Development and preclinical pharmacokinetics of a novel subcutaneous thermoresponsive system for prolonged delivery of heparin

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Abstract

Heparin is still widely used for treatment and prevention of thromboembolic diseases. Due to specific physicochemical properties, it requires frequent parenteral injections. In this study we present the development and *in vitro* evaluation of an advanced delivery system for prolonged subcutaneous release of heparin. The delivery system consisted of an *in situ* forming thermoresponsive poloxamer-based platform combined with pH-responsive polyelectrolyte heparin/chitosan nanocomplexes. Thermoresponsive hydrogels were tested for gelation temperature, gel dissolution and *in vitro* heparin release, whereas polyelectrolyte nanocomplexes were physico-chemically characterized, as well as tested for *in vitro* cytotoxicity and *in vitro* heparin release. Hydrogel combined of two poloxamers demonstrated the highest gelation temperature (28.6 °C), while the addition of hydroxypropyl methylcellulose prolonged gel dissolution. On the other hand, nanocomplexes' dispersions, prepared at 1:1 heparin/chitosan mass ratio and in the concentration range 0.375 – 1.875 mg/mL, demonstrated mean diameter <400 nm and zeta potential >34 mV. Pharmacokinetics of selected formulations (thermoresponsive hydrogel, nanocomplexes and a dual system consisting of nanocomplexes incorporated into thermoresponsive hydrogel) were studied in rats. Heparin plasma concentration-time profiles revealed a double-peak phenomenon, probably due to heparin diffusion inside the polymer matrix and gel dissolution. Pharmacokinetic parameters were determined by a non-linear mixed effects modeling approach. It was demonstrated that thermoresponsive hydrogel with heparin/chitosan nanocomplexes enabled the lowest absorption rate of heparin into systemic circulation and provided heparin concentration above the prophylaxis threshold for 5 days. *In situ* gelling thermoresponsive matrix combined with chitosan nanocomplexes present a promising delivery system for heparin, requiring less frequent administration during long-term treatment.

Keywords: thermoresponsive hydrogels; polyelectrolyte complexes; heparin; subcutaneous administration; pharmacokinetic model

1. Introduction

Although being the oldest anticoagulant drug, heparin is still widely used for treatment and prevention of thromboembolic diseases, *e.g.* deep vein thrombosis and pulmonary embolism. Its potent anticoagulant activity is expressed through formation of a protease inhibitor complex with antithrombin. Due to its specific physicochemical properties, parenteral route remains to be the most convenient way of heparin administration. Moreover, heparin is essentially administered in clinics by frequent injection as it has short half-life and low bioavailability (Hirsh et al., 2001; Krishnaswamy et al., 2010). While scientists have been intensively working on development of non-invasive heparin formulations, there is an urgent need for less frequent heparin injections, followed by longer duration of therapeutically effective drug plasma concentration and thus improved patients' quality of life.

The goal of this study was the design and *in vitro* and *in vivo* evaluation of a subcutaneous heparin delivery system which would allow patient-friendly drug administration. As demonstrated in our previous papers (Radivojša et al., 2013; Radivojsa Matanovic et al., 2015), the combination of temperature-responsive poloxamer-based hydrogels and pH-responsive nanocomplexes of heparin and chitosan seems to be an excellent platform for prolonged subcutaneous delivery of heparin. Researches in the field of thermally induced gelling systems have been of continuous interest, probably due to their wide spectra of applications, easy preparation, good acceptability and biocompatibilty (Bajpai et al., 2008; Liu et al., 2007; Matanović et al., 2014). Various materials exhibit low viscosity water solutions at ambient temperatures and form hydrogels upon an increase in temperature, however poloxamers have been widely used because of their low toxicity, easy gel preparation methods, good compatibility with active pharmaceutical ingredients and pharmaceutical excipients (Barichello et al., 1999; Dumortier et al., 2006; Escobar-Chavez et al., 2006; Ur-Rehman et al., 2010). These poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymers (PEO-PPO-PEO) show sol-gel transition probably due to packing of micelles and micelle entanglements as a response to the temperature increase (Cabana et al., 1997).

In this study, poloxamer-based thermoresponsive hydrogel formulations were developed and thoroughly evaluated *in vitro* by gelation temperature, rheometrical tests, rate of gel dissolution and drug release. We also developed and physico-chemically examined polyelectrolyte complexes between negatively charged heparin and positively charged chitosan (Hep/Ch NC). These complexes present the associations formed between oppositely

charged polyions due to self-assembly electrostatic interactions (Boddohi et al., 2009). The formation of Hep/Ch NC was evaluated with special regard to dispersion pH and polymers concentration since these parameters were addressed as crucial for generation of optically homogenous and stable nanodispersions (Mao S et al., 2006; Sun et al., 2008). Hep/Ch NCs were also visualized and tested for *in vitro* heparin release as well as for *in vitro* cytotoxicity. Finally, the feasibility of the dually responsive mixed delivery system for heparin was studied by subcutaneous administration in rats. Obtained heparin plasma concentration profiles were analyzed using a non-linear mixed effects modeling approach in order to determine pharmacokinetic parameters.

Thus, the objective of the present study was the design and thorough evaluation of a novel and combined dually responsive subcutaneous delivery system for heparin. To our knowledge, this is the first report on preclinical pharmacokinetics of advanced heparin delivery system combined of thermoresponsive matrix and polyelectrolyte nanocomplexes.

2. Materials and Methods

2.1. Materials

Heparin Braun® 5000 IU/ml sodium salt (B. Braun Melsungen AG, Germany) and chitosan hydrochloride (Kraeber&Co GmbH, Ellerbek, Germany) with a MW 30–400 kDa and a degree of deacetylation of 87.2% were used for preparation of Hep/Ch NC. Thermoresponsive hydrogels were prepared from Lutrol® F127 (Poloxamer 407, P407) and Lutrol® F68 (Poloxamer 188, P188) (BASF, Ludwigshafen, Germany), as well as hydroxypropylmethylcellulose (Methocel K4M Premium, HPMC) from Colorcon, (Dartford Kent, UK). For quantitative determination of heparin, Azure A chloride (Standard Fluka, for microscopy) was purchased from Sigma-Aldrich (Steinheim, Germany). Other reagents were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of Heparin/Chitosan nanocomplexes (Hep/Ch NC)

Hep/Ch NC dispersions with mass ratio Hep:Ch 1:1 and heparin concentration of 0.375 (NC1); 1.125 (NC2) and 1.875 mg/mL (NC3) were prepared by polyelectrolyte complexation method. Stock solutions of chitosan hydrochloride, in the concentration of 0.6 mg/mL; 1.8 mg/mL and 3.0 mg/mL were prepared by dissolving a certain amount of chitosan hydrochloride in distilled water. Stock solutions of heparin in the concentration of 1.0 mg/mL; 3.0 mg/mL and 5.0 mg/mL were prepared by dilution of Heparin Braun® in distilled water. In order to prepare Hep/Ch NC dispersions appropriate volume of Ch solution was transferred

into a glass and placed on magnetic stirrer (800 rpm) at room temperature. The appropriate volume of Hep solution was added in droplets into Ch solution and the dispersion was mixed for one hour. Two more nanodispersions with intermediate heparin concentrations were prepared and characterized as well. Obtained dispersions were kept in a refrigerator until use.

2.3. Physicochemical characterization of heparin/chitosan nanocomplexes

The NC1, NC2 and NC3 dispersions were characterized by pH value, mean particle diameter, polydispersity index (PDI) and ζ-potential (ZP). The average particle size and PDI were determined by photon correlation spectroscopy with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) at 25 °C and a scattering angle of 173°. ZP measurements were performed using a Zetasizer Nano ZS by electrophoretic laser Doppler anemometry at 25 °C, applying the auto mode. For data analysis, the viscosity (0.88 mPa s) and the refractive index (1.33) of distilled water at 25 °C were used.

