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RESEARCH PAPER

Novel Self-assembled, Gel-core Hyaluosomes for Non-invasive Management of Osteoarthritis: *In-vitro* Optimization, *Ex-vivo* and *In-vivo* Permeation

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ABSTRACT

Purpose Hyaluronic acid (HA) is an imperative biomaterial with desirable rheological properties to alleviate symptoms of osteoarthritis. Nevertheless, scantly percutaeous permeation of this macromolecule handicaps its effective use for orthopedics and triggers intra-articular injection as the only surrogate. This study presents novel self-assembeld HA-based gel core elastic nanovesicles, (hyaluosomes; GC-HS), for non-invasive transdermal delivery of HA.

Methods GC-HS were prepared with 1% HA using simple film hydration technique. Their size, zeta potential, percentage entrapment efficiency (% EE), elasticity, and *ex-vivo* transdermal permeation were evaluated compared to conventional liposomes CL. Structure elucidation of the formed novel system was performed using light, polarizing and transmission electron microscopy. *In-vivo* permeation of GC-HS through knee joints of female Sprague Dawley rats was compared to CL and HA alone.

Results GC-HS showed nanosize (232.8 ± 7.2), high negative zeta potential (−45.1 ± 8.3) and higher elasticity (size alteration 5.43%) compared to CL. This novel system has self-penetration enhancing properties compared to CL and plain gel. GC-HS showed self-assembled properties and high physically stable for at least 6 months at 4°C. *Ex-vivo* permeation of HS was significantly higher than CL and plain HA gel alone. *In-vivo* study exhibited significant six folds increase in transdermal permeation of HA to knee joints from GC-HS compared to plain HA gel.

Conclusion Novel GC-HS are promising nanogels for topical management of osteoarthritis surrogating the need for intra-articular injection.

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KEY WORDS elastic nanovesicles · gel-core · hyaluronic acid · osteoarthritis · transdermal

ABBREVIATIONS

CL	Conventional liposomes
CLG	Conventional liposomal gel
GC-HS	Gel core hyaluosomes
HA	Hyaluronic acid
HS	Hyaluosomes
SC	Stratum corneum

INTRODUCTION

Transdermal drug delivery is a non-invasive approach that can be utilized to bypass the variables of drugs oral absorption and the pain and side effects associated with injections. The major challenge with transdermal drug delivery is the barrier nature of skin that limits the entry of most of the drugs. Several approaches and delivery systems have been investigated and utilized to overcome the barrier of stratum corneum (SC) to attain higher transdermal permeability (1–3). Among explored systems, phospholipid based ones offer captivating properties for transdermal delivery due to their biocompatibility and ease of mixing with the skin lipids (4). There has been considerable interest on the use of liposomes for skin delivery (5). However, it was proved that conventional liposomes are of little value for transdermal delivery, due to their lower ability to deeply penetrate the skin (6). They were rather remaining confined to the upper layers of the stratum corneum.

It was reported that flexibility of liposomes could result in enhancing their skin penetration (7–9). Furthermore, vesicles with high flexible membranes could deliver macromolecules to deeper layers of skin as compared to rigid liposomes (10).

Being flexible, they were capable of adapting their shape and volume when passing through the SC (8). Modulating vesicles composition; sequences of new vesicles with flexible membranes were developed in order to improve the dermal or transdermal delivery of drugs (4). Transfersomes are liposomes containing edge activators such as sodium cholate and Tween 80 (8, 9, 11). Edge activators are often single-chain surfactants that destabilize lipid bilayers of the vesicles and provide flexible membrane (10). It has been reported that transfersomes have efficiently penetrated the intact skin with therapeutic concentrations of different drugs (7, 10, 11). Furthermore, vesicles membrane elasticity could be increased by addition of the ethanol to the lipids. These vesicles were called ethosomes and they are composed of phospholipids, water and ethanol (8, 12). Ethanol allows them to penetrate easily into the deeper layers of the skin by fluidizing the lipid domain of SC (13).

The most recent approach in liposomal systems that has not so far been employed in skin delivery is the gel core liposomes. They are phospholipid bilayered vesicles entrapping hydrogel polymer inside their core (4). Therefore, they gather the advantages of both phospholipids and hydrogels. In addition, the polymer incorporated inside the core supports the lipid bilayer preventing their rapid degradation in the body and early drug release. Different complicated approaches are described in the literature for the preparation of gel core liposomes, including preparation of a microgel then coating it with the phospholipid bilayer (14, 15) and preparation of liposomes incorporating a polymer in a sol form, gelation of the core are then induced (16, 17).

Hyaluronic acid (HA) is used widely in numerous medical applications as it is biodegradable, biocompatible, non-immunogenic and non-inflammatory biomaterial. It is found in all tissues and body fluids but present in large quantities in the soft connective tissues. HA has excellent water-binding capacity; therefore it is responsible for lubrication of eyes, joints, and skin tissues (18). Its high solution viscosities and unique viscoelastic properties enables its use for orthopedy (19). A lot of studies have been performed to formulate intra articular injections of HA for treatment of knee osteoarthritis (20).

