA-Lys nanoparticles (c).

groups was recorded at 3443 cm^{-1} in FTIR spectrum of HA (Figure 3a). Two bands at 1720 and 1648 cm^{-1} corresponded to carbonyl stretching bands of carboxylic acid and amide, respectively. Ether bands were assigned at 1151 and 1034 cm^{-1} (Silverstein, Webster, 2000; Pavia, Lampman, Kriz, 2010). On the other hand, Lys (Figure 3b) presented a stretching band at 3170 cm^{-1} corresponding to amine groups. Two bands were assigned at 2981 and 2894 cm^{-1} due to symmetric and asymmetric CH_2 stretching, respectively. A carbonyl stretching band of carboxylic acid was observed at 1690 cm^{-1} . Bands at 1588 and 1148 cm^{-1} were assigned to N–H in-plane bending and C–N stretching of amine, respectively (Silverstein, Webster, 2000; Pavia, Lampman, Kriz, 2010).

In general, FTIR spectrum of HA-Lys nanoparticles (Figure 3c) showed similar bands of raw materials. However, the carbonyl stretching band of carboxylic acid of HA was not assigned for HA-Lys nanoparticles. In addition, FTIR spectrum of HA-Lys nanoparticles demonstrated a band at 2625 cm^{-1} attributed to quaternary ammonium salt. As has been discussed elsewhere (Pavia, Lampman, Kriz, 2010), all ammonium salts display a broad band between 2200 and 3300 cm^{-1} due to a combination of bands of NR^{4+} group. In that sense, these data confirm the ionic interaction proposed for self-assembled nanoparticles of HA-Lys involving both carboxyl group of HA and amine group of Lys.

Nuclear magnetic resonance spectroscopy

Figure 4a represents signals of ^1H NMR spectrum

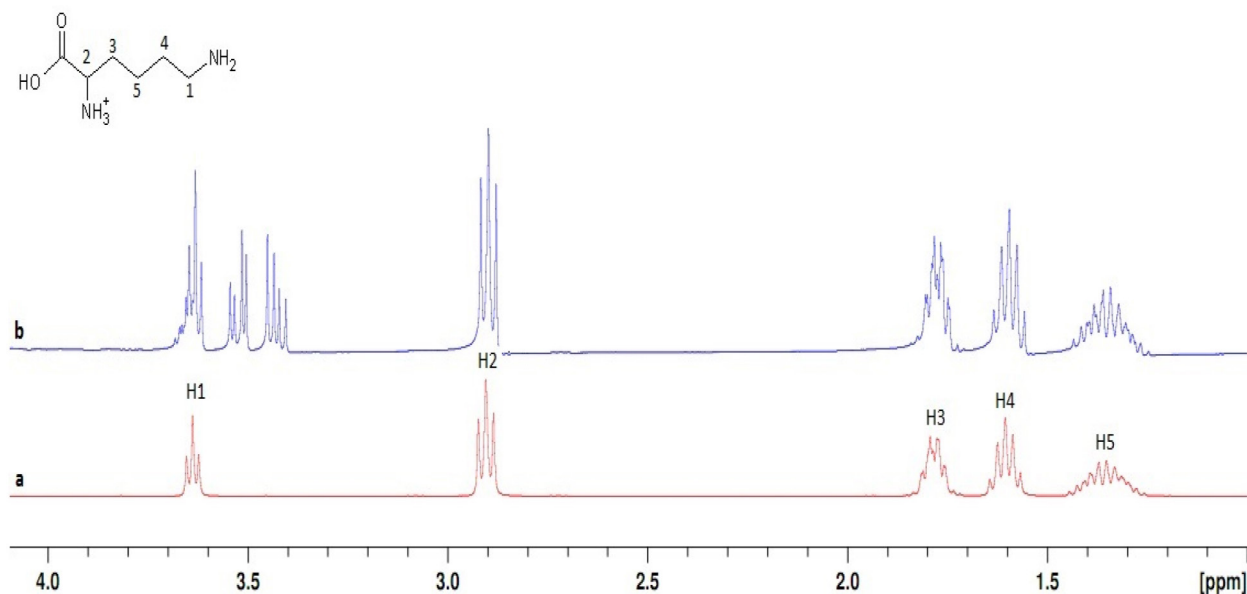


FIGURE 4 - ^1H NMR spectra of Lys (a) and HA-Lys nanoparticles (b) at 400.13 MHz in D_2O .