2.4. SEM micrographs

The morphology of the nanocomplexes was examined using SEM (Supra 32 VP, Zeiss, Oberkochen, Germany). A SEM was used with an acceleration voltage of 0.8 kV and a secondary detector. A drop of NC1 dispersion was deposited on conductive double-sided adhesive tape (diameter 12 mm, Oxon, Oxford Instruments, UK) and left to air-dry.

2.5. *In vitro* cytotoxicity

2.5.1. Cell culture and treatment

Human keratinocyte cells (cell line NCTC2544, ICLC, University of Genoa, Italy) were grown in MEM complemented with 1% (v/v) non-essential amino acids, 1% penicillin/streptomycin mixture, 1% 2 mM l-glutamine, and 10% (v/v) fetal bovine serum (Gibco, Invitrogen, USA). They were cultured as adherent monolayers at 37 °C in a humidified 5% CO_2 atmosphere, and regularly subcultured with trypsin/EDTA when they reached 80 to 90% confluence. Cell culture reagents were from Sigma, Germany unless otherwise indicated. The morphology of cells was examined by inverted light microscope (Olympus CKX41, Japan).

2.5.2. *In vitro* cytotoxicity studies

Cytotoxicity studies were performed by determination of the effect of heparin/chitosan nanocomplexes on cell proliferation using the MTS assay (Cell titer 96 Aqueous One Solution

Cell Proliferation Assay; Promega, Madison, WI) according to manufacturer's procedure. The assay is based on conversion of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, an inner salt, into the soluble coloured formazan product by mitochondrial dehydrogenase enzymes in metabolically active cells. The cells were seeded at a density of 0.5×10^4 cells per well in 50 μL culture medium into a 96-well plate and left overnight to adhere. Afterwards the cells were treated with 50 μL of the test formulations, Hep solution (c = 1.125 mg/mL), Ch solution (c = 1.125 mg/mL), NC1 and NC2.

The cell proliferation was assessed 24 h and 48 h after the addition of the formulations and 3 h before the end of the treatment period, 10 μL of MTS solution were added to each well. The absorbance of formazan was measured at 492 nm using a Safire2 microplate reader (Tecan, Switzerland). The results were expressed as the absorbance ratio of treated to control cells, and cell proliferation was calculated as cell proliferation = $(A_S - A_{S0})/(A_C - A_{C0})$ where A_S is the absorbance of the treated cells (sample), A_C the absorbance of untreated cells (control), A_{S0} the absorbance of test formulation in cell-free medium, and A_{C0} the absorbance of the medium alone.

2.6. Thermoresponsive hydrogels preparations

Appropriate amounts of P407, P188 and HPMC (Table 1) were added to cold distilled water, heparin solution or Hep/Ch nanodispersions while keeping constant magnetic stirrer agitation (500 rpm) and low temperature (5 – 10 °C) in order to prepare thermoresponsive hydrogels. Obtained solutions were kept for 24 h in a refrigerator until use.

Insert Table 1

2.7. Rheological studies

Rheological tests were used to investigate internal structural changes of thermoresponsive hydrogels under gradual heating. The measurements were performed with a Physica MCR 301 rheometer (Anton Paar, Graz, Austria), using a cone-plate measuring system CP50-2. Oscillatory measurements were performed to define the storage (elastic; G') and loss (viscous; G") moduli, which are calculated as $G' = (\tau/\gamma) \times \cos \delta$ and $G'' = (\tau/\gamma) \times \sin \delta$, where τ is the shear stress, γ is the deformation, and δ is the phase shift angle.

For the determination of gelation temperature (T_g) the changes in storage (elastic) and loss (viscous) moduli values were monitored as a function of temperature (10 – 40 °C, heating rate 0.015 °C/s), with a constant shear deformation (strain) of 0.2 % and frequency of 1 Hz. The sol – gel transition temperature was defined as the temperature point at which storage modulus was at its maximum after the abrupt leap due to the temperature increase.

2.8. Hydrogel dissolution

The interaction between administered poloxamer hydrogel and extracellular fluid will result in gel dissolution. Hydrogel dissolution profiles and *in vitro* release profiles of heparin from nanodispersions NC1 – NC3 and hydrogel formulations F1/NC1 – F3/NC3 were obtained simultaneously, applying the method from our previous study (Radivojša et al., 2013). Briefly, 1 mL of each cold solution was transferred into graduated test tube (1 cm diameter) and placed in a 37 °C water bath until a semi-solid gel was formed. After gelation, 2 mL of the release medium pre-equilibrated at 37 °C was layered over the surface of the gel. To simulate physiological conditions after subcutaneous administration phosphate buffer saline (PBS pH 7.4) was used as a release medium. At regular time intervals, the samples were gently manually stirred, the volume of undissolved gel was evaluated visually, and 0.5 mL of the release medium was replaced by an equal volume of the fresh medium. The differences in volume of the hydrogel between points yielded the amount of the hydrogel dissolved during that time period.

2.9. *In vitro* heparin release from NC1 – NC3 and F1/NC1 – F3/NC3

In vitro heparin release study was conducted using a membraneless model, according to the procedure described in the hydrogel dissolution test. At certain time intervals, 0.5 mL of the release medium was withdrawn from a sample and replaced by 0.5 mL of the fresh medium. The amount of heparin in the withdrawn samples was determined by Azure A colorimetric method (Karewicz A et al., 2010). All release experiments were performed as triplicates.

2.10. *In vivo* study

All the procedures for rats care and management performed in this research complied with those required by Italian laws (D.L.vo 116/92) and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC). The protocol of the

animal studies was approved by the Ethical Committee of University of Trieste (num. 1332VOI12) and adhered to the Principles of Laboratory Animal Care.

2.10.1. Animals

Nineteen Sprague-Dawley rats weighing 400 – 450 g were used for animal experiments. The rats were fasted with free access to water, 12 h before the administration of heparin samples.

2.10.2. *In vivo* study protocol

2.5 mL of heparin (90 IU/mL) formulation were administered subcutaneously in the rat neck area. The animals were divided into four groups to which heparin solution (S, group I); thermoresponsive hydrogel in heparin solution (F1/S, group II); thermoresponsive hydrogel in Hep/Ch nanodispersion (F1/NC2, group III); and Hep/Ch nanodispersion (NC2, group IV) were injected. Blood (450 μL) was obtained serially using the "cannulated" tail artery method at each time and directly mixed with 50 μL of sodium citrate (0.11 M). This method allows several consecutive blood withdrawals in the same animal, with a very low level of stress in the animal, permitting to reduce intra animal variability, and hence, it results in a reduction of the number of animals needed for the study (G. Cadelli, 2007; Passerini et al., 2012). The blood samples were immediately centrifuged at 2000g at 4 °C for 15 minutes. Plasma was separated, frozen at - 80 °C, and stored at this temperature until analysis. Blood samples were collected at baseline (30 minutes before drug administration) and at predetermined time intervals after administration (1, 2, 4, 6, and 8h in group I; and 1, 2, 4, 8, 24, 48, 72, 96, and 120h in groups II - IV)

2.10.3. Determination of heparin concentration in blood plasma

Concentration of heparin in plasma samples was determined by factor Xa (FXa) chromogenic assay Berichrom® Heparin Kit (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) according to manufacturer's instructions using Sysmex® CS-2100i analyzer (Siemens AG, Erlangen, Germany). The reagent is not influenced by platelet factor IV complexing of heparin. Detection limit of the assay is 0.05 IU/mL and precision 4.0-7.0% (within day) and 6.9-7.1% (between days) at nominal concentration of 0.5 IU/mL.