HA was recently examined as a vehicle for skin delivery (21, 22) and may also have potential as a depot system (23). However, only few studies considered HA permeation through the skin. Brown *et al.* (24) found that HA was transported across the epidermis and metabolized in the skin when studying penetration of tritium-labeled HA gel. Kage *et al.* (25) reported low penetration of HA through intact skin when examining the permeation of HA tetrasaccharides through hairless mouse skin. These studies indicate that HA alone hardly penetrates intact skin may be due to its hydrophilicity and high molecular weight. Therefore, a carrier is needed in order to efficiently deliver across the skin. Recently, Chen *et al.* (26) utilized a skin penetrating peptide conjugated with phospholipids in order to enhance topical delivery of HA. *Ex-vivo* results showed enhancement of HA skin penetration but the drug was localized in SC and epidermis. However, minimal penetration into the dermis and limited penetration (almost less than 0.1% from the applied dose) into the receptor compartment were obtained.

Delivery of high molecular weight HA into orthopedic joints was reported to restore the desirable rheological properties and alleviate some of the symptoms of osteoarthritis (27). Nevertheless, no study has so far considered improving transdermal permeation of HA. All the utilized formulations in that regard are intra articular injectable ones with their associated pain and complications. Therefore, the aim of this study is to design a novel system that is able to deliver HA transdermally to the joint tissues in order to restore rheological properties while avoiding invasive techniques. In attempt to gather the positive effect of flexible liposomes, gel core vesicles and the viscoelastic properties of HA on the transdermal delivery, this study is the first one to tailor novel self-assembled gel core elastic vesicles, namely, hyaluosomes (HS) for management of osteoarthritis. Optimization and full *in-vitro* characterization for liquefied and gelled nanovesicles would be carried out. *Ex-vivo* permeation experiments were carried out using rat skin. *In-vivo* permeation was assessed as well on rats by topical application on skin covering knee joints.

MATERIALS AND METHODS

Materials

Hyaluronic acid (HA, $M_W = 0.8-1.17^{\times}10^4$ Da) was obtained from Shiseido (Japan). Stain All and methanol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Lipoid S100 (Phosphatidylcholine from soybean) was kind gift from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sigma Chemicals Corporation, U.K. Tween 80 and all other reagents were of analytical grade.

Preparation of Liquefied Hyaluosomes (HS)

The term hyaluosomes utilized in this study was defined as elastic nanovesicles prepared using phospholipids, an edge activator, and HA. Hyaluosomes (HS) were prepared by thin layer evaporation technique. Briefly, both Lipoid S100 (PL) and Tween 80 (as edge activator) were dissolved in chloroform. The solvent was then removed under reduced pressure in a rotary evaporator at 58°C, to obtain a thin film on the flask wall. Evaporation was continued for 2 h after the dry residue appeared, to completely remove all the solvent. Obtained dry lipid films were then hydrated with the hydrating solution containing 2 mg/ml (0.2%) HA. For ethanol

modified ethanol (HS2) 0.2% HA in water:ethanol 80:20 was used as hydrating medium. The hydrated film was subjected to water bath heating at 58°C for 10 min and vortexing for 2 min; this cycle was repeated four times. The prepared vesicles were then extruded through polycarbonate membranes with pore size of 200 nm at room temperature with the aid of a Liposome Extruder (Model ER-1, Eastern Scientific LLC, USA). The dispersion was hermetically sealed and stored at 4°C (7). Conventional liposomes (CL) were also prepared by the same procedure using cholesterol instead of edge activator; and used for comparison.

Preparation of Self-Assembled Gel Core Hyaluosomes (GC-HS)

Novel self-gelling vesicular systems (GC-HS1, GC-HS2) were prepared by the aforementioned methodology and ratios using 10 mg/ml (1%) hyaluronic acid was used as a hydrating medium. Conventional liposomal gel (CLG) was prepared for comparison by same concentration of HA. A series of the prepared formulations with their detailed composition is shown in Table I.

Physicochemical Characterization of Vesicles

Particle Size, Zeta Potential and PDI

The particle size (PS), Polydispersity index (PDI) and zeta potential (ZP) were determined by dynamic light scattering (DLS) technique using Zetasizer Nano ZS. (Malvern, Instruments Ltd., Malvern, UK). For measurements, formulations were properly diluted (0.1%*v*/*v*) with filtered distilled water to avoid multiscattering phenomena. Each sample was measured three times over 10 min. All measurements were conducted at 25°C. All samples were measured in triplicates and results were represented as mean value ± SD.

Table I Detailed composition of the prepared formulations

Formula code	Composition	
	Additives in the lipid bilayer (PL:additive ratio, 85:15)	Hydrating medium
HS1	Tween 80	0.2% HA
HS2	Tween 80	0.2% HA in Water:Ethanol 80:20
CL	Cholesterol	0.2% HA
GC-HS1	Tween 80	1% HA
GC-HS2	Tween 80	1% HA in Water:Ethanol 80:20
CLG	Cholesterol	1% HA

Measurement of Elasticity

Elasticity of the prepared HS and GC-HS was measured by extruding vesicles through a polycarbonate filter of 200 nm pore diameter using a conventional liposome extruder (28). Percentage alteration in size after extrusion was then calculated using the following equation:

$$\%\textit{Alteration in size} = \frac{\textit{size before extrusion} - \textit{size after extrusion}}{\textit{size before extrusion}} * 100$$

Experiments were carried out in triplicates to obtain a mean value ± SD.