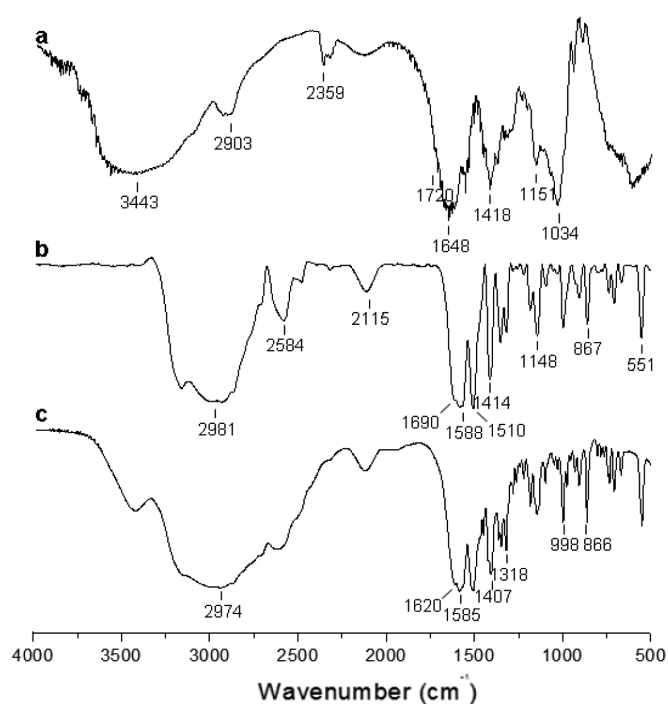


FIGURE 3 - FTIR spectra of HA (a), Lys (b) and HA-Lys nanoparticles (c).

of Lys that corresponded to its characteristic groups. The signal assignment at $\delta 3.64\text{ ppm}$ (H1) of ^1H NMR spectrum of Lys was attributed to the chemical shift of CH_2 group attached to primary amine. A signal at $\delta 2.90\text{ ppm}$ was assigned to CH group attached to carbonyl group (Silverstein, Webster, 2000; Pavia, Lampman, Kriz, 2010). Similar signals were observed in ^1H NMR spectrum

of HA-Lys nanoparticles (Figure 4b), however typical signals of HA were assigned at δ 3.2–3.8 ppm which were attributed to the chemical shift of CH groups attached to oxygen (Pavia, Lampman, Kriz, 2010). The presence of few signals related to HA can be explained by its lower amount in the final nanomaterial.

Figure 5 shows details of regions between δ 3.55–3.75 ppm (**H1**) and δ 2.70–3.10 ppm (**H2**) of ^1H NMR spectra of Lys and HA-Lys nanoparticles. Chemical shifts can be observed in both regions (**H1** and **H2**) when final HA-Lys nanoparticles were compared to raw material (Lys). In addition, no chemical shift was recorded to other hydrogens related to signals **H3**, **H4**, and **H5** (data not shown). These chemical shifts in particular regions of ^1H NMR spectrum of HA-Lys nanoparticles confirmed that interaction between HA and Lys occurred by participation of amine groups of Lys.

Lysine presented a ^{13}C NMR spectrum (Figure 6a) consistent with the literature (Auxilio *et al.*, 2008) in which typical signals were recorded at δ 174.5 ppm (**C1**) that corresponded to carbonyl carbon and δ 54.5 and 39.2 ppm (**C2** and **C3**, respectively) that were assigned to carbons near to amine groups.

In ^{13}C NMR spectrum of HA-Lys nanoparticles (Figure 6b), two additional signals were observed that were attributed to carbons attached to oxygen atoms due to the presence of HA in the final nanomaterial.

Figure 7 demonstrates a detail of the region between δ 38–55 ppm of ^{13}C NMR spectra of Lys and HA-Lys nanoparticles. As previously observed in ^1H NMR analysis, chemical shifts were also registered to **C2** and **C3** carbons when ^{13}C NMR spectrum of HA-Lys nanoparticles was compared to ^{13}C NMR spectrum of Lys. No chemical shift was assigned to other carbons (data not shown). These data also confirmed that interaction between HA and Lys occurred by involving amine groups of Lys.