2.10.4. Pharmacokinetic analysis

Pharmacokinetic data were analyzed using a non-linear mixed effects modeling approach as implemented in NONMEM (version 7.3; Icon plc, Dublin, Ireland). Model building steps were managed by PsN (version 4.2.0, http://psn.sourceforge.net) and Xpose (version 4.4.1, http://xpose.sourceforge.net). Fortran subroutines were compiled with Intel Visual Fortran compiler (version 11.0, Intel, Santa Clara, CA). Following initial exploration of heparin plasma concentration-time profiles we observed a secondary peak with formulations F1/S, F1/NC2 and NC2, while a single peak was observed with heparin solution (formulation S). Double-peak phenomenon was described by a model of absorption from two Depot compartments (Fig. 1). The disposition model used was a one-compartment model with first-order elimination and zero-order endogenous heparin production. The model was implemented in NONMEM using ADVAN6 subroutine. The estimated parameters were volume of distribution (Vd), clearance (CL), absorption rate constants from Depot 1 and Depot 2 (K_{a1} and K_{a2}, respectively), fraction of the dose absorbed from Depot 1 and Depot 2 (f_1 and $1\text{-}f_1$, respectively) and absorption lag-time from Depot 2 (t_{lag2}). It should be noted that absolute bioavailability (f) cannot be estimated with data following subcutaneous dosing alone. Consequently, Vd and CL should be interpreted as apparent volume distribution and apparent clearance following subcutaneous dosing of heparin solution (formulation S). Nevertheless, relative bioavailabilities of formulations F1/S, F1/NC2 and NC2 compared to formulation S were estimated (f_r-F1/S, f_r-F1/NC and f_r-NC2, respectively). Additionally, we estimated formulation effects on K_{a1}, f_1, K_{a2} and t_{lag2}.

Random effects incorporating inter-animal variability were included as exponential terms assuming log normal parameter distributions. Additive, proportional and combined (additive + proportional) error models were evaluated for residual (intra-animal) variability in heparin concentration. Parameters were estimated using the first-order conditional estimation method with interaction between the two levels of random effects. The modeling strategy aimed at a final model with minimal structural and variability parameters needed to adequately describe the data. The model adequacy was evaluated by standard diagnostic plots of predicted vs. observed concentration and weighted residuals vs. observed concentration or time. Additional criteria were convergence of minimization, the number of significant digits more than 3, gradients in the final iteration in the range between 10^{-3} and 10^{2} and acceptable parameter shrinkage. Alternative models were compared by the likelihood ratio test. The criterion for selection of a model was a change in minimum value of the objective function of at least 3.84 per one additional parameter, corresponding to $p<0.05$. Model selection was also based on stability and plausible parameter estimates. Stability was determined as convergence to the

same solution with dispersed initial parameter estimates. Precision of parameter estimates of the final model was evaluated by bootstrap.

Insert Figure 1

3. Results and Discussion

3.1. Preparation and characterization of Hep/Ch NC

Hep/Ch NC were prepared by simple self-assembly complexation method. They were evaluated for mean diameter, PDI, ZP, and pH (Table 2). All dispersions were formed at 1:1 Hep/Ch mass ratio since our previous studies revealed that 1:1 is the most appropriate Hep/Ch mass ratio for the preparation of optically homogenous and stable nanodispersions (Radivojša et al., 2013). According to the results from Table 2, characteristics of polyelectrolyte complexes correlated very well with polymer concentration. Namely, the Hep/Ch NC dispersion with the lowest heparin concentration had the smallest nanocomplexes and PDI, while pH was the highest. Since Hep/Ch mass ratio was the same, the difference in ZP was only minor. Stable nanocomplexes (ZP > 30 mV) with mean diameter < 400 nm were formed in all 5 nanodispersions due to appropriate pH value (between 3.1 and 6.5) (Sun et al., 2008). The increase in Hep/Ch NC mean diameter with an increase in polymer concentration can be explained by a larger number of polymer molecules which interact, and thus form larger number of PECs, leading to increased aggregation tendency. The dispersion pH continually decreased from the lowest to the highest polymer concentration probably due to excess presence of chitosan molecules in the dispersion.

The Hep/Ch NC1 were visualized by SEM (Fig. 2). The nanocomplexes were of undefined shape and smooth morphology, evenly distributed but forming some aggregates.

Insert Table 2

Insert Figure 2

3.2. Cytotoxicity studies

Biocompatibility of NC dispersions was tested by determination of cell proliferation within *in vitro* cytotoxicity studies. Fig. 3 summarizes the cell proliferation as a fraction of untreated control (cells in a medium) following 24 h and 48 h exposure to Hep and Ch solutions as well as to NC1 and NC2.

As evidenced in Fig. 3, all tested formulations demonstrated no cytotoxicity during the whole study time. However, after 24 h exposure Hep solution showed the highest (107.1%) and NC2 the lowest cell proliferation (77%). After 48 h exposure the highest cell proliferation was observed for NC1 (75.9%) and the lowest for Hep solution (60%). Actually, between 24 h and 48 h exposure Hep solution demonstrated a noticeable decline in cell proliferation compared to control. We assumed that this was due to the potential interactions between negatively charged heparin and positively charged proteins from cell medium which impeded cell growth and proliferation as exposure was longer. On the other hand, the other three formulations displayed similar cell proliferation after 24 h exposure which slightly decreased after 48 h exposure. The cell proliferation of around 80% after 24 h treatment for all three formulations containing chitosan is attributed to its precipitation which probably partly hindered the CO_2 inflow to the cells and consequently impaired their functions. Namely, immediately after the addition of the formulation containing chitosan to the cell medium with pH 7.4, chitosan deprotonated and hence precipitated. This phenomenon is clearly seen at direct microscopic observations of 24 h and 48 h treatment (Fig. 4b and 4c) where chitosan precipitation can be observed as black dots.

Insert Figure 3

Insert Figure 4

3.3. Determination of gelation temperature (T_g)

The main characteristic of each thermoresposive polymer solution is its lower critical solution temperature (LCST) at which sol-gel transition occurs. In this study oscillatory rheometry was performed in order to determine gelation temperatures of the tested thermoresponsive formulations, *e.g.* hydrogel formulations obtained with water (F1-F3) and with Hep solution (F1/S-F3/S). The measurement was conducted by monitoring the storage (G') and loss (G") moduli as a function of time. At lower temperatures the loss modulus dominated over the storage modulus. With an increase in temperature a sharp rise in both

moduli occurred, G' outstripped G", and at specific point both moduli reached plateaus. Since storage modulus reflects the solid-like component of a system, we assumed that the sol-gel transition process started when storage modulus exhibited drastic increase, and finally was completed when G' reached plateau. Thus, the gelation temperature corresponded to the temperature point at which G' reached its maximum and afterwards remained constant despite the increase in the temperature. Our results revealed that the same hydrogel compositions demonstrated practically equal T_g regardless of whether they were obtained in water or Hep solution. The addition of P188 and HPMC to P407 formulations however shifted T_g, so that F2 and F2/S had higher T_g (28.6 °C) than F1 and F1/S (25.1 °C) and F3 and F3/S (25.1 °C). The effect of P188 can be explained by lower proportion of less hydrophilic PPO units in P188 than in P407, whereas the addition of HPMC increased the viscosity of a solution which facilitated the entanglements between poloxamer micelles, causing gelation at lower temperatures (Jeong et al., 2012).