Morphological Characterization

Morphology of the prepared nanovesicles was determined by transmission electron microscopy (TEM). Samples were firstly diluted with distilled water and dropped onto a carbon-coated copper grid and left for 1 min to allow vesicles to adhere on the carbon substrate. The excess dispersion was removed with a filter paper. Negative staining using a 2% phosphotungstic acid solution (*w*/*w*) was directly made on the deposit for 45 s. Then the air dried samples were directly examined under the TEM (29, 30).

Phase Behavior and Structure Elucidation

An initial analysis of the nature and structure of the designed self-gelling HA based system was done using microscopical analysis. Confirmation of the formation of gel core HS was done using by means of a projection microscope fitted camera (Olympus, Germany). Briefly, formulations were treated with Triton X-100 and their phase behavior was investigated before and after addition of Triton X-100 (17). Moreover, a polarized light microscope was used in order to reveal more details on the structure of the prepared self-assembled GC-HS without any dilutions. Undiluted samples of the examined systems were investigated for the presence of liquid crystals.

Detection of HA in Solutions with Stain All

HA is a multi-negative charged biopolymer and it can form stable complexes with many cationic dyes. Some reported methods for its analysis and quantification in solutions and biological fluids are based on this property (31–34). Stain All is a reported assay method for determination of the HA in different dilution folds of solutions and biological fluids (32, 34). It was adopted in this study for HA determination. Stain All is the short name for 1-ethyl-2-{3-(1- ethylnaphtho[1,

2d]thiazolin-2-ylidene)-2-methylpropenyl}- naphtho[1, 2d]thiazolium bromide. It can bind with glycosaminoglycans and form complexes whose optical properties are different from those of the free dye (32).

The solution of the dye (0.1 mg/ml) was prepared by dissolving 5 mg of Stain All in 43 ml of water and 7 ml of methanol (32). HA solutions in concentrations range from 0.25 to 5 μg/ml containing Stain All dye (final concentration 0.03 mg/ml) were prepared. A calibration curve was constructed from the absorbance at 640 nm of HA/dye complex formed in such solutions. The absorbance dependence on concentration is linear in the specified range of concentrations with regression equation $y=0.3603x+0.1586$ and r^2 value that equals to 0.9975.

Moreover, at concentrations higher than about 2 μg/ml the color change can be clearly detected by the naked eye as the typical magenta color of the free dye turns blue/cyan upon addition of HA.

Determination of Entrapment Efficiency

Percentage entrapment efficiency (%EE) of the prepared HS was determined by separation of vesicles from the solution, after properly diluting the sample, using centrifugation (15.000 rpm for 15 min at 4°C). Supernatant was collected and used for determination of un-entrapped HA using Stain All assay method and measuring UV absorbance of HA/dye complex at 640 nm. The entrapped drug was calculated by the following equation:

$$\%EE = \frac{total\ drug - free\ drug}{total\ drug} * 100$$

Ex-vivo Skin Permeation and Deposition Study

In this experiment, the ability of the prepared liquefied (HS1, HS2) and the novel self-assembled gel core hyaluosomes (GC-HS1, GC-HS2) in enhancing HA transdermal penetration and dermal retention was investigated. Conventional liposomes (CL, CLG) and corresponding HA 0.2% aqueous solution and 1% aqueous gel were used for comparison. Dorsal skin of male Sprague Dawley rats (200±25 g) was used. Rats were subjected to hair removal using depilatory cream to keep skin integrity, and then sacrificed. Full thickness (1.2–1.4 mm) skin was excised, stored at −20°C and defrosted immediately prior to use.

The *ex-vivo* experiment adopted in the current study was performed according to the open hydration protocol described in the literature (35, 36). Skin pieces were fixed to the modified Franz diffusion cells 24 h prior to the experiment, with the stratum corneum side up to the donor compartment and the dermis side to the receptor fluid. The donor compartment was kept dry and open to atmosphere (35), in order to maintain a transepidermal hydration gradient generating the driving force for skin penetration of elastic vesicles (37). Afterwards, the receptor content was replaced by a fresh medium (9 ml distilled water) and 200 μl of the tested formulations were applied to skin surface, which had an available diffusion area of 1.4 cm^2. Formulations were kept non occluded throughout the experiment. The receptor medium was kept at 37° ±1°C with 100 rpm shaking throughout experiments using a shaking water bath. Each test formulation was assessed in five separate cells. Placebo formulations were also tested in order to ensure the absence of any interference in the assay method. The whole volume of the receptor fluid was removed at appropriate time intervals (2, 4, 6, 8, and 24 h) and immediately replaced with fresh medium. Samples were quantified for HA concentration using Stain All assay method and measuring UV absorbance of HA/dye complex at 640 nm.

For dermal localization studies, the amount of HA retained in the skin samples was determined. At the end of the above mentioned *ex-vivo* transdermal permeation test (24 h), the skin mounted on the diffusion cell was removed. The residual formulation adhering to the skin was removed with a spatula and then the skin surface was washed with distilled water. For extraction of HA from skin pieces 10 ml methanol : water mixture, ratio 1:1 were added to the skin in a glass vial and shaked over night at room temperature (26). The obtained dispersions were centrifuged for 10 min. at 10000 rpm. Supernatants were withdrawn, suitably diluted and HA content was determined spectrophotometrically at 640 nm using Stain All assay method according to Kutsch H. *et al.* (34).