Taking into account FTIR and NMR data, nanoparticles of HA-Lys were successfully prepared by self-assembling involving ionic interaction between carboxyl group of HA and amine group of Lys. This result is consistent with studies that previously reported ionic interrelations of self-organized nanoparticles of lecithin and chitosan (Sonvico *et al.*, 2006) and polyarginine and hyaluronic acid (Oyarzun-Ampuero *et al.*, 2011).

CONCLUSION

Hyaluronic acid-lysine nanoparticles were successfully prepared by a simple one-step dripping procedure as confirmed by SEM analysis. A nanometer-sized material with suitable stability in aqueous medium was obtained. FTIR and NMR data confirmed that self-assembled nanoparticles were formed by ionic interaction

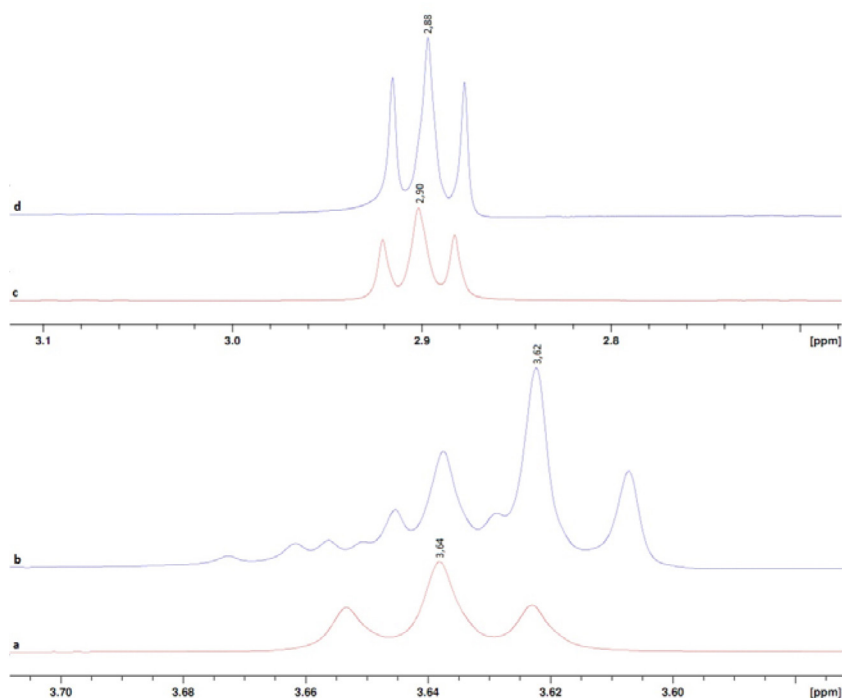


FIGURE 5 - Details of regions between δ 3.55–3.75 ppm (**H1**) and δ 2.70–3.10 ppm (**H2**) of ^1H NMR spectra of Lys (a) and HA-Lys nanoparticles (b) at 400.13 MHz in D_2O .