3.4. Thermoresponsive hydrogel dissolution

Poloxamer-based hydrogels undergo dissolution in an aqueous environment due to water penetration into gel network, leading to unpacking of the micelles, polymer hydration and finally gel dissolution. Dissolution profiles of thermoresponsive hydrogels obtained with NC1, NC2 and NC3 Hep/Ch NC dispersions are presented in Fig. 5a. It can be concluded that F2 formulations showed the fastest gel dissolution (3 days), followed by F1 formulations (4 days), while F3 formulations demonstrated much longer gel dissolution (9, 14, and 15 days for F3/NC1, F3/NC2, and F3/NC3, respectively). We assumed that swelling of HPMC chains decreased the rate of medium diffusion into the gel matrix, resulting in lowered rate of dissolution. However, the time required for complete gel dissolution varied among F3 formulations, so that F3/NC1 dissolved first, followed by F3/NC2 and the last was F3/NC3.

3.5. *In vitro* heparin release

A membrane-free model, which enables direct contact between the gel surface and the release medium, was applied for *in vitro* drug release studies, as described in our previous work (Radivojša et al., 2013). In this study, *in vitro* heparin release was tested for NC1-NC3 dispersions and F1/NC1-F3/NC1, F1/NC2-F3/NC2, and F1/NC3-F3/NC3 thermoresponsive hydrogels. The results are shown in Fig. 5b and 5c.

The *in vitro* release study for all tested formulations was conducted over 20 days. As presented in Fig. 5b, final heparin recoveries were 69%, 62%, and 57% for NC1, NC2, and

NC3, however, the release rate differed among nanodispersions. After one hour, NC1 released ~85% of the final heparin recovery, NC2 31.5%, and NC3 only ~16%. After two days of the study, NC1 released ~85% of the final heparin recovery, NC2 79.5%, and NC3 ~58%. It can be concluded that the release was strongly influenced by polyelectrolyte concentration, so that NC1 demonstrated immediate release pattern, whereas heparin release from NC2 and NC3 was prolonged for 2 and 3 days, respectively. This can be explained by pH-responsive behaviour of Hep/Ch NC. Namely, the initial pH values of NC1, NC2, and NC3 were 5.18, 4.18, and 3.94, respectively (Table 2). According to the difference in the initial pH, after the addition of the release medium (pH 7.4), the increase in pH of the three systems was also different and thus, various proportion of chitosan molecules deprotonated and precipitated. With the addition of the fresh medium after sampling, pH was gradually increasing, resulting in dissociation of nanocomplexes and heparin release due to chitosan deionisation. In the cases of NC2 and NC3, larger amount of heparin within nanocomplexes was removed from the medium after each sampling which probably caused the lower final heparin recovery.

On the other hand, the results presented in Fig. 5c revealed that all thermoresponsive formulations (F1/NC-F3/NC3) prolonged heparin release. We assumed that these combined systems released heparin in a combined mode, driven by hydrogel dissolution, nanocomplexes-dissociation, and drug diffusion. As F3 formulations demonstrated the slowest gel dissolution (Fig. 6a), the heparin release profiles from these hydrogels had the slowest rate. Moreover, F3/NC2 and F3/NC3 showed lower final heparin recovery than F1 and F2 formulations obtained with NC2 and NC3.

Insert Figure 5

3.6. *In vivo* study

Time course of heparin concentration following subcutaneous administration of various heparin formulations is presented in Fig. 6. With all heparin formulations, except heparin solution, a low secondary peak was observed. The secondary peak is presumably associated with dissolution of the hydrogel after 24 to 48 hours, while the absorption rate was controlled with heparin diffusion inside the polymer matrix, which is hindered with incorporation of chitosan nanocomplexes. With administration of NC2 as solution we presume that the secondary peak is associated with entrapment of heparin as chitosan is precipitated due to increase of pH at the site of administration. Pharmacokinetic parameters of the compartmental

model are summarized in Table 3. Goodness of fit plots (Fig. 7) and individual profiles (Supplementary data) demonstrate that the model adequately describes the data. Compared to solution, bioavailability of heparin was improved 2 to 3 times with all three formulations (parameter f_r). The model parameters were used for simulation of the *in vivo* absorption profiles of heparin. Typical absorption profiles are presented in Fig. 8. Comparison of the estimated absorption rate from Depot 1 (K_{a1}) between F1/S and F1/NC2 is in accordance with the expected decrease of diffusion rate with formulation of heparin as nanocomplexes, as well as with nanocomplexes dissociation and release of heparin when pH was increased. The absorption from Depot 2 was postponed for 40 h and 44 h for formulations with nanocomplexes (t_{lag2}-F1/NC2 and t_{lag2}-NC2, respectively), while it was approximately 24 h for F1/S (t_{lag2}-F1/S). Absorption half-lives of heparin from Depot 1 were 1.3 h, 2.5 h, 8.2 h, and 3.0 h for S, F1/S, F1/NC2, and NC2, respectively. Absorption rate from Depot 2 (K_{a2}) was much lower resulting in a flat secondary peak. With F1/S and F1/NC2 the majority of heparin (>70%) was absorbed very slowly from Depot 2 (absorption half-life was 60 and 63 h, respectively) which maintained plasma heparin concentration above the prophylaxis threshold value of 0.1 IU/mL for five days.

Insert Table 3

Insert Figure 6

Insert Figure 7

Insert Figure 8

4. Conclusion

In this paper, *in vitro* and *in vivo* evaluation of an advanced subcutaneous delivery system for heparin has been presented. The delivery system was based on thermoresponsive poloxamer matrix with pH-responsive polyelectrolyte nanocomplexes. Heparin release from these systems can be controlled through several mechanisms, *e.g.* dissolution rate of the polymer matrix, heparin diffusion inside the polymer (size of nanocomplexes, medium penetration, density of polymer mesh) and dissociation rate of nanocomplexes. With preclinical testing in rats we were able to demonstrate that the developed systems can provide prophylactic levels of heparin for several days. Based on the results of *in vivo* study it can be

concluded that dually responsive systems seem as an attractive approach for subcutaneous delivery of heparin, requiring less frequent administration during long-term treatment.

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Figure captions

Fig. 1. A pharmacokinetic model of the absorption of heparin after subcutaneous administration of tested formulations.

Fig. 2. SEM micrograph of Hep/Ch nanocomplexes' dispersion (c_{hep} = 0.375 mg/mL, NC1).

Fig. 3. Cell proliferation after 24 h and 48 h exposure to Hep and Ch solutions, NC1 and NC2 Hep/Ch NC dispersions. The results are presented relatively to the proliferation of untreated control cells. Data are expressed as mean ± S.D. (n = 6).

Fig. 4. Inverted light microscope observations of tested formulations following 24 h or 48 h treatment: a) Untreated control cells, b) Hep/Ch NC1, c) Hep/Ch NC2. Scale bar is 100 μm.

Fig. 5. a) Hydrogel dissolution; b) heparin release from nanocomplexes' dispersions NC1-NC3; and c) heparin release from thermoresponsive hydrogels F1/NC1-F3/NC3 during 20 days.

Fig. 6. Pharmacokinetic profiles of heparin in rats following subcutaneous administration of heparin solution (S), heparin-chitosan nanocomplexes (NC2), thermoresponsive hydrogel in heparin solution (F1/S) and thermoresponsive hydrogel in dispersion of heparin/chitosan nanocomplexes (F1/NC2). Symbols are measured concentrations in individual animals (n=5) and thick lines represent fits of the typical (population) prediction of the compartmental model. Thick dotted and dashed lines are the estimated base line heparin concentration and the prophylactic threshold value of 0.1 IU/mL, respectively.

