International Journal of Pharmaceutics 426 (2012) 256–262

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Low molecular weight heparin gels, based on nanoparticles, for topical delivery

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ARTICLE INFO

Article history:
Received 2 November 2011
Received in revised form 20 January 2012
Accepted 21 January 2012
Available online 30 January 2012

Keywords:
Low molecular weight heparin
Gel
Topical delivery
Nanoparticles

ABSTRACT

A commercial suspension of nanoparticles (Eudragit® RS 30D) was used to manufacture a gel for topical application. Gels were prepared by mixing a polycationic polymer (Eudragit® RS 30D) and a low molecular weight heparin (LMWH), an antithrombotic agent. Gels formed spontaneously at a ratio of 1:1 as a result of electrostatic interactions between the polyanionic drug and the polycationic polymer. Different types of heparin were used: Bemiparin, Enoxaparin (Lovenox®), Nadroparin (Fraxiparin®) and Tinzaparin (Innohep®). Several LMWH concentrations were tested. Rheological measurements were performed to investigate the gel behavior. Gel formation was confirmed by dynamic rheological measurements as the elastic modulus (G') was higher than the viscous one (G''). The amount of heparin incorporated into the gel matrix was determined. A maximum of incorporation (100%) was reached using a heparin solution of 600 IU/mL. The release kinetics of LMWH from the gel were also studied. Regardless of the LMWH used in the formulation, a biphasic release profile was observed. Accordingly, a burst effect was observed. Afterwards, the release rate became steady. The penetration of the LMWH through the dermal barrier was also investigated.

1. Introduction

A simple definition of gel is a soft, solid or "solid like" material which may contain both solid and liquid components. Gels are viscoelastic materials formed by a cross-linked three-dimensional network and a solvent. The solvent is the major component in the structure. The solid-like appearance is obtained by the entrapment of a large amount of solvent within the solid matrix (Sangeetha and Maitra, 2005).

Gels can be classified according to the cross-linking type: thermally irreversible or chemical gels and thermo reversible or physical gels (Rogovina et al., 2008). Gels of first type are covalently cross-linked networks, and cannot be dissolved. Physical gels are formed by non-covalent interactions such as Van der Waals interactions, hydrogen bonding or ionic interactions.

Depending on the solvent, gels can further be classified as organogels, aerogels and hydrogels.

(i) Organogels are semi-solid systems containing a large amount of an organic solvent (Murdan, 2005; Vintiloiu and Leroux, 2008) such as hexane, cyclohexane, octanol, vegetable oils and long-chain synthetic esters. Only a few organogels have been investigated for drug delivery. They include in situ formed gels from L-alanine derivatives (Murdan, 2005), lecithin gels (Avramiotis et al., 2005), microemulsion-based gels (Hadidi et al., 2009), poly(acrylic)acid (Jones et al., 2007, 2008) and sorbitan monostearate gels (Murdan, 2005).

(ii) Aerogels are structures in which solvent (organic or inorganic) is replaced by a gas. Silica aerogels (Wagh and Ingale, 2002) and carbon aerogels are the most studied aerogels. Silica aerogels and polysaccharide-based aerogels have been proposed for drug delivery of various drugs such as gentamicine (Zeng et al., 2007), ibuprofen and paracetamol (Mehling et al., 2009).

(iii) However, in terms of drug delivery, hydrogels are by far the most studied systems. The solvent included in the polymer matrix may be water or another aqueous physiological fluid (for instance 0.9% NaCl in water). Nowadays hydrogels are developed for tissue engineering (Yamaoka et al., 2006) and drug delivery purposes. A basic method to prepare hydrogels is by dissolving hydroxy propyl methyl cellulose (HPMC) in an aqueous medium before incorporating hydrophilic drugs (Labouta and El-Khordagui, 2010). Physically cross-linked gels have been attracting increasing interest because the use of toxic cross-linking agents is avoided; different cross-linking techniques based on crystallization or hydrogen bonds have been investigated. Poly(acrylic acid) and poly(methacrylic acid) form complexes with poly(ethylene glycol) (Baranovskii et al., 2011). Protein interactions and antigen–antibody

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doi:10.1016/j.ijpharm.2012.01.044

interactions may also allow the preparation of physical gels (Hennink and van Nostrum, 2002). Ionic interactions between polycations and polyanions are another way to manufacture physically cross-linked gels: alginate or dextran hydrogels have been widely studied for drug delivery applications (Babak et al., 2008; Hamidi et al., 2008).

Despite their name, low molecular weight heparins (LMWHs) can be considered as high molecular hydrophilic polyanions (Lanke et al., 2009). Most of the currently available LMWH are depolymerised porcine mucosal heparin preparations, prepared by chemical or enzymatic digestion methods (Fareed et al., 1998). Their molecular size generally ranges from 3000 to about 12,000 Da. These polyanions are a mixture of sulphated glycosaminoglycans (Betz et al., 2001). LMWHs are used to prevent blood clotting, so they could be used to treat superficial thrombosis. Indeed, by topical administration, LMWH could increase local thrombolysis and resorption of haematomas. There are some dosage forms already on the market, including Thrombophob® gel in the USA and Lioton® 1000 gel in Switzerland. LMWH have also shown some effects in skin, such as anti-inflammatory activity and pain relief (Song and Kim, 2006).