In-vivo Study

The experiment was performed on 27 female Sprague Dawley rats (200±25 g) divided into six groups. Just before the experiment, the rats' hair was removed over the exposed area (knee joint and leg). Formulations were applied topically on the rats' skin on the knee joint and allowed to dry. To avoid leakage from the exposed leg and to get the comparable dose application in all rats, all exposed rats were sedated with ether inhalation for almost 1 min during the application. All animals were treated in accordance with the institutional laboratory animal care approved ethical guidelines.

Groups I, II, and III were investigated for a single dose application for short exposure time. Each group consisted of three rats; they were exposed to topical application of a weight equivalent to 1 mg HA of GC-HS, Eth-GC-HS, and 1% HA aqueous gel, respectively. After 6 h exposure, rats were sacrificed (26).

On the other hand, groups IV, V, and VI were investigated for multiple applications of higher HA dose and for longer

exposure time. Each group consisted of six rats; they were also exposed to GC-HS, Eth-GC-HS, and 1% HA aqueous gel, respectively. Only, the right legs were subjected to the tested formulations while the left ones were kept as control. The multiple dose study was performed by applying a weight equivalent to 2 mg HA once daily for 2 consecutive days. Rats were then sacrificed after 48 h of the first application (38).

The skin was cleaned from formulation residues if any. Skin from the application site and joint tissue samples were collected. For extraction of HA from samples 10 ml methanol: water mixture (ratio 1:1) were added to the skin in a glass vial and shaked over night at room temperature (26). Obtained dispersions were centrifuged for 10 min. at 10000 rpm. Supernatants were withdrawn, suitably diluted and the drug content was determined spectrophotometrically at 640 nm using Stain All assay method according to Kutsch H. *et al.* (34).

Stability Studies

In order to determine the physical stability of selected gel formulations, they were stored at 4°C for up to 6 months. At predetermined time intervals, the particle sizes of the vesicles, PDI and %EE were measured.

Statistical Analysis

Data analysis was carried out using Microsoft Excel 2010. Results were expressed using mean and standard deviation. Statistically significant differences were determined using two-tailed and unpaired student's *t*-test. $P < 0.05$ was described as the level of significance.

RESULTS AND DISCUSSION

Preparation and Characterization of HA Based Elastic Nanovesicular Systems

Liquefied Hyaluosomes

As mentioned previously, CL were of little value for transdermal drug delivery. Therefore, in this study, modified elastic liposomes were prepared. Incorporating Tween 80 in vesicles' bilayer and using 0.2% HA as hydrating medium, modified HA-based elastic vesicles namely, hyaluosomes (HS1) were prepared. In an attempt to further improve elastic properties of the vesicles, ethanol-modified hyaluosomes (HS2) were prepared by incorporation of ethanol to the hydrating medium.. Preliminary screening of optimal concentration of Tween 80 and ethanol was performed. Results revealed that a ratio of 85:15 (PL: Tween ratio) and 20% ethanol was the optimum giving the best size and elasticity. The thin film-technique was adopted in this study as it was reported to help entrapment of hydrophilic drugs, due to formation of a thin-film with large surface area which enables the complete hydration of the vesicles (39). These liquefied HS were characterized compared to CL containing same drug concentration (CL).

The vesicles size was in the nano-size range with the size distribution (polydispersity index; PDI) 0.2-0.3 and a negative zeta potential. The obtained negative zeta potential might be due to the negatively charged lipids and the presence of HA (26). Compared to CL, HS1 and HS2 have demonstrated significant ($p < 0.05$) lower size, and higher entrapment efficiency (%EE) (Table II). Lower (particle size) PS of HS might be attributed to the incorporation of Tween that can achieve higher vesicular curvature (40). Higher %EE of elastic vesicles compared to conventional liposomes has also been reported by other researchers (41, 42).

Self-Assembled Gel-Core Hyaluosomes (GC-HS)

To circumvent drawbacks of liquid status upon skin application we have to convert such nanovesicles into gel form. The distinct viscoelastic properties of HA were utilized in this study to achieve that aim in addition to form gel core vesicles in one step. Moreover, HA was recently reported to have clear and unique potential in enhancement of skin delivery and dermal localization of drugs incorporated inside (21, 23). Combining the advantages of modified elastic vesicles together with unique properties of HA in skin delivery is anticipated to enhance transdermal permeation of HA together with its retention inside the deeper layers of the skin acting as depot. Moreover, being core gelled may improve vesicles stability, reducing their degradation and early leakage of entrapped HA; thus enhancing transdermal delivery.

Applying the same preparation protocol and using HA 10 mg/ml (1%), novel self-gelling HA-based systems were formulated by our group. This novel formulation can be described as self-gelled system incorporating elastic nanovesicles (Table II) that were core gelled with HA.

Elasticity was a very important property for deformable vesicles that make them different from conventional liposomal vesicles. Upon extrusion, a stress was exerted on the vesicles to be enforced to pass through membrane pores. Elastic vesicles could spontaneously undergo deformation to pass through pores avoiding structure rupture, keeping their size and integrity. Therefore, % size alteration is inversely proportional to elasticity. Table II showed the % size alteration of the prepared novel systems after extrusion. As shown in Table II, the prepared GC-HS exhibited significant higher elasticity compared to CL. This might be attributed to the edge activator mechanism that could temporarily transform the bilayer structure to a single layer allowing vesicles to pass through small pores, and then retain their shape (43).