Fig. 7. Goodness of fit plots for the compartmental pharmacokinetic model of heparin with measured heparin concentrations in individual animal (symbols), line of identity (thick solid line) and LOESS local smoother (thick dashed line).

Fig. 8. Typical absorption profiles of heparin following administration of heparin solution (S), heparin-chitosan nanocomplexes (NC2), thermoresponsive hydrogel in heparin solution (F1/S) and thermoresponsive hydrogel in dispersion of heparin/chitosan nanocomplexes (F1/NC2). Note that fraction absorbed is relative to the cumulative amount of heparin absorbed.

Fig. 1

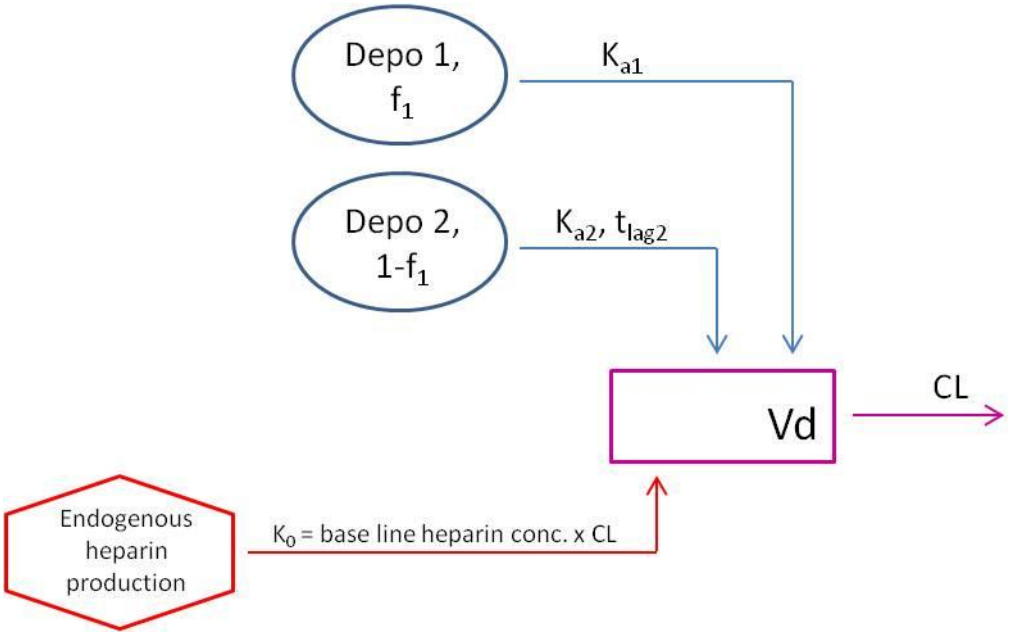

Depo 1,
f_1
K_{a1}
Depo 2,
1-f_1
K_{a2}, t_{lag2}
Vd
CL
Endogenous
heparin
production
K_0 = base line heparin conc. x CL

Fig. 2

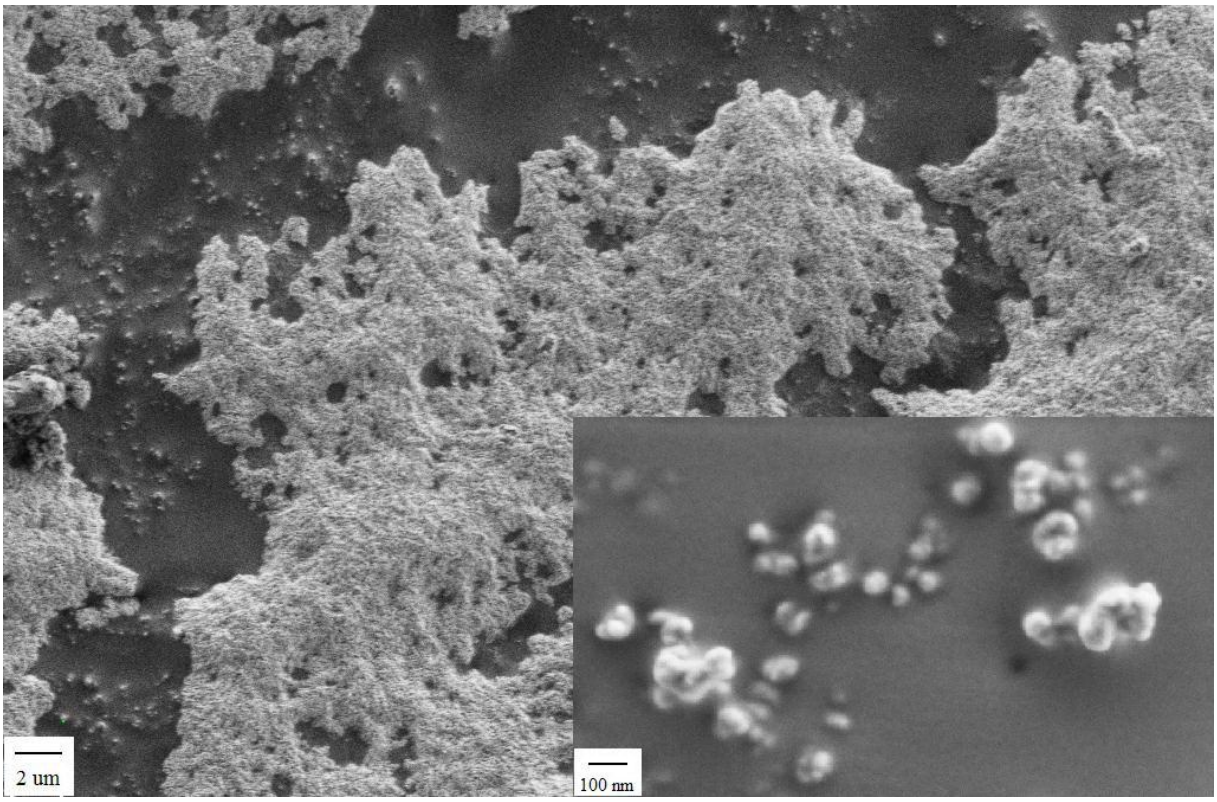


Fig. 3

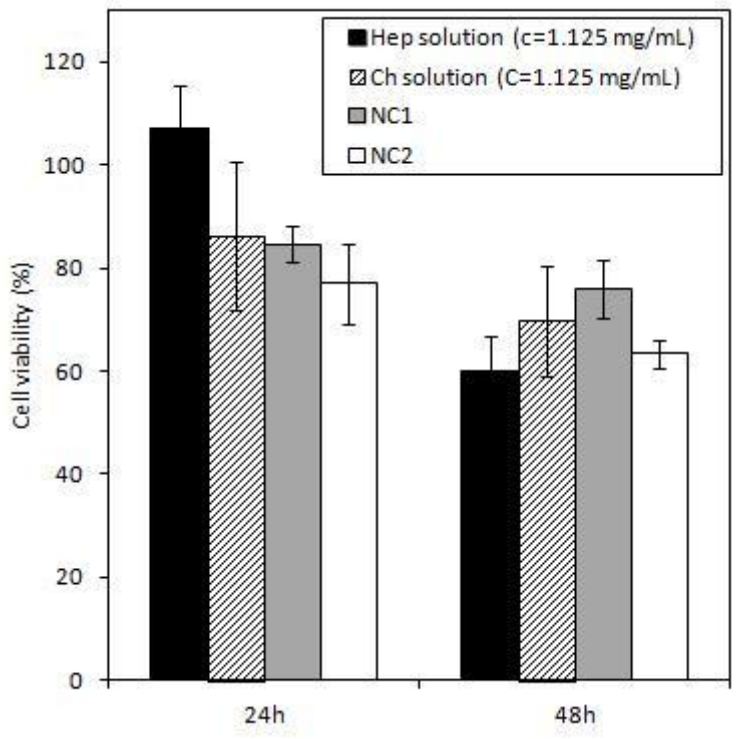

Hep solution (c=1.125 mg/mL)
Ch solution (C=1.125 mg/mL)
NC1
NC2
Cell viability (%)
120
100
80
60
40
20
0
24h
48h

