Production of LMWH-conjugated core/shell hydrogels encapsulating paclitaxel for transdermal delivery: In vitro and in vivo assessment

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Topical delivery
Tape stripping

Abstract
Topical applications that reduce systemic toxic effects while increasing therapeutic efficacy are a promising alternative strategy. The aim of this study was to provide an enhanced transdermal delivery of low molecular weight heparin (LMWH) through the stratum corneum by using cationic carrier as a novel permeation enhancer. Recent studies have shown that heparin-conjugated biomaterials can be effective in inhibiting tumor growth during cancer treatment due to their high ability to bind growth factors. Paclitaxel (PCL) was co-encapsulated into the same cationic carrier for the purpose of improving of therapeutic efficacy for a combined cancer treatment with LMWH.

In vitro and in vivo studies showed that the LMWH and PCL release was significantly affected by polymer molecular weight and block composition. Skin penetration tests have indicated that larger amounts of LMWH were absorbed from LMWH-gel conjugate through SC, than aqueous formula. However, it was found that the plasma transition of LMWH released from gel conjugate was lower compared to the plasma concentration of LMWH released from aqueous solution. It is recommended that PCL-loaded LMWH-conjugated core/shell hydrogels can be used as promising drug release systems for transdermal applications that can improve therapeutic efficacy and reduce side effects in a combined cancer treatment.

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1. Introduction
The biomedical applications in the new generation materials aim to improve the functional characteristics in order to reinforce their interaction with the biological systems [1,2]. Hydrogels have become the focus of the researchers due to their unique characteristics such as high water content, softness, flexibility and biocompatibility. Especially, physically cross-linked gels are rather preferred in clinical practices, because of the hydrophilic to hydrophobic ratio [6,7]. Hydrogels can solubilize proteins, peptides and water-insoluble antitumor-acting agents, as well as they can form complexes with hydrophilic bioactive agents through different interactions [8–10].

LMWH, which is a multifunctional glycosaminoglycan, has frequently been used in clinical practices as a potential anticoagulant [11]. Due to its advantageous biological activities, the heparin’s coupling with biomaterials offers very interesting results [12–14]. Some studies have shown that LMWH may be effective in stopping tumor growth by being able to bind to angiogenic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in tumoral cells [15]. Since LMWH's high negative charge density and molecular size (3–12 Da) cause interaction with the cell membranes, its oral absorption is limited, and it has usually been used as a systemic drug [16]. However, in the case of systemic administration, the problem of very short half-life of heparin, due to its low hydrolytic stability, emerges [17]. For this reason, encapsulation of LMWH with polymeric drug carriers may allow controlled release by increasing the stabilization of heparin against hydrolysis [18]. Recently, polymeric drug carriers for LMWH have been identified in the literature. Cationic polymers have recently been proposed as an alternative to provide slower LMWH release due to the strongly binding of the polymer to heparin [19–21]. Zhao et al. synthesized a series of block copolymers of oligo (ethylene glycol) methyl ether methacrylate (OEGMEMA) and 2-(methylcrotylloxy) ethyl trimethyl ammonium chloride (METEA) in which contain cationic block (METEA) at different ratios. They have prepared poly-ion micelle complexes from these block copolymers in the presence of heparin and...
have reported that they can be used in applications such as the release of growth factors and absorption of heparin-bound drugs into the cell [13].

The release of the drug in a suitable, non-disturbing and sustainable manner for the patient are the key features of any drug release system. The transdermal applications are a treatment approach that can meet these expectations well. However, the most important problem in transdermal drug applications is the low permeability of the skin. Despite the excellent therapeutic potential of LMWH very few articles have been published on the transdermal application of LMWH. The passive transdermal release of LMWH is still difficult due to its relatively large molecular weight, negative charge and hydrophilic nature [22–24]. Other active methods, such as sonophoresis [25], iontophoresis [26], ultrasound [27] and microneedles [28], as well as heparin-carrier polymeric systems such as liposomes [29,30] and nanoparticles [31] have been used to enhance the penetration effect of LMWH into the skin. However, there may be limitations to the use of these methods. Pasteuriza et al. investigated the transdermal release of heparin from the gel matrix produced by electrostatic interaction between LMWH and Eudragit® RS 30D, a polycatonic commercial polymer. They have observed the biphasic release kinetics that is not dependent on the amount of LMWH in the gel formulation. The transdermal transition of LMWH was examined through rat skin and it has been suggested that LMWH may be an alternative for treating topical thrombosis and haematomas, especially in local problems and through the skin [32].