Nevertheless, transdermal delivery of LMWH is difficult due to their molecular weight, negative charge and hydrophilicity (Lanke et al., 2009). In addition, the low permeability of skin makes the transdermal delivery of most drugs difficult (Mitragotri and Kost, 2001). Several attempts have been to promote transdermal penetration of LMWH. Liposomes have been suggested as a potential vehicle for transdermal delivery of LMWH (Betz et al., 2001; Song and Kim, 2006). Other active methods such as sonophoresis (Benson and Namjoshi, 2008; Förster et al., 2009), iontophoresis (Lanke et al., 2009; Pacini et al., 2006), ultrasounds (Mitragotri and Kost, 2001) and microneedles (Wu et al., 2007) have been able to achieve LMWH penetration into the skin.

The aim of this study is to prepare a hydrogel containing LMWH with proven permeation properties. It was hypothesized that the incorporation of LMWH into a gel (i) may protect the drug from the skin enzymes, (ii) may favor the penetration of the drug based on the cationic charge and a high gradient concentration opening tight junctions as previously demonstrated with chitosan (Hamidi et al., 2008) and (iii) improve bioavailability based on the first two properties.

The gel we proposed to develop is based on ionic interactions between this polyanionic drug and Eudragit® RS 30D, a cationic polymethylmethacrylate copolymer grafted with trimethyl ammonioethyl methacrylate chloride (Dittgen et al., 1997). Eudragit® RS 30D is commonly provided as aqueous pseudolatex generally used in the coating of solid dosage forms (Bodmeier et al., 1996). It is commercially available as an aqueous suspension of polymeric nanoparticles with a mean size about 130 nm.

The rheological behavior of this gel and the ability to release LMWH in vitro and in vivo were investigated in this study.

2. Materials and methods

2.1. Materials

Four commercial low molecular weight heparins (LMWH) were used as model drugs. Sodium bemiparin powder (MW 3600 Da) was kindly donated by ROVI Pharmaceutical Laboratories (Madrid, Spain). Calcium nadroparin [Fraxiparin® (MW 4300 Da)], sodium enoxaparin [Lovenox® (MW 4500 Da)] and sodium tinzaparin [Inohep® (MW 6500 Da)] (Gray et al., 2008) marketed by GlaxoSmithKline (Marly-le-Roi, France), Sanofi-Aventis (Paris, France) and Leo Pharma (Vernouillet, France), respectively were obtained from commercial sources.

Eudragit® RS 30D (copolymer of ethyl acrylate, methyl methacrylate and a low content of a methacrylic acid ester with quaternary ammonium groups (trimethylammonioethyl methacrylate chloride)) was generously given by SCPI (Beauvais, France).

The reagents used for the measurement of the anti-Xa activity were supplied by Diagnostica Stago (Asnières-Sur-Seine, France). Other reagents were of analytical grade.

Male Wistar rats (mean body weight 700 ± 100 g, Janvier, Le Genest-Saint-Isle, France) were housed in air-conditioned quarters under a photoperiod schedule of 12 h light/12 h dark, fasted overnight with water ad libitum. New Zealand rabbits (mean body weight of 2500 ± 250 g, Charles Rivers Laboratories, L'Arbresle, France) housed in separate cages were fasted overnight with water ad libitum. Guidelines and legislative regulations on the use of animals for scientific purposes were followed.

2.2. Gel preparation

Gels were manufactured with various concentrations of LMWH (400, 600, 800, 1000, 2000, 3000, 4000 and 5000 IU/mL): they were prepared by mixing, under magnetic stirring (200 rpm for 3 h) at room temperature, an equal volume of an aqueous solution of LMWH with the Eudragit® RS 30D suspension.

2.3. Gel characterization

2.3.1. Rheological study

The rheological measurements were performed with a Rheo Stress AR 600 rheometer (Thermo Electron Corporation, Saint Herblain, France) equipped with a cone-and-plate geometry (plate diameter 35 mm and cone angle 2°). A solvent trap was used to minimize the water evaporation. All samples were equilibrated for 30 min before rheological measurements. All experiments were carried out with enoxaparin from Sanofi-Aventis unless otherwise specified.

Viscoelasticity properties were determined by applying an oscillating shear stress for a given frequency of 1 Hz. The storage modulus G' and loss modulus G'', as well as the loss angle δ ($\tan\delta = G''/G'$) were measured for each stress and frequency. When $G' > G''$ (which means $\tan\delta < 1$ or $\delta < 45°$, the elastic properties are more pronounced than viscous ones and conversely. So, the values of $\tan\delta$ (or δ) are good and precise indicators of the viscoelastic nature or the gel. The lower the $\tan\delta$ or δ values, the more elastic and cross-linked the gel.