Fig. 4

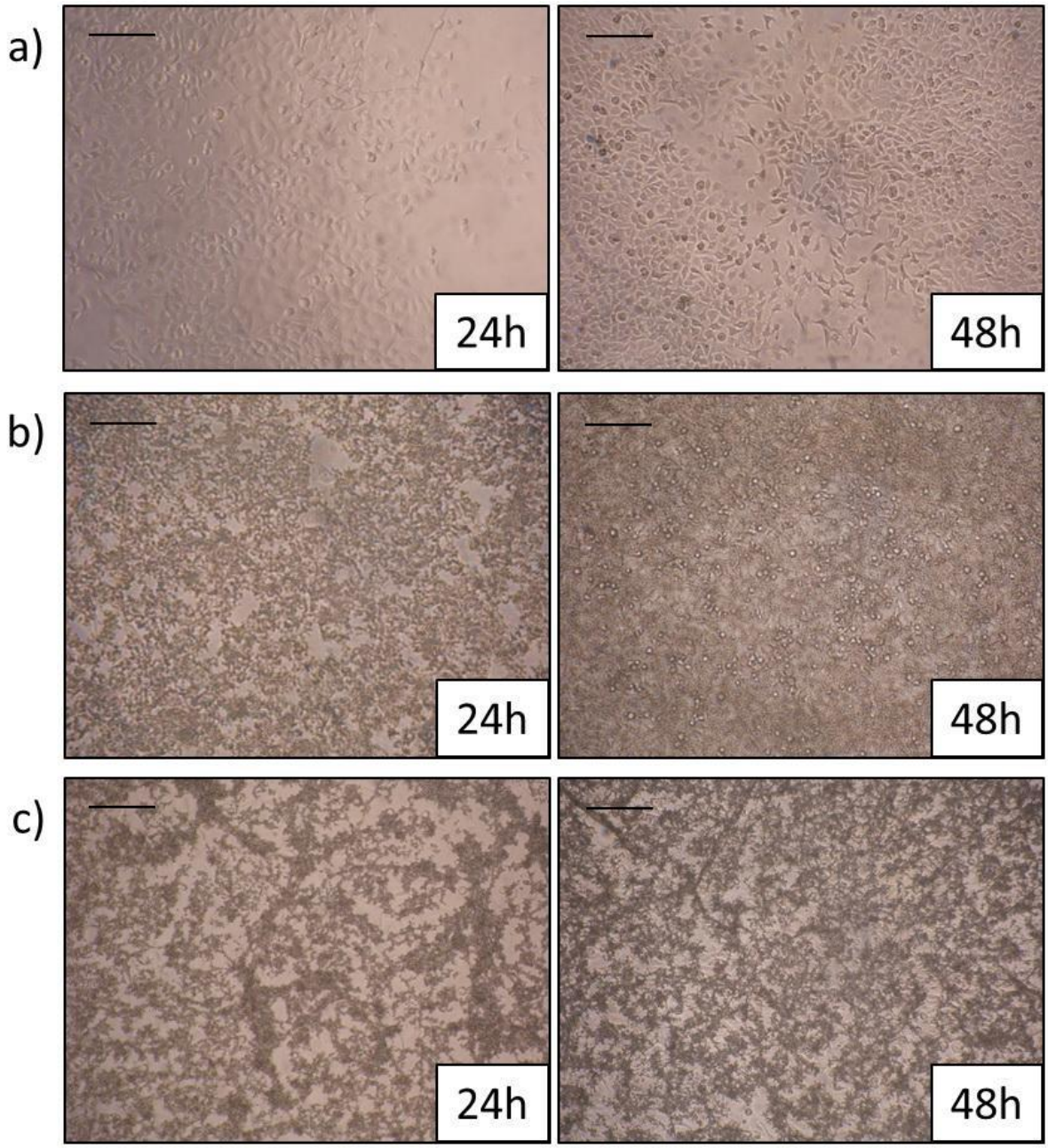
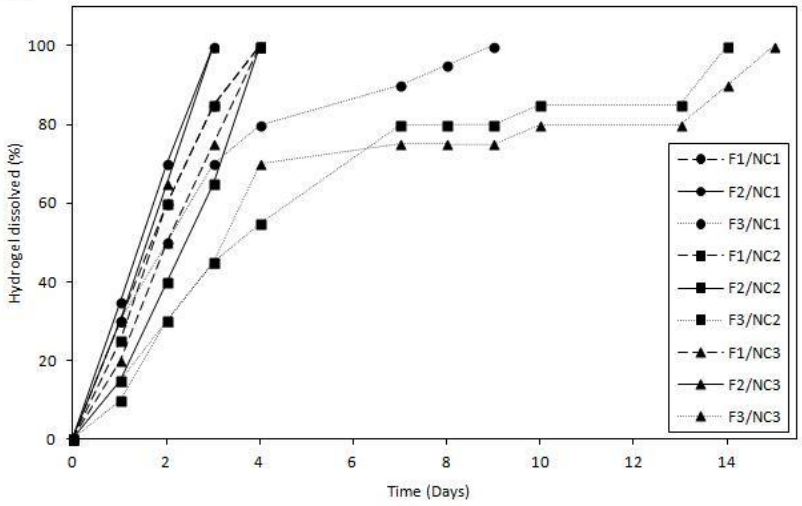
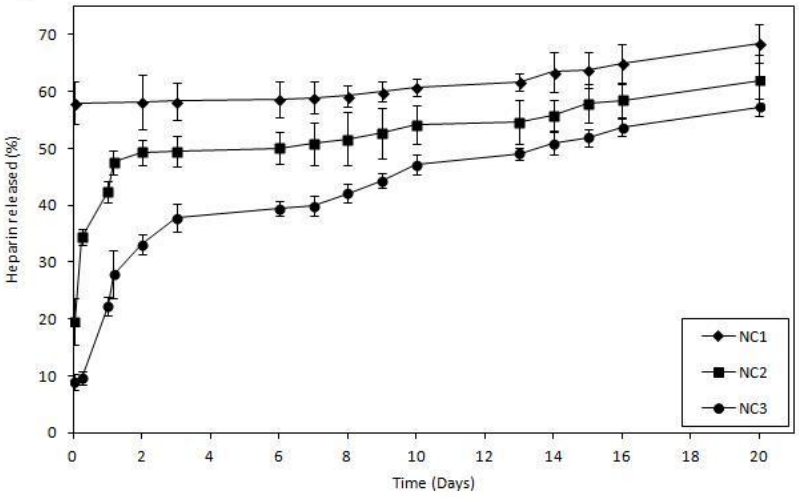


Fig. 5

a)


Hydrogel dissolved (%)
Time (Days)
F1/NC1
F2/NC1
F3/NC1
F1/NC2
F2/NC2
F3/NC2
F1/NC3
F2/NC3
F3/NC3

b)


Heparin released (%)
Time (Days)
NC1
NC2
NC3

c)