The most recent studies have begun to use LMWH as a component in tumor-targeted releasing systems since it has been shown that LMWH triggers the death of abnormal cells in the body by interacting with transcription factors. The use of anti-proliferative and anti-mitotic drugs in the prevention of the conversion of the actinic keratosis disease, which is also known as the thickening of the skin, to cancer is having been investigated [33]. Paclitaxel (PCL) is known to be an anti-cancer agent with proven efficacy in the treatment of ovarian and breast cancers [34]. Because of the fact that PCL is not soluble in water, its clinical use is limited and it is currently formulated intravenously, and therefore, numerous side effects of PCL have been reported [35]. For this reason, PCL release systems such as liposomes [36], micelles [37] and polymer conjugates [38] are being investigated. The blocking of various biological and immunological processes by PCL has been the driving force in the search for different route of administration [39,40]. For this purpose, the transdermal release of the PCL has begun to be investigated with priority in its utilization for the treatment of various skin diseases, such as psoriasis or cancer chemotherapy [41,42]. The polymeric systems comprising controllable hydrophilic/hydrophobic components has been drawing attention for the transdermal release of PCL, such as liposomes [29,30] and nanoparticles [31] have been investigated [33]. Paclitaxel (PCL) is known to be an anti-cancer drug, which is also known as the thickening of the skin, to cancer is having been investigated [41,42]. The polymeric systems comprising controllable hydrophilic/hydrophobic components has been investigated with priority in its utilization for the treatment of various skin diseases, such as psoriasis or cancer chemotherapy [41,42].

2. Materials and methods

2.1. Materials

The monomers, all other chemicals and solvents were purchased from Sigma-Aldrich. Tetrahydrofuran (THF) were purified by following the conventional drying and distillation procedures prior to use [43]. PCL was purchased from Tocris Bioscience (Bristol, UK). The commercial LMWH, sodium enoxaparin, sodium tinzaparin (MW 4500 Da) and sodium dalteparin were obtained from Fluka. Monomers were passed over a column of basic alumina to remove the hydroquinone methyl ether inhibitor and stirred over calcium hydride, the less volatile 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) inhibitor was added and stored at −20 °C. The monomers were each distilled under reduced pressure before transferring into the reaction vessel by cannula under a dry nitrogen atmosphere.

1-Methoxy-1-trimethylsiloxy-2-methyl-1-propene (MTS) was distilled and stored at −20 °C in a graduated Schlenk flask under dry nitrogen prior to use. Tetra-n-butylammonium ibenzoate (TBABB) was prepared by the procedure of Dicker et al. as reported in the literature [44]. All glassware and transfer needles were dried by storing in an oven overnight at 140 °C, prior to use.

Nine 16-week-old male Wistar rats weighing about 250 g used in this study were provided from Süleyman Demirel University Experimental Animal Research Center (Isparta, Turkey). The rats were housed in polysulphone and sterilizable transparent cages during the experiment, at a temperature of 24 ± 1 °C, under a 12:12 light-dark cycle and in a regularly ventilated place. All animal procedures were conducted after ethical committee permission with 22 August 2013 date and 01 number by Süleyman Demirel University Animal Experiments Local Ethical Committee (SDU-HADYEK) (21438139-156).

The antiXa assay which are a method for determining the concentration of LMWH in blood plasma was performed on plasma samples with antiXa assay kits purchased from Diagnostica Stago (Asnières-Sur-Seine, France) [45,46].

2.2. Methods

2.2.1. Synthesis of the PDPA-b-PDMA-b-PDPA triblock copolymers

The PDPA-b-PDMA-b-PDPA triblock copolymers were prepared by group transfer polymerization. Initially, TBABB catalyst (100 mg) was added from a sidearm under a nitrogen purge into a 250 mL three-necked round bottom flask and then THF (125 mL) and MTS (0.15 mL) were transferred via cannula to the flask. After stirring for 15 min, first monomer (DPA, 3.0 mL) was transferred to the mixture and an exotherm was monitored with a contact thermocouple attached to the side of the reaction vessel. The reaction mixture was stirred and allowed to cool to ambient temperature (approximately 40 min) and then a 5 mL of the reaction mixture was extracted via syringe for gel permeation chromatography (GPC) and proton nuclear magnetic resonance spectroscopy (1H NMR) analysis.