In addition, the presence of HA gel entrapped inside the core and surrounding vesicles might further increase the

Table II Physicochemical characteristics of the different prepared vesicular systems

Formula code	Vesicles size* (nm)	PDI*	Zeta potential (mV)	Size after* extrusion (nm)	Size alteration (%)	Entrapment efficiency* (%)
HS1	180 ± 1.7	0.3 ± 0.05	−41.2 ± 8.3	165.2 ± 2.74	**	53.9 ± 3.6
HS2	169 ± 2.8	0.2 ± 0.05	−52.1 ± 8.2	156.0 ± 3.25	**	48.9 ± 4.2
CL	361 ± 15.1	0.4 ± 0.1	−39.0 ± 5.8	284.1 ± 15.2	21.2	37.2 ± 2.7
GC-HS1	233 ± 7.2	0.2 ± 0.01	−45.1 ± 8.3	220.15 ± 5.4	5.4	41.0 ± 1.8
GC-HS2	226 ± 10.1	0.3 ± 0.1	−46.1 ± 9.2	218.5 ± 6.9	3.2	32.6 ± 4.6
CLG	1011 ± 36.6	0.8 ± 0.4	−40.2 ± 4.4		***	23.6 ± 2.2

*$n = 3$

** Size was lower than the diameter of the extrusion membrane.

*** Not applicable due to extremely large particle size.

elasticity of HS. GC-HS1 and GC-HS2 showed size alteration 5.4 and 3.2% respectively compared to CL that showed size alteration 21.23%. This demonstrated higher elasticity might further increase the penetration to deeper layers of the skin and enhance transdermal permeation (7, 12). Moreover, entrapment of the vesicles inside HA external gel matrix offers an additional opportunity to increase the localization of the drug in the skin layers serving as depot (23).

Although there was a decrease in the *% of HA entrapped* inside the vesicles when increasing its concentration from 0.2 to 1% (Table II), 3 to 4 times increase has been observed in *HA amount entrapped* inside the vesicles when using 1% HA. Considering that, in both HA concentrations the same amount of PL was utilized, this might indicate that this amount entrapped is the maximum HA amount that could be tolerated by the vesicles. On the other hand, the highly viscous HA might be adsorbed on the surface of the vesicles during their formation lowering the possibility for more HA to be entrapped inside vesicles core.

Morphological examination *via* TEM confirmed formation of HS with an obvious gel core, GC-HS1 and GC-HS2 (Fig. 1a and b). As shown in the obtained photomicrographs, a perfect vesicle spherical structure was obtained with homogenous size distribution. Moreover, no aggregates appeared in the micrographs indicating the advantage of being in a gel form. The gel structure minimized the vesicles aggregation and fusion after preparation. It is worthy to notify that those formulations were prepared spontaneously forming stable self-gelling formulations. On the other hand, CL had not such self-gelling properties. CLG pointed out problems in forming homogenous dispersion; this was confirmed by the large PDI value (PDI 0.8 ± 0.4) and malformed vesicles observed with TEM (Fig. 1c). Moreover, results inferred the formation of vesicles with extremely large size (1011 ± 36.6 nm). Therefore this formulation was excluded from further investigations.

Confirmation of the formation of gel core vesicular structure was done by examining formulations under light microscope before and after addition of triton X-100. Spherical structure of GC-HS was maintained in formulations treated with triton-X100 (Fig. 2). On the other hand, the vesicular structure was totally vanished after treating placebo formulation with triton-X100. Triton-X100 dissolves the lipid bilayer so when the core was gel, the spherical structure of the core could be retained as in GC-HS. Hence, placebo formulation

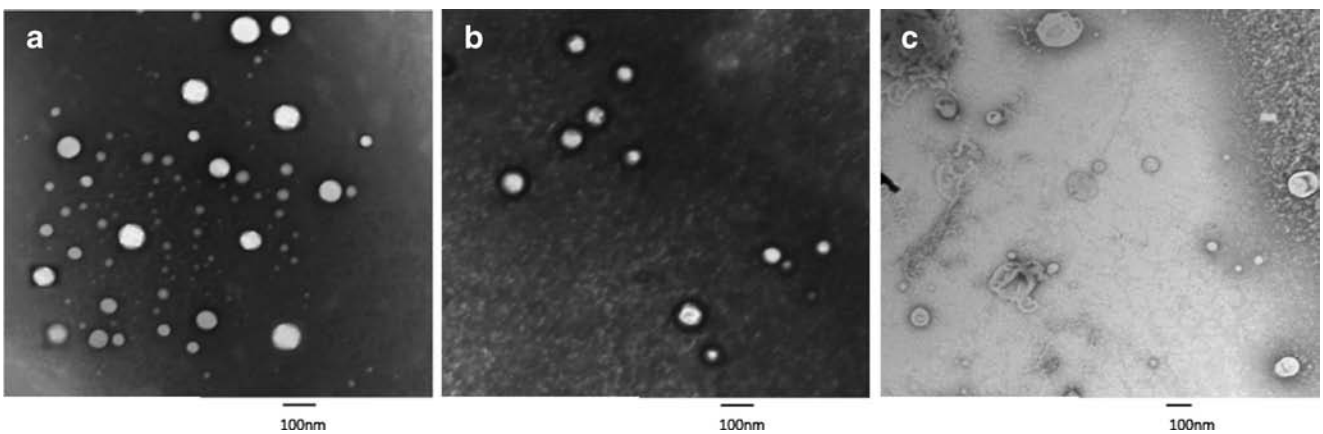

Fig. 1 Transmission electron micrographs of the prepared gel core hyaluosomes (**a**) GC-HS1 and (**b**) GC-HS2 compared to conventional liposomes (c) CLG.