Two kinds of oscillatory tests were successively performed:

(1) A stress sweep was carried out at a frequency of 1 Hz in a stress range of 0–100 Pa to determine the gel formation kinetic. Various gels (corresponding to 1000–5000 IU/mL of LMWH) were studied in order to determine the influence of LMWH concentration on viscoelastic behavior. Measurements were performed at 20 °C and 32 °C in order to study possible changes in stability or consistency as a function of temperature. These stress sweeps also allowed the linear viscoelastic region (i.e. where moduli are independent of the stress) to be identified.
(2) A frequency sweep was performed by varying the angular frequency from 0.01 to 100 Hz, at a constant shear stress of 1 Pa at 20 °C (this value fell within in the linear viscoelastic region).

2.3.2. Incorporation of LMWH into the gel network

The amount of LMWH involved in the cross-linking with Eudragit® RS 30D was determined indirectly by measuring the amount of free LMWH contained in the aqueous phase, which was recovered after centrifugation of the gel. Thus, immediately after

preparation, gels were centrifuged at 42,000 × *g* for 20 min at room temperature. The supernatant was recovered and the concentration of LMWH was determined by a turbidimetric assay. This method is based on the quantitative precipitation at 1:1 stoichiometry occurring between sulphate and carboxyl groups of heparin at pH 6.8 and the amine groups of cetylpyridinium chloride (Javot et al., 2009). All experiments were performed in triplicate. Briefly, 500 μL of supernatant were reacted for 1 h at 37 °C with 500 μL of acetate buffer (1 M, pH 5), followed by the addition of 2 mL of cetylpyridinium chloride (2.9 mM) in NaCl aqueous solution (168.8 mM). Precipitation was assayed by spectrophotometry at 500 nm (Uvikon 922, Kontron, Eching, Germany). The LOQ and LOD for this analytical method are 0.85 and 0.25 IU/mL respectively. The cross-linked drug was expressed as the percentage of LMWH incorporated in gels with respect to the initial LMWH concentration.

2.3.3. Release study

In vitro release kinetics of LMWH were studied with gels that had been dried at room temperature for 48 h. Gel drying was performed to ensure that a constant mass was used for the release study. Briefly, 50 mg of dried gel were poured into 20 mL of phosphate-buffered saline (KH_2PO_4 4.4 mM, Na_2HPO_4 045.1 mM, NaCl 100 mM, pH 7.4 adjusted by H_3PO_4) at 37 °C under magnetic stirring at 200 rpm. Dissolution tests were performed in sink conditions. At selected times (5, 15, 30 min and 1, 2, 4, 8 and 24 h) aliquots of 1.5 mL were withdrawn and replaced by fresh buffer. Phosphate-buffered saline of different ionic strengths (NaCl 0.1, 0.5 and 1.0 M) was also tested. The drug released in vitro was evaluated by the turbidimetric assay described above and the results are presented as the percentage of drug released with respect to the initial LMWH concentration in the gel.

2.3.4. LMWH localization in the skin after topical application: tape stripping

Hairless male Wistar rats ($n = 3$) were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg). One hundred microliters of LMWH gel was applied to the square test area (2 cm × 2 cm) on the ventral skin with an approximate LMWH amount of 65 IU/cm^2. Application time was fixed at 1 h. Before performing the tape stripping, the animals were sacrificed with a lethal dose. Excess formulation was removed by a cotton swab. To determine the LMWH concentration in the stratum corneum (SC), 20 tapes (Scotch® Crystal Clear Tape, 3M, Cergy-Pontoise, France) were stuck onto the test area and carefully peeled away. The tapes were pooled in 10 mL of phosphate buffer at pH 7.4. Then samples were vortexed for 5 min to extract the LMWH from the SC. The resulting solution was centrifuged at 42,000 × *g* for 20 min. LMWH extracted from the SC was assayed by the turbidimetric method described above.

2.3.5. LMWH quantification in plasma after topical application

The gel was applied on the hairless shaved skin of male New Zealand rabbits ($n = 6$). Eight hundred microliters of LMWH gel were applied onto a square test area (6 cm × 4 cm) of the dorsal skin with an approximate amount of 90 IU/cm^2 LMWH under occlusive conditions. Blood samples were collected from the marginal ear vein at selected times (2, 4, 6, 8, 12 and 24 h) into vials containing sodium citrate. Samples were then centrifuged for 10 min at 3000 × *g* to obtain clear plasma which was frozen at −20 °C until further analysis. Guidelines and legislative regulations on the use of animals for scientific purposes were followed.