After that, the second monomer (DMA, 10 mL) was added via cannula and a second exotherm was recorded (5.2 °C). The resulting mixture was stirred until it cooled down to room temperature. After extraction of a 5 mL aliquot from the polymerizing PDPA-b-PDMA reaction mixture (as described above) the third monomer (DPA, 2.5 mL) was added via cannula for producing an ABA triblock copolymer (PDPA-b-PDMA-b-PDPA). The reaction mixture was again stirred at room temperature until the exotherm had abated (approximately 50 min). After extraction of a final 0.5 mL aliquot for GPC analysis, the reaction was stopped by addition of 2 mL of methanol prior to recovery using a rotary evaporator (Laborota 4001, Heidolph, Germany). The resulting triblock copolymer was dried on a vacuum line (Nuve EV 018, Turkey) at room temperature for 24 h after removing PDPA and PDPA-b-PDMA contaminations as in the previous work [43]. Table 1 summarizes 1H NMR and GPC data of triblock copolymers in high yield (~98%).

2.2.2. Selective quaternization of the PDMA residues

The PDMA residues of each of these triblock copolymers were selectively quaternized. For this purpose, polymer was dissolved in THF at ambient temperature. A stoichiometric amount of methyl iodide (MeI) (based on the DMA content of the individual block copolymer) was added and the resulting mixture was stirred for 24 h. After purification
by precipitation in n-hexane, the filtrate was then dried in a vacuum oven at 55 °C for 48 h. The extent of quaternization was assessed by \(^1\)H NMR spectroscopy [47].

2.3. Copolymer characterizations

2.3.1. Gel permeation chromatography (THF eluent)

The molecular weights (\(M_n\)) and polydispersity index (\(M_w/M_n\)) of all polymers were determined by GPC calibrated with respect to PMMA standards (ex. Polymer Labs, with \(M_n\) ranging from 680 g mol\(^{-1}\) to 218,600 g mol\(^{-1}\)). The GPC consisted of an Agilent Isu Pump, a refractive index detector, both Mixed ‘D’ and Mixed ‘E’ columns (ex. Polymer Labs). The GPC eluent was high-performance liquid chromatography (HPLC) grade THF stabilized with butylhydroxytoluene (BHT), at a flow rate of 1.0 mL min\(^{-1}\).

2.3.2. Nuclear magnetic resonance spectroscopy

The block copolymer compositions of all triblock copolymers were determined by using a Bruker 400 MHz Avance NMR instrument in deuterium oxide (D\(_2\)O) by adjusting the solution with 1 M potassium hydroxide (KOH) to between 7.0 and 8.0, the aqueous triblock copolymer solutions could form a physical gel spontaneously in a few minutes. To detect gel formation which occurs above the critical minimum concentration (\(\%\)) of 10\%, visual observation of mobility during tube tilting method was utilized. For drug loading, LMWH and PCL were mixed with acetic acid solution of copolymer and drugs were encapsulated simultaneously with the gelation. Drug encapsulation efficiency was determined by measuring the amount of free LMWH in the supernatant after centrifugation (Mikro 220, Hettich, Kirchhellen, Germany). at 24,320 × g for 20 min at room temperature LMWH concentration was determined by using Azure II colorimetric method [29]. To the test, 50 mL of supernatant samples were reacted with Azure II solution (0.01 mg/mL) and were then assayed spectrophotometrically (Schimadzu UV-LC1800) at \(\lambda = 595\) nm using a standard curve. All measurements were performed in triplicate.

The drug encapsulation efficiency and loading capacity was defined as follows:

encapsulation efficiency (%) = \(\frac{\text{total amount of drug} - \text{drug in supernatant}}{\text{total amount of drug}} \times 100\)

loading capacity = \(\frac{\text{total amount of drug} - \text{drug in supernatant}}{\text{total weight of gel}} \times 100\)

2.5. In vitro drug release study

The in vitro release kinetics of LMWH and PCL in hydrogel formulations were assessed using a dialysis tube diffusion technique. Briefly, 50 mg of drug loaded gel were put into a dialysis bag (Molecular weight cut-off 12–14,000 Da) and immered in 50 mL of phosphate buffer (0.01 M pH 7.4) at 37 °C under stirring at 200 rpm. At specified time intervals, 0.5 mL of release medium was collected from each sample and replaced with the same volume of fresh phosphate buffer. The cumulative release amount of LMWH and PCL was determined by using the Azure II colorimetric assay as described above and HPLC using a Shimadzu system (Shimadzu, LC 20A Model), respectively. The chromatographic analysis was performed using ultraviolet-visible (UV–vis) detection (254 nm) and isocratic elution (45:55, v/v) from water and acetonitrile nitrile at a flow rate of 1 mL/min for 16 min at ambient temperature. A 50 μL sample was injected onto the reversed-phase C18 column (Phenomenex Gemini 5 μm C18, 250 × 4.60 mm) for all the analyzed samples. All drug release data were averaged over three measurements.