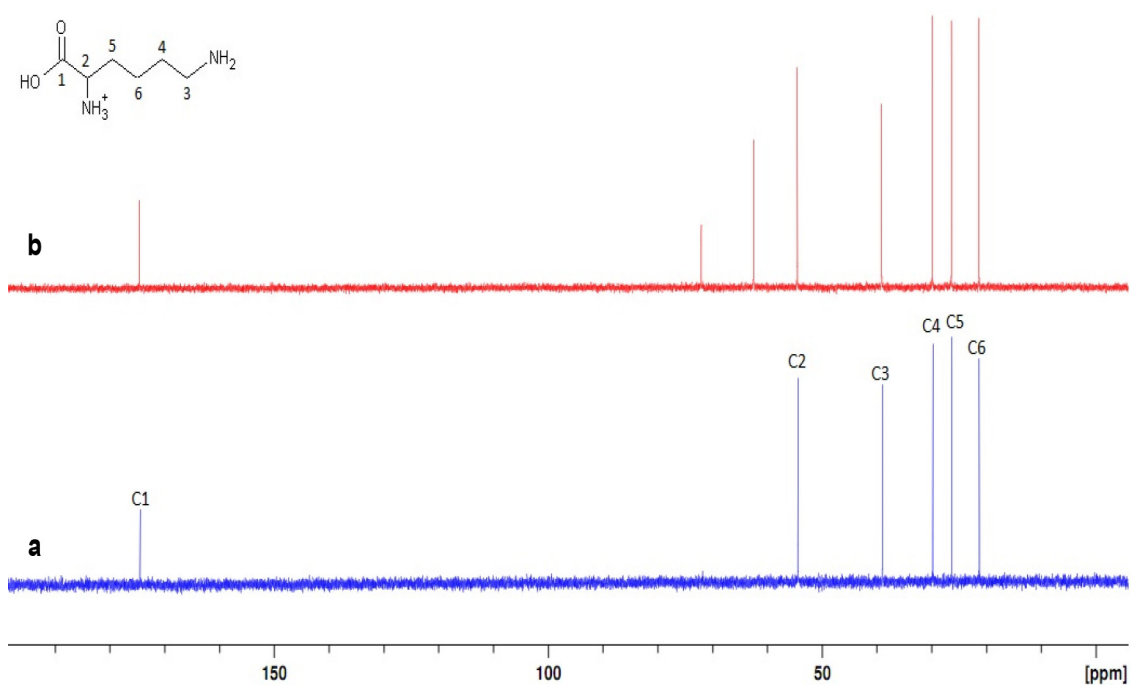


FIGURE 6 - ^{13}C NMR spectra of Lys (a) and HA-Lys nanoparticles (b) at 100.61 MHz in D_2O .

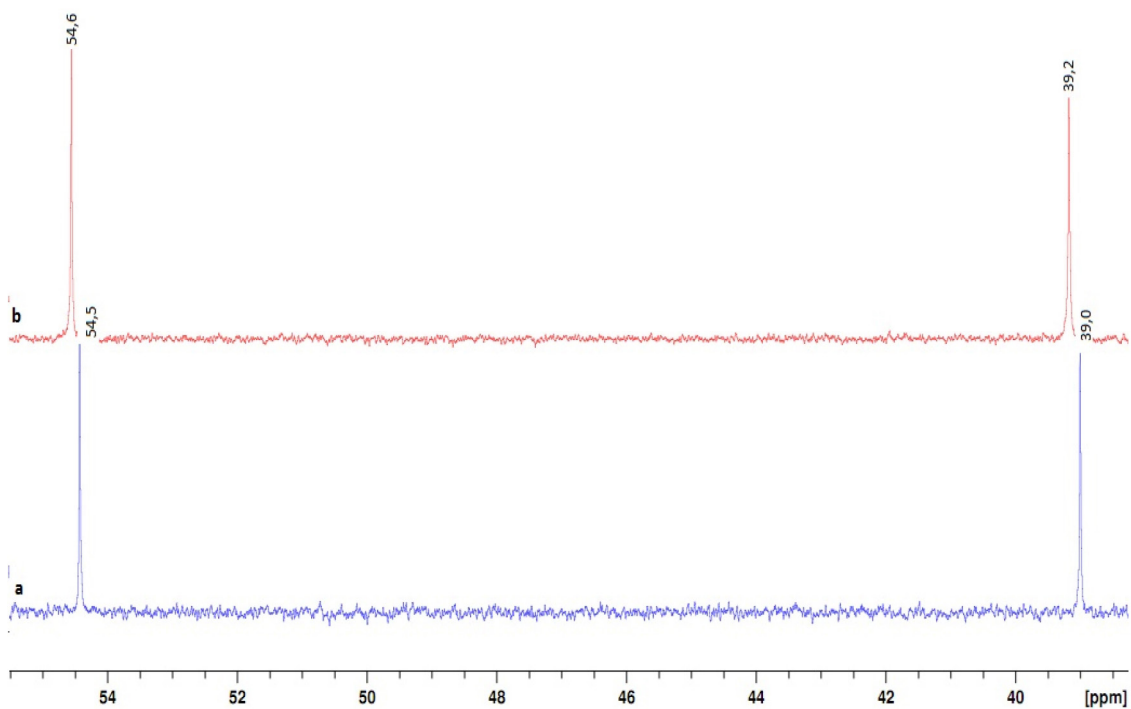


FIGURE 7 - Details of the region between δ 38–55 ppm (C2 and C3) of ^{13}C NMR spectra of Lys (a) and HA-Lys nanoparticles (b) at 100.61 MHz in D_2O .

between carboxyl group of HA and amine group of Lys. In summary, these physicochemical features suggest that HA-Lys nanoparticles can be further used as a topical dermal filling.

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