The concentration of LMWH (bemiparin in this experiment) was determined automatically (STA Compact automate, Diagnostica Stago, France) with a chromogenic assay for factor Xa (Stachrom® heparin, Diagnostica Stago). Plasma samples (25 μL) were mixed with 50 μL of antithrombin III solution. This solution was mixed with 100 μL of factor Xa and incubated for 30 s at 37 °C. Factor Xa substrate (100 μL) was added to the solution and incubated at 37 °C. The absorbance was determined at 405 nm every 2 s for 10–30 s of incubation. A linear relationship between absorbance/min and the concentration of bemiparin in the range of 0.1–0.8 anti-Xa IU/mL was obtained. The LOQ and LOD are 0.06 and 0.02 IU/mL respectively. This method has already been validated in our laboratory by Hoffart et al. (2006).

3. Results and discussion

3.1. Gel properties

The manufactured gels had a brilliant white semisolid appearance. Macroscopically the gels appear as smooth gels although a slight sandy feeling was noticed upon skin spreading. It was also observed macroscopically that the gels were much more fluid for lower concentrations of LMWH. Indeed, the gel consistency depended on the amount of LMWH added to the Eudragit® RS 30D suspension. In case of a formulation prepared with 400 IU, a milky suspension, rather than a gel, was obtained. When the amount of LMWH was increased, the gel consistency increased up to a maximum of about 1000 IU. Above 3000 IU the gel consistency declined. These observations suggest that gel formation could be explained by electrostatic interactions between the positive charges carried by the polymer (Eudragit® RS 30D) and the negative charges of the active ingredient, as already reported by Hoffart et al. (2006) in the case of LMWH nanoparticles. In other words, it appeared that a larger amount of negatively charge carried by LMWH would form stronger gels with the constant amount of charge in the Eudragit® RS 30D.

3.2. Rheological behavior

The linear viscoelastic region was located between 0.1 and 5 Pa at a constant frequency of 1 Hz at 20 °C.

The elastic or storage, G' (reflecting the solid-like component of viscoelastic behavior) and loss or viscous, G'' (reflecting the liquid-like component) modulus were monitored during the gel formation (Tamburic and Craig, 1995). For all concentrations (except 400 IU/mL) G' was always higher than G'', as recorded in Table 1. As indicated previously, the very low value of tan δ (∼0.18) which correspond to a very low value of δ (∼10°) for all concentrations except 400 IU/mL, show a very good elastic behavior of the gel, which means the formation of a cross-linked gel. According to macroscopic observations previously described, the gel was formed at concentrations higher than 400 IU/mL: the elastic and viscous moduli increased with LMWH concentration to finally reach a maximum at 1000 IU/mL. Lucero Muñoz et al. (1998) have prepared hydrogels containing a constant amount of Eudragit® RS 30D and various proportions of HPMC. They have shown an increase in apparent viscosity when the proportion of HPMC was increased. This observation was expected but may be compared to our study since in both cases the amount of Eudragit® RS 30D was fixed.

Table 1
Viscoelastic parameters for gels with different LMWH concentrations. The results are expressed as a mean ($n = 3$).

LMWH concentration (IU/mL)	G' (Pa)	G'' (Pa)	δ (°)	Tan δ (1 Hz)
400	0.09	0.181	62.28	1.922
600	836	148	10.03	0.173
800	2534	556.8	12.39	0.190
1000	4609	860.8	10.58	0.143
3000	1617	262	14.02	0.289
5000	668.7	225.5	18.63	0.443

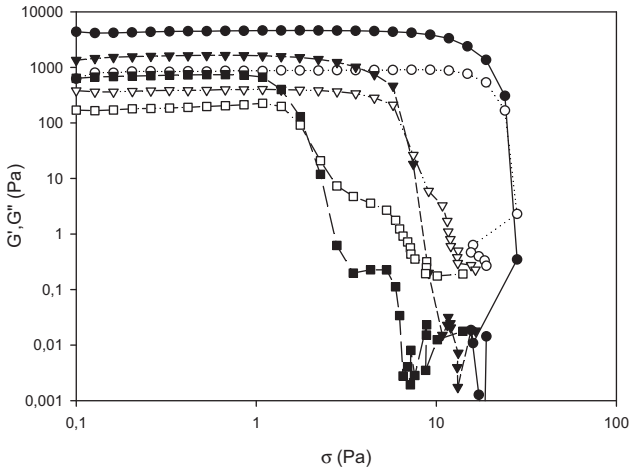

Fig. 1. Evolution with time of a stress sweep representing G' at 2 h (●), 3 h (▼), 5 h ( ) and G'' at 2 h (○), 3 h (▽), 5 h ( ) as a function of stress (Pa) of enoxaparine gels prepared with 1000 IU/mL of LMWH. Results are expressed as mean ± SD (n = 3).