2.6. Ex-vivo transdermal permeation tests

Skin permeation tests were performed by using excised hairless abdominal skin which was surgically separated from each rat. To this end, the rats were sacrificed and the subcutaneous fat and muscle tissues carefully removed from their abdominal site; the remaining full-thickness skin was washed with phosphate buffer solution (pH 7.4). Before and at the end of the penetration experiment skin sections were checked in terms of integrity.

The full thickness skin sample was mounted between the donor and acceptor chamber of a jacketed Franz-type diffusion cell, providing a diffusion area of 1.77 cm\(^2\) and a receptor compartment volume of 12 mL. After that skins were allowed to equilibrate with the pH 7.4 phosphate buffer for 1 h at 32 °C under stirring. To perform kinetic studies, 0.2 mL of LMWH-conjugated hydrogel formulations and LMWH control (0.2 mL of aqueous solution, containing same amount of LMWH) were applied onto the skin surface with a syringe, respectively. At selected time intervals, 0.5 mL samples were withdraw and replaced with fresh receptor medium at equal temperature. Each sample was diluted to a suitable concentration for UV–vis detection. The above treatment were repeated three times for each formulation.

2.7. In vivo permeation studies: tape stripping

Tape stripping is a method widely used to obtain more information about drug concentration within skin [29,32,42]. A volume of 0.2 mL of the LMWH gel formulations were applied to cover the hairless abdominal test area of the Wistar rats under ketamine/xylazine anesthesia (ketamine 87 mg/kg and xylazine 13 mg/kg). 24 h after treatment, a number of adhesive tape (Scotch® Clear, 3 M, USA) completely covering the treated area were adhered and carefully peeled away. To extract LMWH, the removed tape strips were transferred into 10 mL of phosphate buffer at pH 7.4 and then vortexed for 30 min and then centrifuged.

<table>
<thead>
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<th>Code</th>
<th>Comonomer composition (mol%)</th>
<th>(M_n^a) (g mol(^{-1}))</th>
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<th>DP(^c)</th>
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</table>

\(^a\) As determined by GPC (light scattering detector).
\(^b\) As determined from \(^1\)H NMR spectroscopy.
\(^c\) As calculated from both \(^1\)H NMR spectroscopy.
At the same time, the amount of LMWH penetrated into deeper epidermal layers following 24 h of exposure was determined. To this end, rats were sacrificed and the tape-stripped skin was removed surgically. The obtained skin samples was cut into small pieces and pooled in a tube containing 10 mL of phosphate buffer. The resulting solution was sonicated for 20 min, vortexed again for 30 min and centrifuged at 10,000 g for 30 min. The amount of LMWH extracted from tapes and remaining skin samples was determined by Azure II colorimetric method as mentioned in the above section.

Same study was conducted also to evaluate the transdermal delivery of PCL. To determine the amount of drug released into the phosphate buffer medium through the skin samples, aliquots were taken at predetermined intervals and replaced with an equal volume of phosphate buffer. The aliquots were kept in the refrigerator until analyzed by HPLC. All experiments were repeated 3 times.

2.8. Analysis of LMWH in plasma after topical application

To monitor the concentration of LMWH in the blood plasma anticoagulant factor Xa assay was used (STA Compact automate, Diagnostica Stago, France). Blood samples (0.1 mL) were collected via tail vein over a 48 h period (4, 8, 16, 24 and 48 h) into tubes containing sodium citrate at a ratio of 1:9 of blood and centrifuged. Subsequently, samples were centrifuged to separate the serum and plasma at 18,620 × g for 25 min and the supernatants stored at −80 °C until analyzed (Wise Cryo., Germany).

All the procedures on animals were performed in accordance with ethical principles and legislative regulations on the use of animals for scientific purposes.

3. Results and discussion

3.1. Cationic triblock copolymer syntheses and characterizations

The synthesis of a series of ABA triblock copolymer precursors was successfully accomplished through group transfer polymerization (GTP), initiated by a monofunctional initiator. The number average molecular weight (Mn) and Mw/Mn values of all copolymers were determined by GPC and the results are given in Table 1. As expected from GTP chemistry, all synthesized block copolymers showed controlled molecular weight and narrow molecular weight distribution. The GPC traces of each step in the synthesis of a PDPA-b-PDMA-b-PDPA triblock copolymer (for representative polymer, PDPA13-b-PDMA24-b-PDPA13) are given in Fig. 1B.