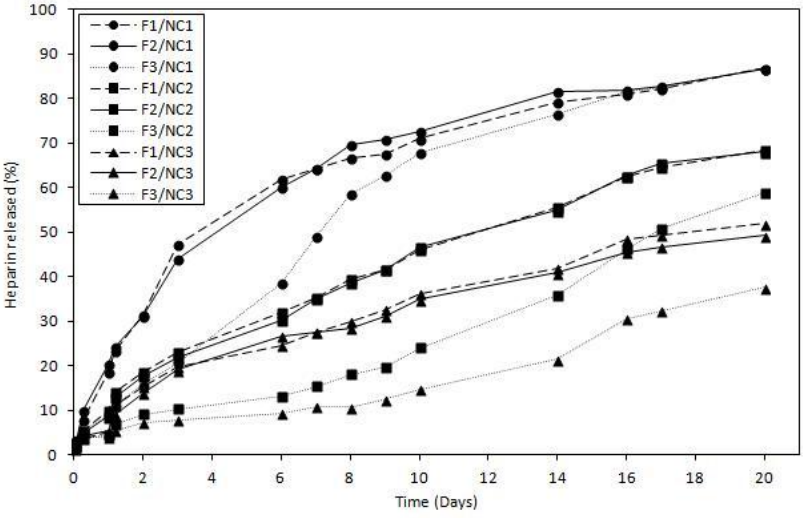

Heparin released (%)
Time (Days)
F1/NC1
F2/NC1
F3/NC1
F1/NC2
F2/NC2
F3/NC2
F1/NC3
F2/NC3
F3/NC3

Fig. 6

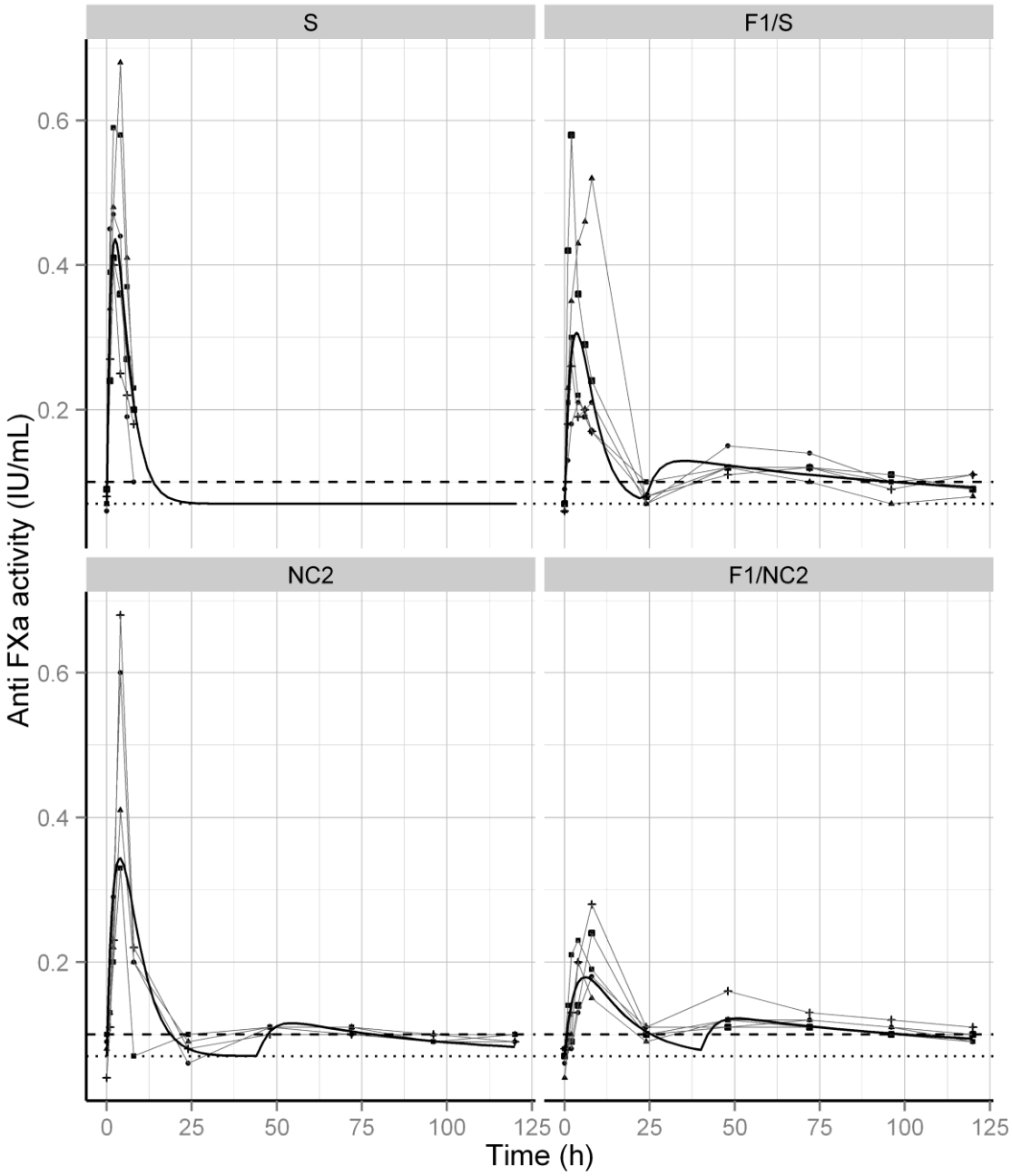

S
F1/S
NC2
F1/NC2
Anti FXa activity (IU/mL)
0.2
0.4
0.6
0
25
50
75
100
125
Time (h)

Fig. 7

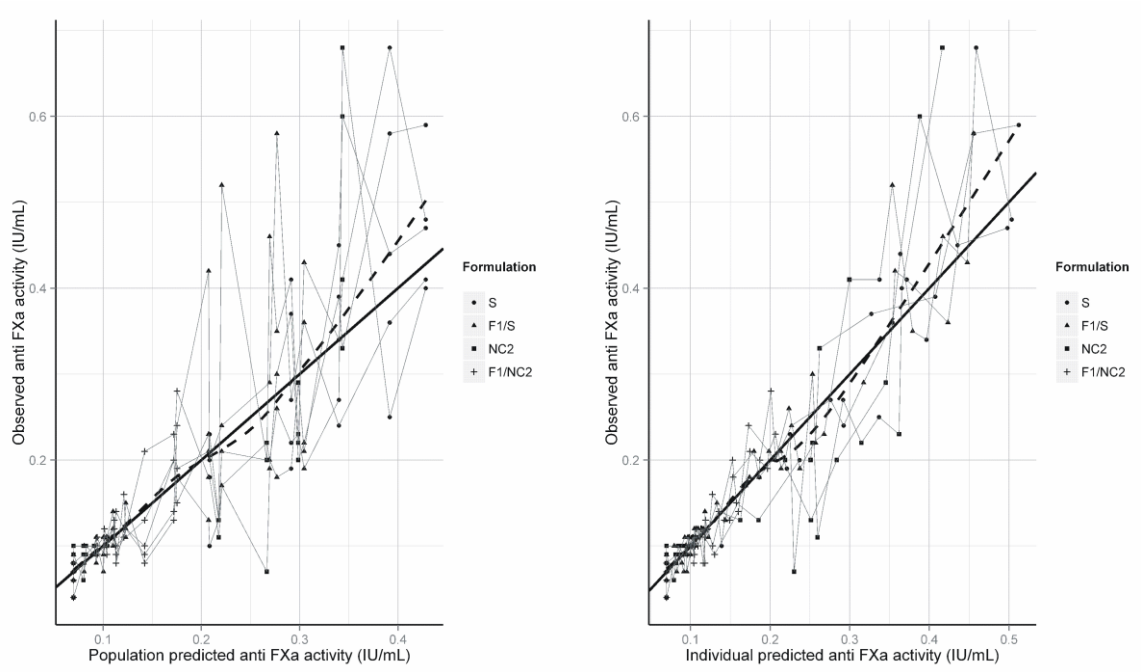

Observed anti FXa activity (IU/mL)
0.6
0.4
0.2
0.1
0.2
0.3
0.4
Population predicted anti FXa activity (IU/mL)
Formulation
S
F1/S
NC2
F1/NC2
Observed anti FXa activity (IU/mL)
0.6
0.4
0.2
0.1
0.2
0.3
0.4
0.5
Individual predicted anti FXa activity (IU/mL)
Formulation
S
F1/S
NC2
F1/NC2