In our case, the increase in the ionic interactions between the two oppositely charged polymers plays the role of the increasing proportion of HPMC. Gels prepared with higher concentrations of LMWH (3000 IU/mL) showed a decrease in these parameters (G', G'', δ). This behavior could be explained by a progressive saturation of the positive charges present in the polymer by the LMWH sulphate groups. When saturation was attained (1000 IU/mL), the strongest gel was obtained as shown in Table 1 ($G' > G''$). The most concentrated gels (600–5000 IU/mL) can be considered as typical three-dimensional networks established by interparticular bounds. Fig. 1 shows the gel formation at a concentration of 1000 IU/mL as a function of time and stress. It can be observed that gel is already structured after 2 h of stirring since $G' > G''$. Nevertheless, an additional hour of stirring (3 h total) leads to the complete formation of the gel since G' and G'' values are at their maximum. Total stirring of 5 h before performing the G' and G'' measurements does not change the gel overall structure. However, the resistance to stress was better for the 3-h mixing time since the breakdown point was around 40 Pa whereas it was only about 20 Pa for 5 h. This observation confirms that a mixing time of 3 h for the two oppositely charged polymers corresponds to the optimal preparation conditions which were adopted for all the gel preparations used in this study. The 40 Pa threshold is very high, enough to insure very good stability at rest. Results of literature show that a yield about 1–2 Pa is often sufficient to stabilize colloidal systems as emulsions against sedimentation or creaming (Benna-Zayani et al., 2008).

When different LMWH concentrations (1000, 3000 and 5000 IU/mL) were compared at 20 °C (Fig. 2a), it appears that the gel prepared with the lowest LMWH concentration presented a more solid-like behavior (G' of 1000 IU/mL is the highest value of the graph). This gel is also the most resistant to strain. The magnitude order of solid–liquid behavior is 1000 > 3000 > 5000 IU/mL. As also shown in Fig. 2a the resistance to rupture is lower for the most concentrated gels (i.e. 20 Pa and 10 Pa for the 3000 and 5000 IU/mL, respectively). At 32 °C (Fig. 2b), gels present the same solid–liquid behavior order but a more solid-like appearance for the three concentrations, which could be explained by the increase in viscosity of Eudragit® RS 30 D with temperature. Indeed, Bonacucina et al. have observed an increase of G' with gels prepared with Carbopol. This module was varied from 79 to 300 Pa at 20 and 60 °C, respectively (Bonacucina et al., 2006). Regardless of concentration, G' and G'' were higher at 32 °C than 20 °C. The gels also showed a more resistant network for all concentrations compared with 20 °C.

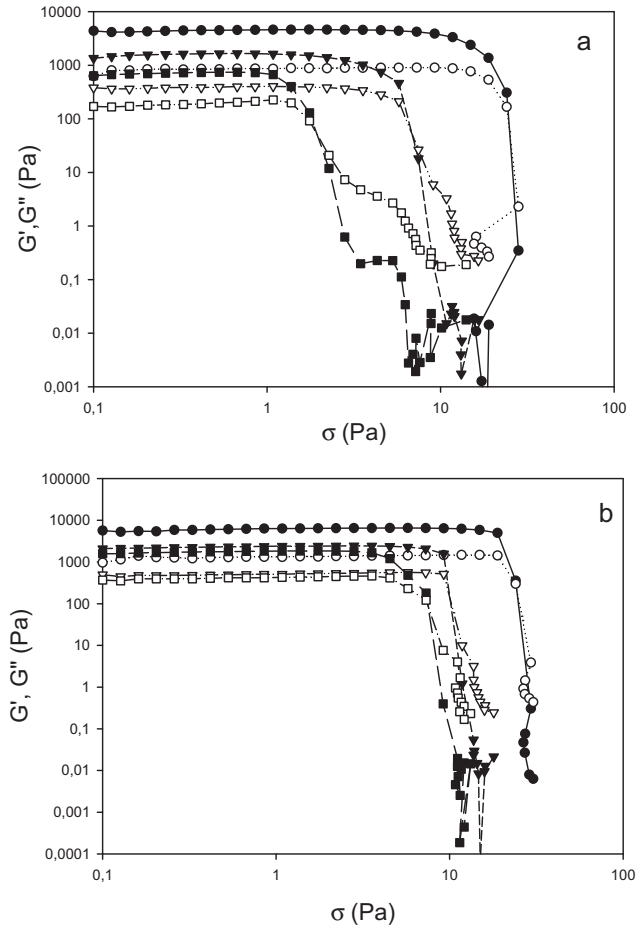

Fig. 2. Comparison of stress sweep performed at 20 °C (a) and 32 °C (b). G' (1000 IU/mL (●), 3000 IU/mL (▼), 5000 IU/mL ( )) and G'' (1000 IU/mL (○), 3000 IU/mL (▽), 5000 IU/mL ( )). Results are expressed as mean ± SD (n = 3).

A frequency sweep test was performed at constant stress of 1 Pa. G' and G'' were measured. As observed in Fig. 3, both the dynamic storage modulus (G') and the viscous modulus (G'') did not depend on the frequency. Similar behavior was observed at 32 °C (data not shown). This confirms the viscoelastic behavior of the gels prepared, irrespective of their concentration (Ma et al., 2008).