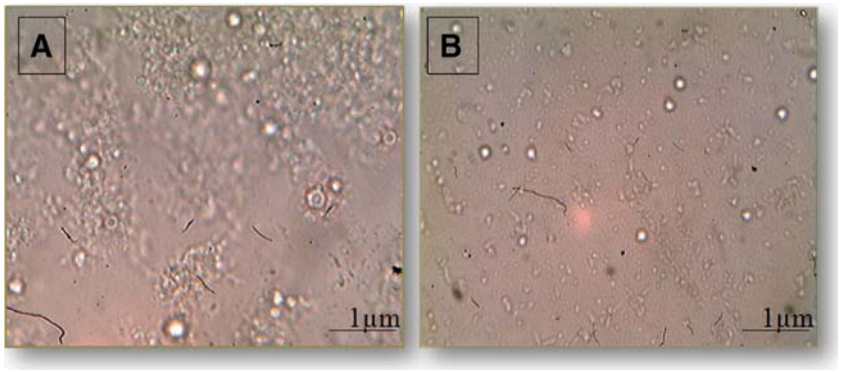

Fig. 2 Light microscopy photomicrographs of gel core hyaluosomes (GC-HS1), (**a**) before treatment with Triton X-100 (**b**) following treatment with Triton X-100.

cannot preserve its spherical shape after lipid layer removal (Fig. 3).

Polarizing Microscopy

A polarized light microscope was used in order to investigate the gel structure details of this novel system and the presence of liquid crystals. Polarizing microscopy is a technique reported to discriminate isotropic (monophasic) from anisotropic systems which are characterized by crystal formation [223]. The optical properties of isotropic systems are not direction dependent and with only one RI the propagation direction of light passing through this material is not influenced. When the investigated formula presents structures that reflect the incident light, it is an evidence of the presence of liquid crystals (Fig. 4b and c) in comparison to HA sample (Fig. 4 a) that was free of any crystals Liquid crystals are described as a state of matter between solids and liquids, it means that, they are fluid like liquids but are organized like solids, being called mesophases (44). These organization contributes to the highly stability of systems.

The formation of liquid crystals could be induced by some components present in this system, such as surfactants (44). Photomicrographs obtained for GC-HS1 and GC-HS2 indicated the formation of a peculiar system that is not a simple gel and not a genuine liquid crystal, but a gel system that incorporates liquid crystals inside. These are commonly observed defects of the smectic phase lamellar structures that are formed around the inner core of the vesicles (45, 46). This characteristic structure can make the system formed more stable by reducing the possibility of vesicles aggregation and fusion. It is noteworthy that HS sample treated with triton (Fig. 4d and e) exhibited significantly lower crystals, confirming explanation o dissolved bilayer proposed by TEM.

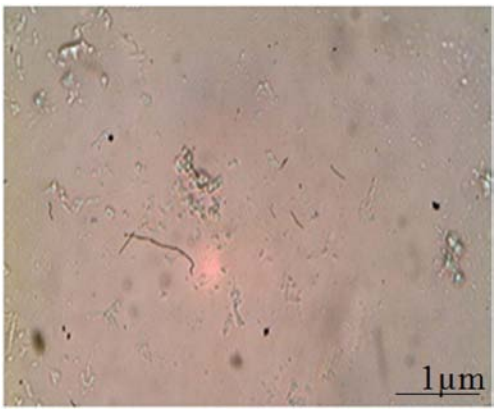

Fig. 3 Light microscopy photomicrograph of placebo vesicles after treatment with Triton X-100.

Ex-vivo Skin Permeation and Deposition Studies

Both liquefied HS and the novel self-assembled GC-HS were tested for their efficiency in enhancing transdermal permeation and dermal localization of HA compared to corresponding CL and drug aqueous dispersions. Results indicated that HA skin uptake and accumulation were significantly ($p<0.05$) enhanced by both HS and CL compared to corresponding drug solutions (Fig. 5). Regarding transdermal permeation, only HS either in liquefied or gel state were able to significantly ($p<0.01$) enhance the transdermal permeation of HA to the receptor fluid compared to the corresponding drug aqueous dispersions (Fig. 5 and 6). In contrast, HA permeation to the receptor compartment from CL and CLG was not enhanced and almost similar to that from corresponding drug aqueous dispersions (Fig. 5 and 6). These results are in agreement with other studies reporting the ability of elastic vesicles to penetrate skin more deeply compared to conventional liposomes (7, 8).

Increasing the concentration of HA from 2 to 10 mg/ml significantly ($P<0.05$) enhanced its dermal localization from all gel formulations (GC-HS, CLG, and 1% HA) (Fig. 5). However, the magnitude of improvement of HA localization induced by the novel self-assembeled GC-HS was much more significant ($P<0.01$) than CLG and 1% HA aqueous gel. On the other hand, the amount of HA that reached the receptor fluid increased significantly ($P<0.01$) only in the novel self-assembled GC-HS. The amount of HA that was delivered to the receptor compartment from GC-HS1 and GC-HS2 was approximately 5.5 folds ($p<0.01$) higher than that from