Fig. 8

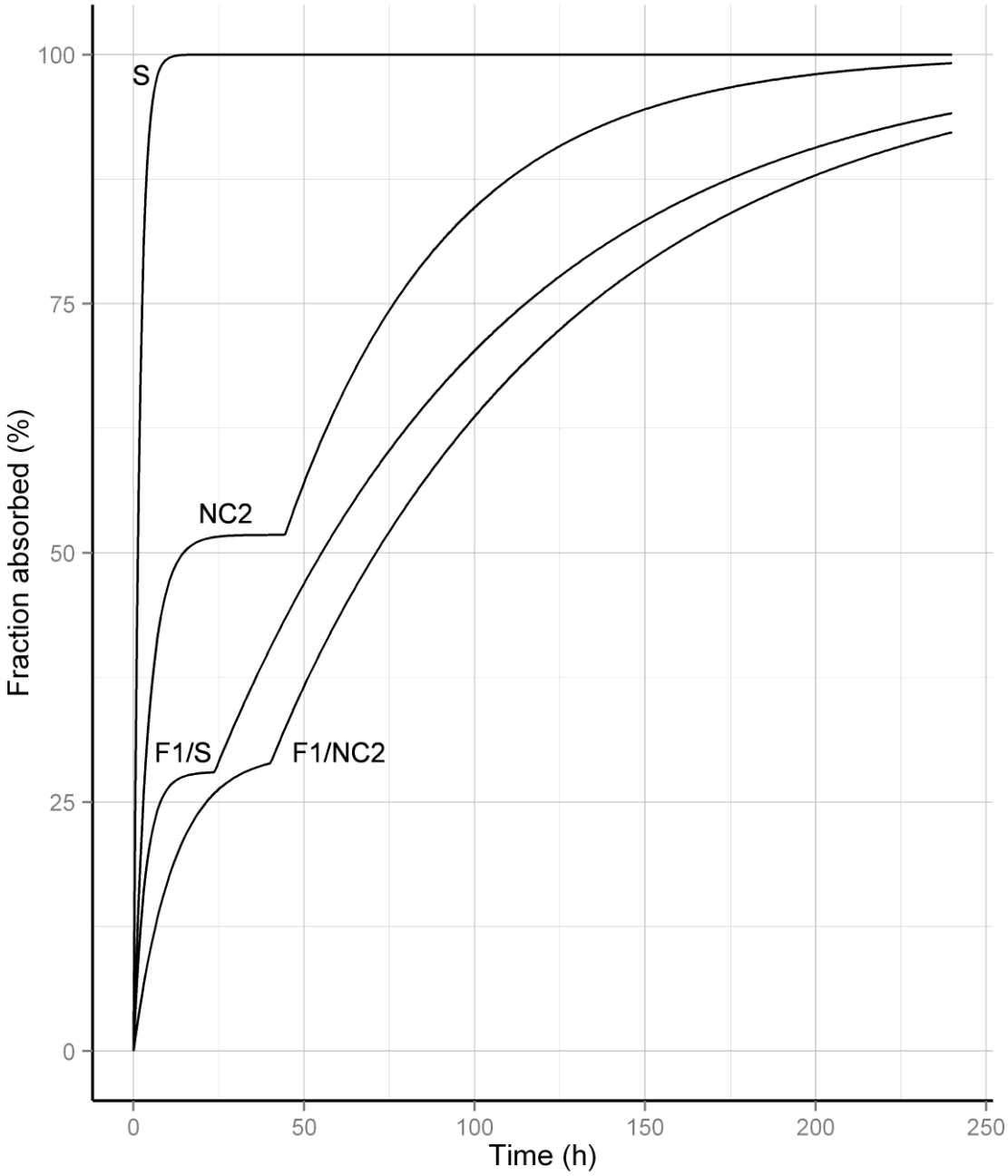

100
75
50
25
0
Fraction absorbed (%)
S
NC2
F1/S
F1/NC2
0
50
100
150
200
250
Time (h)

Table 1

The compositions of thermoresponsive hydrogels.

Hydrogel formulation	P407/P188/HPMC (wt %)	Heparin conc. (mg/mL)
F1	18/0/0	/
F1/S	18/0/0	1.125
F1/NC1	18/0/0	0.375
F1/NC2	18/0/0	1.125
F1/NC3	18/0/0	1.875
F2	18/1/0	/
F2/S	18/1/0	1.125
F2/NC1	18/1/0	0.375
F2/NC2	18/1/0	1.125
F2/NC3	18/1/0	1.875
F3	18/1/1	/
F3/S	18/1/1	1.125
F3/NC1	18/1/1	0.375
F3/NC2	18/1/1	1.125
F3/NC3	18/1/1	1.875

Table 2

The main characteristics of five Hep/Ch NC dispersions.

Heparin conc. (mg/mL)	Mean diameter (nm)	PDI	ZP (mV)	pH
0.375	175	0.252	41.4	5.18
0.75	196.5	0.273	36.1	4.29
1.125	245.5	0.356	34.5	4.18
1.5	286.5	0.396	37.6	4.01
1.875	363.5	0.653	39.5	3.94

Table 3

Estimated pharmacokinetic parameters of heparin following subcutaneous administration in rats.

Parameter	Estimate	RSE (%)	Inter-animal variability[a], RSE, Shrinkage (%, %, %)
Base line (IU/mL)	0.0699	3.5	
CL (mL/h)	90.6	8.2	15.9, 37.0, 32.0
Vd (mL)	318	19.7	35.8, 54.2, 21.4
f_r-F1/S	3.11	11.7	
f_r-F1/NC2	2.77	17.4	
f_r-NC2	1.82	15.9	
Absorption from Depot 1			
K_{a1}-S (h^{-1})	0.552	16.7	
K_{a1}-F1/S (h^{-1})	0.283	50.1	
K_{a1}-F1/NC2 (h^{-1})	0.0845	17.8	
K_{a1}-NC2 (h^{-1})	0.230	18.8	
f_1-F1/S	0.280	22.1	
f_1-F1/NC2	0.299	13.5	10.1, 43.0, 28.2
f_1-NC2	0.518	16.3	
Absorption from Depot 2			
K_{a2}-F1/S (h^{-1})	0.0116	23.5	
K_{a2}-F1/NC2 (h^{-1})	0.0110	17.3	
K_{a2}-NC2 (h^{-1})	0.0206	28.8	
t_{lag2}-F1/S (h)	23.7	9.5	
t_{lag2}-F1/NC2 (h)	40.2	11.0	
t_{lag2}-NC2 (h)	44.4	13.5	
Residual error, Shrinkage	21.0, 8.1	10.3	

[a]Inter-animal variability is reported as CV% estimated as $\sqrt{(e^{\omega} - 1)} \cdot 100$, except for f_1 which is logit constrained as $\ln(f_1/(1-f_1))$ and variability is reported as $SD(f_1) = \sqrt{\omega} \cdot \theta_{f1} \cdot (1 - \theta_{f1})$