The block copolymer compositions were determined by comparing the relative intensities of the individual peaks of each block in the proton NMR spectra recorded in the CDCl3 solvent. To calculate the absolute DP of the first block, the peak integrals of three protons of the MTS at δ 3.5–3.6 initiator at each chain were compared with those of the C-H protons of the isopropyl group of PDPA residues observed at δ 3.0. The DP value of the specified PDPA was used to determine the DP values of the diblock and other co-monomers (PDMA and PDPA) in the triblock copolymer. To this end, the peak integrals of the two —CH proton in isopropylamine of PDPA observed at δ 3.0 and the peak integral of the six dimethyl amino protons of PDMA δ 2.3 are compared. The same comparison was made to determine the DP of the third block of the PDPA-b-PDMA-b-PDPA triblock copolymer. The DP values of the co-monomers in the PDPA-b-PDMA-b-PDPA triblock copolymer determined by this comparison are given in Table 1. A good fit between the experimental and theoretical Mn's, DP's and co-monomer compositions values was obtained.

Cationic block copolymers (PDPA-b-Q-PDMA-b-PDPA) having different quaternization degree were obtained by the selective quaternization of PDMA middle block using an appropriate substoichiometric amount of the Mel. Degrees of quaternization of the PDMA residues were calculated using 1H NMR after vacuum drying of block copolymers. The peak integrals of the quaternized tertiary amine protons of the PDMA residues at δ 3.3 were compared to that of the four methyl groups (δ 1.4) of the diisopropylamino residues for the PDPA-b-Q-PDMA-b-PDPA block copolymers.

Fig. 1. Characterization of representative PDPA13-b-PDMA24-b-PDPA13 triblock polymer (A) Proton NMR spectra in CDCl3; (B) GPC traces of each step in the synthesis of triblock copolymer.
Successful quaternization of representative PDPA$_{13}$-b-PDMA$_{74}$-b-PDPA$_{13}$ was confirmed by $^1$H NMR spectra of the block copolymer which were given in Fig. 2(A). The NMR spectrum (recorded in D$_2$O/ DCl at pH 2) shows the assignment of all peaks corresponding to both blocks of PDPA$_{13}$-b-PDMA$_{74}$-b-PDPA$_{13}$ triblock copolymer. At this pH, the PDPA block is protonated and positively charged and hence molecularly dissolved. By increasing the pH of the solution (pH 11–12), the tertiary amine protons coming from the PDPA block at about 1.2 are not seen in the spectrum. This indicates the dehydration of the PDPA block at pH 12.

After quaternization of the PDMA block with the MeI in the precursor triblock copolymer, the 100% quaternization of PDMA is evidenced by the absence of the quaternary tertiary amine protons (c*) as shown above (Fig. 2(B). On the other hand, the “c” peak of quaternary amine protons was recorded at δ 3.0–3.2 in spectrum. The block composition is determined by comparing the “c” peak integral with the “g” peak integral of PDPA. Accordingly, the block composition of the cationic derivative is the same as the preceding block copolymers. This indicates that the PDMA block is fully quaternized. On the other hand, the degree of quaternization of the partially quaternized derivatives was determined from the integral ratio of the quaternized tertiary amine protons of the PDMA residues at δ 3.3 vs. six methyl protons at δ 2.3.

3.2. Preparation of drug loaded physical hydrogel

The LMWH-Conjugated Core/Shell Hydrogels were prepared by simply mixing certain amount of polymer and drug together in the aqueous solution at approximately pH 3 followed by pH adjustment to around 9.0. Cationic block copolymers are soluble in acidic media because of the protonation of tertiary amine group of both blocks such as precursor derivatives. When the pH value was adjusted to a pH range of 9–10 by adding KOH solution, PDPA blocks were deprotonated and hence become hydrophobic. In the dilute solution, amphiphilic copolymer self-assembled into flower micelle which consisting of cationic outer layer and hydrophobic PDPA core. At higher copolymer concentrations, pH induced physical gelation occurred via packing of micelles connected through bridges. Gelling is simply indicated by the tube inversion method, which is used to identify gels that maintain a constant shape by reversing the “free-standing gel” tube [6]. Essentially, gelation only results in bridging between neighboring grains in the aqueous solution if the central PDMA-block is sufficiently long at the given copolymer concentrations. In some derivatives, all quaternization grades of free-standing gel were obtained. Table 2 gives the gelling information of 100% quaternized derivatives.

In the triblock copolymer series, (PDPA$_{29}$-b-PDMA$_{42}$-b-PDPA$_{29}$; PDPA$_{13}$-b-PDMA$_{74}$-b-PDPA$_{13}$ and PDPA$_{18}$-b-PDMA$_{62}$-b-PDPA$_{20}$) having...
approximately the same molecular weight, by increasing the outer PDMA block length from 42 to 74, a transition from flowing sol state to non-flowing gel was observed. The impact of the molecular weight also led to a similar result. Table 2 also showed that at a constant block composition, the triblock copolymer chains with higher molecular weight are long enough for bridging, which act as physical crosslinks. The 340 coded triblock copolymer did not exhibit any gelling behavior even at higher concentrations of the triblock copolymer. In some derivatives, all quaternization grades of free-standing gel were obtained. Table 2 gives the gelling information of 100% quaternized derivatives.