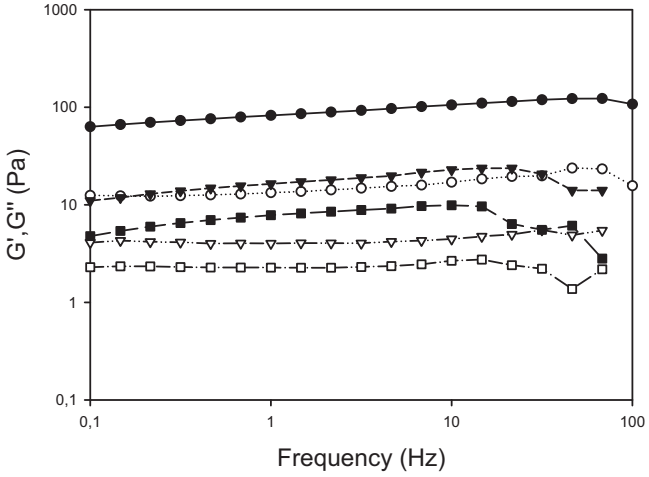

Fig. 3. Frequency sweep performed at 20 °C. G' (1000 IU/mL (●), 3000 IU/mL (▼), 5000 IU/mL ( )) and G'' (1000 IU/mL (○), 3000 IU/mL (▽), 5000 IU/mL ( )). Results are expressed as mean ± SD (n = 3).

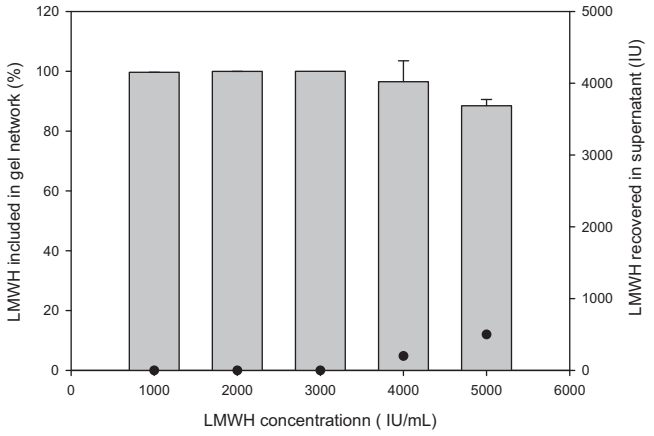

Fig. 4. Amount of cross-linked LMWH with Eudragit® RS 30D gels as a percentage (bars) as well as the amount of LMWH recovered in supernatant in IU (black spots). Results are expressed as mean ± SD ($n = 3$).

3.3. Drug incorporation into the network

As shown in Fig. 4, an increase of LMWH incorporated in the gel network is observed when the amount of LMWH added to the gel during preparation was increased. All the added drug (100%) was incorporated into the gel prepared with LMWH concentrations of 1000–3000 IU/mL.

The percentage of cross-linked drug slightly decreased with higher concentrations, and finally reached 96% and 90% for 4000 and 5000 IU/mL respectively. In other words, all the added LMWH molecules were not involved in the gel network since a small amount (200 and 500 IU for 4000 and 5000 IU/mL respectively) was found in the clear aqueous supernatant after centrifugation. The ionic interactions between the cationic groups in Eudragit® RS 30D and the negatively charged LMWH explain the high amount of LMWH cross-linked to form the gel. It can be considered that the binding sites of the Eudragit® RS are totally occupied by the LMWH molecules up to 3000 IU/mL for a constant amount of Eudragit® RS. A saturation of the binding sites appears at a concentration just below 4000 IU/mL of LMWH since 200 IU of the LMWH were found in the supernatant. However, based on this assumption, adding 5000 IU/mL of LMWH onto the same amount of Eudragit® should lead to approximately 1200 IU of free LMWH, which was not the case. Some steric rearrangement could probably explain this difference. As observed by Song and Kim (2006), a similar behavior was obtained when preparing cationic flexosomes with LMWH. These authors have prepared liposomes with anionic, neutral and cationic lipids and obtained entrapment efficiency close to 92% in their liposomes prepared with cationic lipids. On the other hand, the entrapment efficiency was limited to 30% for neutral and anionic liposomes. They also concluded that an ionic interaction between the negatively charged LMWH and the positively charged liposomes occurred, which may improve the adsorption of the LMWH to the liposomal membrane.