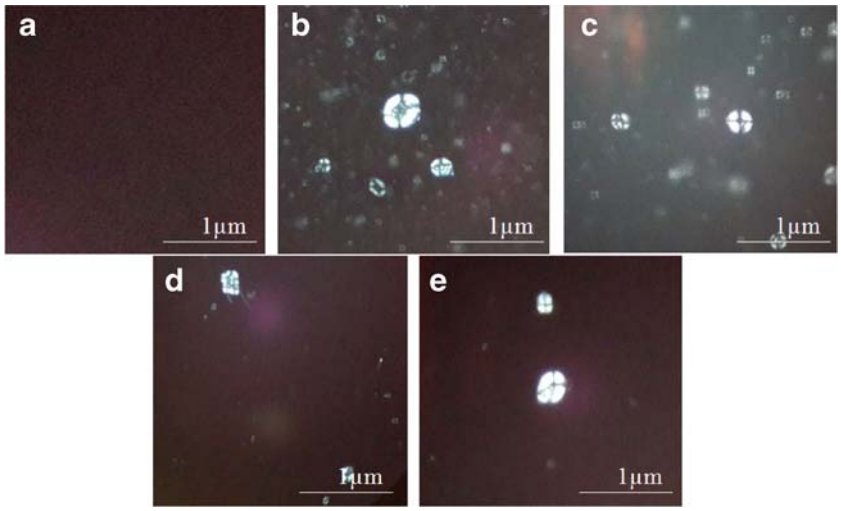

Fig. 4 Photomicrographs of liquid crystal present in self-assembled gel core hyaluosomes evaluated by polarized light microscope. (**a**) HA 10 mg/ml (**b**) GC-HS1 (**c**) GC-HS2 (**d**) GC-HS1 after addition of Triton X-100 (**e**) GC-HS2 after addition of Triton X-100.

1% HA aqueous gel. In addition, as shown in Fig. 6, not only the amount of HA reached the receptor fluid was increased but also the drug delivery rate was significantly enhanced. The amount of HAreached the receptor fluid from GC-HS1 after 2 h was 5.4 folds and 2.6 folds higher than the amount reached from the liquefied HS1 vesicles and 1% HA aqueous gel respectively after 8 h.

The observed enhancement of dermal localization of the drug might be partly attributed to the positive effect of HA on enhancing drug retention inside the skin. It has been reported that due to its high hygroscopic properties, HA may increase the SC hydration and create hydrophilic pathways; thus enhancing the skin permeability. Furthermore, the hydrophobic part in HA chain might facilitate permeation through the SC (23). However, HA alone and CLG prepared with same HA concentration was not able to significantly enhance transdermal permeation. Besides, the magnitude of improvement of HA skin localization induced by the novel self-assembeled GC-HS was much more significant ($P<0.01$) than CLG and HA. This noticeable improvement indicated the augmenting effect of assemblage of elastic vesicles, gel core, and HA properties in this novel formulation. Being highly elastic, the prepared self-assembled gel core vesicles could penetrate the skin through the hydrophilic pathways formed by HA, without losing vesicles integrity. Moreover, being core gelled, provided the system with more stability to withstand surrounding environment preventing vesicles damage and early drug leakage (17). Consequently, more vesicles can be transported with less deformation to the receiver compartment. On the other hand, CL with less flexible bilayers would fracture during transport through skin pores therefore they were unable to reach acceptor fluid.

In-vivo Study

The aim of this study was to evaluate the penetration of HA through the skin and its delivery to the knee joint in rats after single and multiple dose topical applications. The prepared

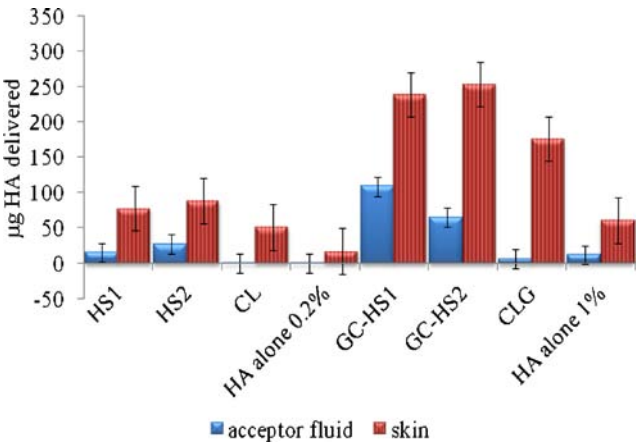

Fig. 5 Improved high molecular weight HA penetration into rat skin and acceptor fluid after 24 h using the different formulations (Table I). The error bars indicate the corresponding standard errors ($n=3$).

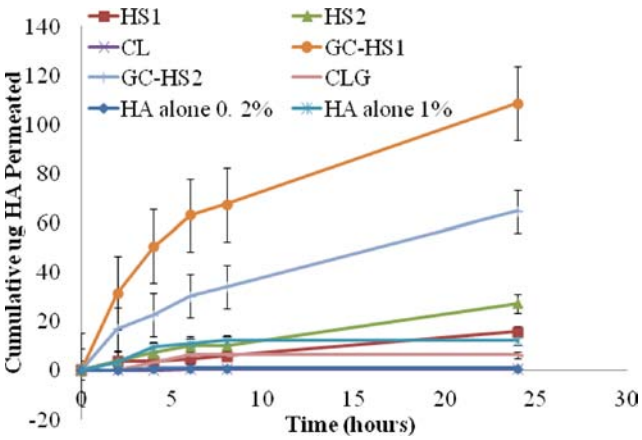

Fig. 6 *Ex-vivo* HA permeation profiles from the different formulations (Table I) through rat skin in Franz diffusion cell at 37°C ± 0.5°C. The error bars indicate the corresponding standard errors ($n=5$).