3.3. In vitro release studies

Drug loading was performed using ABA triblock copolymers which are capable of forming hydrogels. LMWH conjugation was achieved by interaction of its sulfate and carboxylate groups with quaternary ammonium cationic groups of PDMA chains. PCL, a poorly water-soluble drug, was entrapped into PDPA core via hydrophobic interaction. Drugs were loaded to the gel matrix during the gelation.

The release of PCL were conducted as a function of molecular weight and block composition. As can be seen from Fig. 3, the diffusion rate of hydrophobic drug molecules out of the hydrophilic gel matrix can be controlled by changing ratio of hydrophobic to hydrophilic blocks. The drug release rate from 341 and 346 coded triblock copolymers was determined to be the slowest. Both copolymers have the higher molecular weight and longer hydrophobic chains. This is explained by the fact that as the length of the hydrophobic block PDPA increased the rate of drug release reached minimal due to the strengthened hydrophobic interaction with the PCL agent. In addition this, the micelle radius which was measured by dynamic light scattering (DLS) given in Table 2 was supported that gels with a slower release profile have larger hydrophobic cores. At the same time, release profiles was found to depend greatly on the molecular weights of the block copolymers. Fig. 3 shows the release profiles for three gels with different molecular weights. Increase of molecular weight led to a more sustained and prolonged release period due to the increased gel consistency.

Furthermore, to investigate the pH-dependency of the drug release, the experiments carried out in different buffered solutions with pH 7.4.

Table 2

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<th>ABA block copolymer composition&lt;sup&gt;a, b&lt;/sup&gt;</th>
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<th>micelle diameter&lt;sup&gt;d&lt;/sup&gt; (nm)</th>
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</table>

<sup>a, b</sup> As determined by 1H NMR spectroscopy. <sup>c</sup> As determined by DLS on 1.0% aqueous solutions.

Fig. 3. (A) In vitro cumulative percent drug release of PCL from gel formulations (20 wt%) (B) influence of the various molecular weight of the triblock copolymers with 39:61 hydrophilic/hydrophobic length blocks ratio on the release (C) influence of the molecular weight of the triblock copolymers with 26:74 hydrophilic/hydrophobic length blocks ratio on the release (D) Influence of the hydrophobic PDPA block length of triblock copolymers on the release. All release studies were conducted in pH 7.4 buffer solutions at 37 °C at 20% gel concentration. The results were calculated as mean ± SD. (n = 3).
5.5 and 3.0. As we have reported in previous studies, the PCL was fully released within 3 h at pH 3. This fact may be due to protonation of the tertiary amine groups of PDPA block and completely dissociation of micellar gel which resulting in rapidly diffusion of drug molecules into the dissolution media. This finding was consistent with our previous report [43].

The cumulative release of LMWH from cationic hydrogels are shown in Fig. 4. The results showed that heparin was released more quickly in the first 4 h period, followed by a prolonged slower release. The rapid initial release could be attributed to the excess amount of LMWH which interacts weakly with the cationic carrier matrix. The effect of the quaternization degree on the negatively charged LMWH release was also investigated. As the quaternization degree increased, a decrease was occurred in the release rate of LMWH. This result could be related to excess amount of positive charge provide further interaction with drug in the polyelectrolyte complex. Similarly, longer cationic block lengths resulted in efficient trapping of LMWH and hence sustainable slow release. Molecular weight of block copolymer did not significantly affect the rate of release of LMWH.

One of the most important parameters that influences the conformation of polyelectrolyte gels is the salt concentration of surrounding environment. Depending on the salt concentration polymer chains are able to undergo a conformational transition between contracted and expanded states. LMWH release kinetics were performed in solutions containing different concentrations of sodium chloride and compared with results when distilled water is used as release medium. The results are presented in graphical form in Fig. 4(E). High level of salts can prevent the binding affinity between cationic polymer chains and oppositely charged LMWH due to the screening of attractive forces. As a result, the amount of drug released in a certain period of time increased. The amount of drug released in deionized water was found to be the lowest.