3.4. LMWH kinetic release

If the drug is to cross the skin barrier, it must first be released from the gel network. So, the release kinetics of the four commercial LMWH from the gel containing 5000 IU/mL were evaluated (Fig. 5a). This gel was selected because it contains the largest amount of drug included within the gel. Thereafter, the release of the drug was performed for sodium tinzaparin gels with LMWH concentrations between 1000 and 5000 IU/mL (Fig. 5b). Tinzaparin was selected because the four LMWH showed the same release profile at 5000 IU/mL. Tinzaparin was not released at the lowest

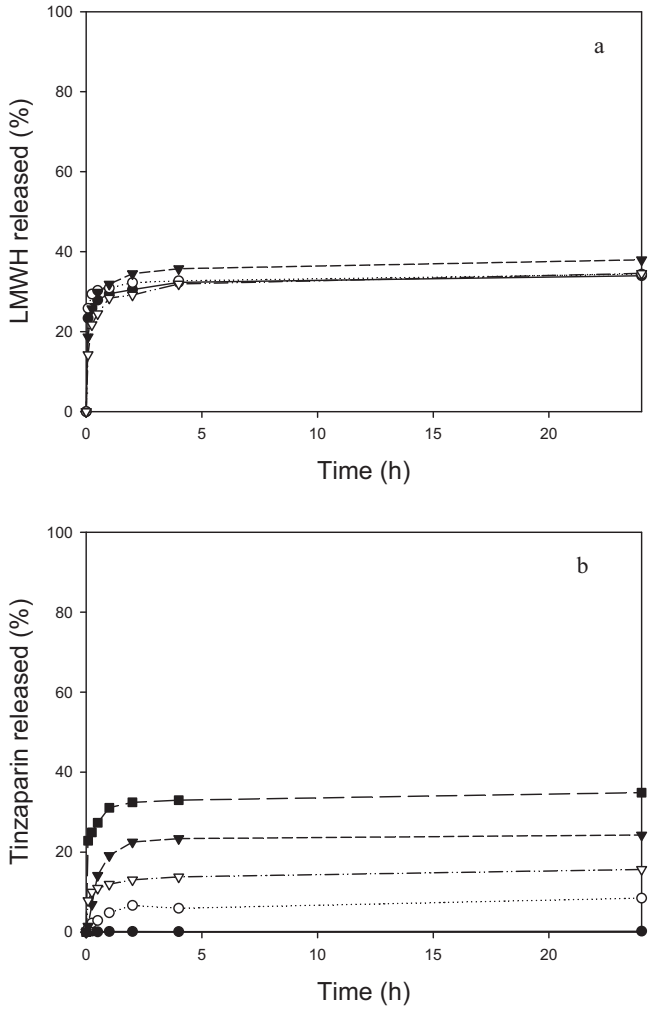

Fig. 5. (a) Release kinetics of four LMWH in gels at 5000 IU/mL. Bemiparin (●), enoxaparin (○), nadroparin (▼) and tinzaparin (▽). (b) Release kinetics of sodium tinzaparin (Innohep®) at different concentrations: 1000 IU/mL (●), 2000 IU/mL (○), 3000 IU/mL (▼), 4000 IU/mL (▽) and 5000 IU/mL ( ). Results are expressed as mean ± SD ($n = 3$).

concentration (1000 IU/mL). When the amount of LMWH in the gels was increased from 1000 to 5000 IU, the percentage of drug released increased from 0 to 32%. More precisely, a drug release of 7.8% (corresponding to 150 IU LMWH) in gels prepared with 2000 IU/mL was observed whereas the drug release was 17.6% (corresponding to 470 IU in the medium) for an initial concentration of 3000 IU/mL. Higher initial concentrations of drug leads to a better drug release: 1100 IU (27.5%) were released from a gel prepared with 4000 IU/mL. The maximum amount of drug released in the dissolution medium was obtained with a gel prepared with 5000 IU/mL: in this case, a maximum release of 32% (corresponding to 1800 IU) was obtained.

From the rheological studies, it was shown that the 1000 IU/mL gels were the strongest ones in terms of viscoelastic behavior. That probably means that the electrostatic interactions were at a maximum, thus preventing LMWH release from the gel network. Nevertheless, gels made with 3000 IU/mL or 5000 IU/mL were also very strong, which finally is a better option in terms of drug release due to a potential concentration gradient. As shown in Fig. 5a, the same double phase profile was obtained for the different commercial LMWH preparations. An initial burst (between 5 and 15%) was observed during the first 30 min of the experiment followed by a plateau, which was reached after about 4 h. Regardless of LMWH

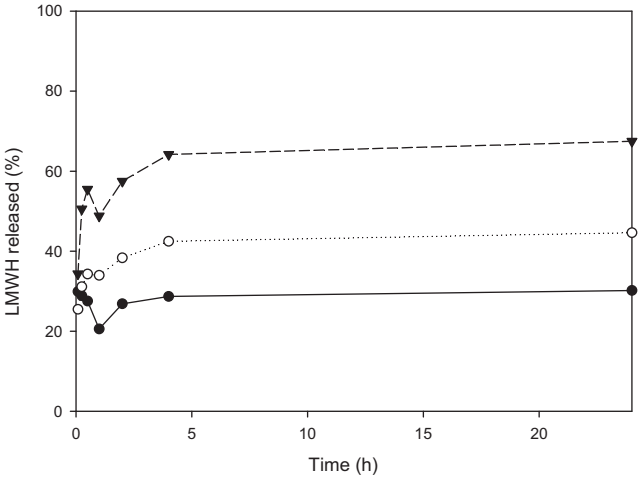

Fig. 6. Release kinetics of LMWH performed in dissolution media of different ionic strength: NaCl 0.1 M (●), NaCl 0.5 M (○) and NaCl 1.0 M (▼). Results are expressed as mean ± SD ($n = 3$).