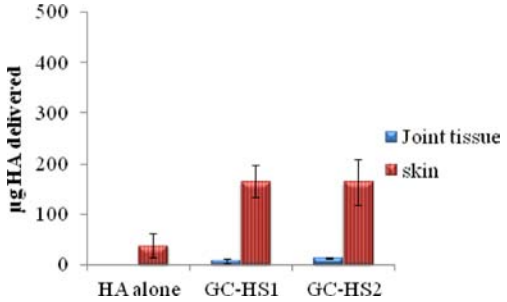

Fig. 7 Total amount of HA accumulated in rats skin and joint tissue after single application of formulations GC-HS1, GC-HS2, compared to HA 10 mg/ml. Dose applied was equivalent to 1 mg HA and rats were sacrificed 6 h post application. The error bars indicate the corresponding standard deviations ($n=6$).

novel self-assembled GC-HS were examined compared to HA alone 10 mg/ml. First, single application (dose) experiment was investigated. An amount equivalent to 1 mg HA was applied to the skin covering the knee joint and then rats were sacrificed after 6 h of the application (rat groups I, II, and III). It was found that HA penetrated to a high degree into the examined skin samples. Enhanced dermal localization of HA from the novel formulations was also observed (Fig. 7). Accumulation of HA inside the skin from the examined formulations GC-HS1 and GC-HS2 was 4.3 and 4.3 folds higher than 1% HA gel after 6 h contact. On the other hand, no drug was found in the examined joints after 1% HA gel application while minimal amounts 7.9 ± 2.4 and 13.1 ± 1.1 µg were found after application of GC-HS1 and GC-HS2 respectively. The low amounts observed in the joint tissues might be due to the applied low dose and the short contact time.

On the other rat groups (IV, V, and VI) dose equivalent to 2 mg of HA and the formulations were applied two times over 48 h (once/day) then rats were sacrificed after 24 h from the second application. A highly significant ($p<0.01$) increase in the amount of HA penetrating through the skin into the rats joint tissue was observed (Fig. 8). Moreover, the amount of HA found in examined joint tissues from GC-HS1 and GC-HS2 was 6. and 5.9 folds higher than HA aqueous gel. These

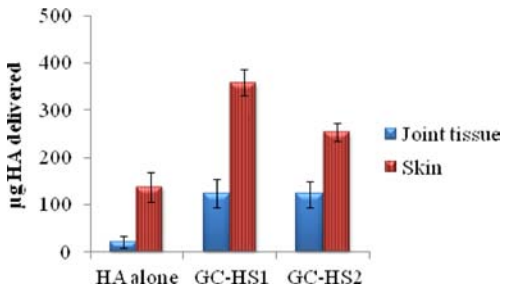

Fig. 8 Total amount of HA accumulated in rats skin and joint tissue after two application (once daily) of formulations GC-HS1, GC-HS2, compared to HA 10 mg/ml. Two doses were applied, once daily, each was equivalent to 2 mg HA and rats were sacrificed 48 h post first application. The error bars indicate the corresponding standard deviations ($n=6$).

Table III Vesicles size and %EE determined for selected formulations when fresh and after storage for 6 months in 4°C

Formulation	vesicles size* (nm)		Entrapment efficiency* (%)	
	Initial	After 6 months	Initial	After 6 months
GC-HS1	233 ± 7.2	269 ± 9.2	$41.\pm1.8$	40.6 ± 3.00
GC-HS2	226 ± 10.1	244 ± 11.1	32.6 ± 4.6	31.5 ± 4.6

*$n=3$

results are very promising and indicate that transdermal route of administration may be useful for HA delivery and allows avoiding unnecessary side effects occurred with intra articular injection.

Stability Study

Results obtained after 6 months storage at 4°C did not observe any destructive effect of storage on most important properties of the tested self-assembled GC-HS and indicated high storage stability of the prepared formulations (Table III). This stability could be attributed to the steric stabilization provided by Tween 80. It has been reported that presence of alkyl chains on the vesicle surface, the hydrocarbon chain of surfactant could penetrate into the phospholipid bilayer, exposing the polyethylene oxide groups on the surface of the vesicles, thereby producing a steric stabilization, which could decrease vesicle fusion (47). In addition, the presence of high molecular weight HA around the vesicles might prevent their aggregation and fusion. Moreover, the incorporation of HA in gel form inside the inner core of the vesicles supports the lipid bilayer preventing their degradation and avoiding drug leakage.

CONCLUSION

On the basis of the ability of high molecular weight HA to alleviate pain of osteoarthritis when reached joint tissues, we have succeeded in formulating a novel self-assembled gel core hyaluosomes that may have potential application in the non-invasive transdermal management of osteoarthritis. The system combines the elastic properties of deformable liposomes together with the stable characteristics of gel core vesicles. Moreover, the presence of suitable concentration of high molecular weight HA additionally potentiate the elasticity and stability of the novel system. On the other hand, we have accomplished a simple highly spontaneous method for the preparation of gel core vesicles avoiding previously reported complicated methods. Hence it may be easy to scale-up and commercialize this novel system.

Conflict of interest The authors report no financial or personal conflict of interest.

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