The effect of LMWH concentration used in the gel formulations on release rate was investigated. For this purpose, the gels were loaded in the range of 2.5–10 mg/mL LMWH. Drug release curves showed that increasing amount of LMWH from 1.25 mg/mL to 2.5 mg/mL the released amount of LMWH increased. However, the rate of LMWH release was slower when the concentration of LMWH is higher than 2.5 mg/mL. The slower release is attributed to the formation of a more rigid gel structure due to the increased electrostatic interaction between the oppositely charged gel matrix and LMWH.

3.4. Ex vivo permeation studies

Fig. 5 shows the permeation profiles of LMWH across excited rat skin versus time from the LMWH formulations. Compared to LMWH

![Graphs and images showing cumulative LMWH release profiles and influence of quaternization degree, length of cationic block, molecular weight, and salt concentration on LMWH release.](image-url)
aqueous solutions, topical gel formulation demonstrated 2.5 times higher penetration ability. This situation is concerning with amphiphilic structure of polymeric carrier which act as a permeation enhancer. Namely, it provides a pathways across the lipophilic stratum corneum (SC) barrier via its hydrophobic block and interact with keratin of the stratum corneum via hydrophilic interaction. In addition, it is explained by high-affinity interaction between positive charges on the polymer and stratum corneum having a net negative charge [29].

Furthermore, penetration experiments were conducted for the PCL agent as well (Fig. 6). Compared with in vitro experiments performed using synthetic membrane, it has been found that the PCL penetration is faster when rat skin was used as a membrane. As the hydrophobic block length increases, the penetration of the drug increased unlike the dialysis membrane. Since the hydrophobic PCL is solubilized in the hydrophobic core of micellar gel, the rate of penetration varies with the hydrophobicity of the drug carrier matrix.

Regarding the results, it is thought that amphiphilic compounds can be effectively absorbed stratum corneum consisting alternating lipophilic and hydrophilic layers. During transdermal penetration, particularly spherical micelles minimizes the lipophilic-hydrophilic interface and thus they may more easily overcome physiological protection response of skin [48]. These amphiphilic structures inserting between well-ordered bilayers of stratum corneum can rearrange themselves and thus can provide new pathways that enhanced drug transport through skin by causing local nanometer-scale defects in lipid molecular packing [49].

3.5. LMWH quantification in plasma after topical application

After topical administration of LMWH, it is undesirable for LMWH to pass directly into the systemic bloodstream. Blood samples was collected from rats at times 4, 8, 16, 24 and 48 h to monitor the passage of LMWH into plasma from the its gel formulation. The plasma anti-Xa activity of LMWH delivered from gel and aqueous solution were [anti-Xa] max = 2.0 IU/mL and [anti-Xa] max = 0.1 IU/mL, respectively. It has been determined that LMWH is locally limited, and only small amount of drug passes through blood vessels, therefore, no systemic side effect of LMWH may be expected. Similar findings have been also reported many researchers. The authors proposed these gels as good candidate for the topical application of LMWH. The therapeutic plasma concentration of LMWH was reported to be 0.6–1.0 IU/mL. Accordingly, the plasma concentration of LMWH after cationic gel administration by the transdermal route is considerably lower than its therapeutic activity.

Fig. 7 shows the anti-Xa activities of LMWH released from gel and aqueous solution for 48 h after transdermal application. While the plasma concentration of the LMWH released from the cationic gel was fairly constant, the free LMWH plasma concentration first increased

![Fig. 5. Rat skin permeation profiles of LMWH-conjugated hydrogel and LMWH aqueous solution.](image)

![Fig. 6. Influence of length of hydrophobic block on the permeation rate of PCL through the rat skin in LMWH-conjugated hydrogels.](image)
and then decreased. It is clear that LMWH application with cationic gel has a longer and slower release. The low absorption rate of LMWH loaded in micellar cationic gel and lack of systemic circulation indicate that triblock cationic gel may be a suitable candidate for transdermal release of LMWH.

3.6. Localization of LMWH in the skin after local administration

In researches conducted on transdermal release of drugs, it is important to determine the drug amounts within the stratum corneum. “Tape stripping”, which is often used for this purpose, is a method to determine the amount and localization of drug retained on the skin without disturbing the skin integrity. 48 h after LMWH-loaded gel application, the amount of LMWH retained on stratum corneum, which cannot penetrate subcutaneously, was determined by the method of sticking a tape on the region treated with the gel. Gel-applied region was covered completely with tapes and then the tapes were removed and LMWH was extracted by putting the tapes in phosphate buffer solution (pH 7.4). When LMWH amounts were analyzed by Azure II method, the amount of LMWH retained in “stratum corneum” was found to be higher than that of LMWH loaded gel for aqueous solution (Fig. 8).