type, release of drug was incomplete since the plateau was stable between 4 and 24 h. The burst drug release may allow the drug to be rapidly incorporated into the skin. Furthermore, a gradient could be formed between the gel and the skin to promote the complete and progressive release of the drug. This burst could be explained by the release of LMWH weakly or not involved in the electrostatic interactions with the cationic groups of the polymer. On the other hand, the non-total release of drug could be explained by the strong electrostatic interactions between drug and polymer. Similar behavior was observed by Hoffart et al. when preparing Eudragit® nanoparticles loaded with LMWH. They found an important burst and an incomplete drug release explained by the strong electrostatic interaction between the negatively charged LMWH and the positively charged polymer (Hoffart et al., 2006). To confirm this hypothesis, the LMWH release was measured (with a gel prepared with 5000 IU/mL of drug) in dissolution media of different ionic strength (NaCl 0.1, 0.5 and 1.0 M). As observed in Fig. 6, drug release increases with the increase of ionic strength to reach a maximum of 65% in NaCl 1 M. This result confirms the strong influence of ionic interactions between drug and polymer in the formation of the polymer network. Ionic strength has an important influence on drug release. For example, Bodmeier et al. (1996) observed that increasing the NaCl concentration in the buffer decreased the polymer hydratation and consequently reduced the drug release. On the other hand, Holgado et al. (2008) observed an increase of morphine release on increasing the ionic strength of the medium: they found that ionic strength values higher than physiological range also produced a faster release of the drug. A high affinity of hydrophilic drug for the Eudragit® hydrogel could also contribute to a non total drug release.

3.5. LMWH localization in the skin after topical application

The method of tape stripping was used to determine the amount of LMWH deposited in the SC. The SC is the first barrier of skin. This method is an easy and non-invasive technique to study the drug permeation into the skin. The analytical method allows 80% of the applied drug to be recovered. LMWH solution was used as control, and it was found that the absorption of LMWH through the SC was 2-fold higher with the LMWH solution than with the gel as shown in Table 2. Actually, this reflects the low release of LMWH from the gel as it was observed in vitro (Fig. 5b). A similar observation was reported by Song et al. They obtained a higher amount of LMWH (25%) in the skin, when LMWH was applied as a solution rather than a cationic flexosomes (10%). A slight increase of drug transported by the cationic flexosomes was also observed in viable skin (Song and Kim, 2006). In our case, 32% of LMWH was found in the SC. This result is in agreement with the amount of drug released during the in vitro test (also 32%). It can be hypothesized that a gradient of drug is established that allows the LMWH to cross the skin barrier. The cationic charge of the gel could improve the intradermal retention of the drug due to the negative charge in the skin surface at physiological pH (Song and Kim, 2006).

Table 2
LMWH (Tinzaparin) recovered in the SC after topical application.

Formulation used	Administered dose (IU/cm^2)	LMWH recovered in the SC (%)
LMWH solution (5000 IU/mL) ($n = 1$)	65	68
LMWH gel (5000 IU/mL) ($n = 3$)	65	32 ± 5

3.6. LMWH quantification in plasma after topical application

For topical application of LMWH, drug should not pass into the systemic blood circulation to avoid a systemic effect. After 24 h of occlusion application, LMWH was not detected in plasma so no systemic effect would be expected. Similar results have been obtained by Song and Kim (2006). These authors prepared flexible liposomes to deliver LMWH into the skin and observed a small accumulation of drug within the skin without any systemic effect, which they explain by the large size of liposomes which prevents them entering the blood capillaries. A similar effect could be expected for the LMWH gel. Polymer nanoparticles are entrapped in the network by electrostatic bonds. The size of the nanoparticles (around 130 nm) as well as their entrapment in the gel network does not allow the nanoparticles to penetrate in local blood circulation but allows the drug to be accumulated in the skin probably as a result of a concentration gradient. It cannot be excluded that a very low amount of heparin is absorbed since the LOD of our method is 0.02 IU/mL. Nevertheless, if this was the case, such a low absorption would have no therapeutic consequence. Although this result should be confirmed (and also in other animal species), it is a strong argument to continue developing heparin gels for local treatment. Indeed, local heparin would really be very benefit for treating haematomas with no, or very low, systemic absorption.

4. Conclusion

LMWH could be delivered to skin by the topical application of the LMWH gels. The gel containing 5000 IU/mL gel had the best physicochemical properties, allowing the LMWH to cross the skin barrier. Topical delivery of LMWH could be a useful alternative for the treatment of superficial thrombosis and haematomas. Further studies should be performed to study the interaction of drug with skin in more detail.

Acknowledgement

The help of Dr. Gillian Barratt in reviewing the revised manuscript is greatly acknowledged.

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