This was attributed to the fact that it was more difficult for hydrophilic LMWH to pass through the “hydrophobic” stratum corneum barrier. The results of ex vivo skin permeation experiments realized for LMWH aqueous solution is consistent with these findings. On the other hand, the LMWH antiXa activity in blood after 48 h of LMWH administration is quite high in LMWH solution application. LMWH passing the skin barrier appears to have migrated to the blood vessels without accumulating in intradermal tissue [29].

Skin treated with “tape stripping” after 48 h of gel application was surgically removed and the amount of LMWH accumulated in the intradermal tissue was also determined. As seen in Fig. 8, the amount of LMWH released from the LMWH-loaded gel was found to be higher in the intradermal tissues than the amount in LMWH aqueous solution. As a result, it can be stated that LMWH-loaded gel application has a local effect. It is believed that micellar gel having a relatively larger particle size to enter intradermal blood vessels and being absorbed more easily in the skin due to its amphiphilic character will enable LMWH to accumulate in subcutaneous tissues and thus to maintain a saturated concentration level for a long time. Accumulation of LMWH in subcutaneous tissues from LMWH-loaded gel occurs as LMWH is separated from the gel in intradermal tissues due to its low concentration in the

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Fig. 7. Anti-Xa activity versus time plots for (▲) LMWH conjugated gel (●)LMWH aqueous solution.

Fig. 8. Percentage of LMWH per unit area distributed to different parts of the rat skin after 48 h of application of topical formulations in vivo. The results were calculated as mean ± SD. (n = 6).
gel. It has been concluded that LMWH applied via cationic micellar gel accumulates in intradermal tissues and has not entered the systemic circulation.

3.7. Drug release kinetics modeling

In controlled drug delivery systems, the release of the drug from the polymeric matrix after the diffusion of the solvent into the polymeric matrix takes place according to different mechanisms. To describe the mechanism of drug release for the formulations was determined by finding the R^2 value for each kinetic model: first-order, Higuchi and Korsmeyer–Peppas models, corresponding to the release data in each dissolution medium and the results were given in Table 3. The results revealed that the release of PCL and LMWH from gel formulations is most fitted to the Higuchi-diffusion model, according to the correlation coefficient values. Korsmeyer–Peppas model was further used to verify the mechanism of drug release from prepared formulations and the R^2 values were found to be >0.91. Korsmeyer–Peppas model release exponent (n) pointed to non-Fickian type of release mechanism (anomalous transport). In addition, the values n determined of lower than 0.89 represent to the that the drug release is controlled through coupled diffusion and erosion mechanisms.

4. Conclusion

In this research, a series of cationic triblock copolymers were synthesized by using group transfer polymerization method; LMWH and hydrophobic an anticancer drug was incorporated into their core/shell (hydrophobic core-cationic hydrophilic shell) structured hydrogels. This study indicated that gels can be optimized to achieve desired release profile by altering, mainly, molecular weight of copolymers and ratio of hydrophilic block length to hydrophobic block length. The resulting drug-loaded core/shell hydrogels exhibited slower release which can be sustained over a wide period of time for both LMWH and PCL. The ex-vivo studies showed that the permeation of LMWH through rat skin was increased using cationic micellar gels. Furthermore, it was observed that the permeation of the hydrophobic block length results in a lower ex vivo release of PCL through rat skin. After 48 h of in vivo application period, the amount (per unit area of rat skin) of LMWH delivered from gel formulations into deeper layers of skin was 2.5 times more than that from its aqueous solution. At the same time, in vivo permeation studies confirmed that compared to LMWH aqueous solution, plasma concentration of LMWH delivered from gel formulation was lower would not cause systemic toxicity. In vitro release kinetics from all the formulations were best explained by Higuchi and Korsmeyer–Peppas equations via diffusion and erosion mechanism. In conclusion, enhanced transdermal delivery of LMWH via novel cationic core/shell hydrogels would provide a useful way of treating superficial thrombosis and hematuria. Moreover the co-encapsulation of any anti-tumor drug into the same cationic carrier with LMWH could be an alternative for the purpose of improving of therapeutic efficacy in a combined cancer treatment.

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References


Table 3

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<th>Formulation code</th>
<th>Regression (R^2)</th>
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Qt is the amount of drug released at time t, Q_0 is the amount of drug in the solution, and K_1 is the first order release constant. M/M_∞ is the fractional drug release, K_0 is the release rate constant and n is the release exponent. n < 0.5, the release mechanism is Fickian diffusion, which normally represents Higuchi drug release kinetics; 0.5 < n < 1, the release is anomalous or non-Fickian diffusion, n > 1, then the release mechanism is super case II transport.


