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Research paper

# Oral low molecular weight heparin delivery by microparticles from complex coacervation

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**Abstract**

As low molecular weight heparins exhibit limited oral absorption they usually have to be administered parenterally. Their strong negative charge appears to be one of the biggest hurdles to overcome in order to increase oral absorption. Complex coacervation has been proposed as a microencapsulation technique for increased oral drug absorption on the basis of charge compensation. Optimized tinzaparin/acacia gum mixture were coacervated with either gelatin A or B leading to microparticles with monodispersed size distribution, good fluidity and high encapsulation rates (>90%), while mean particle size varied between 5 and 20 μm, respectively, depending on the gelatin type. Tinzaparin was homogeneously distributed throughout the particle matrix and anti-Xa activity was maintained during preparation and storage. Drug release occurred in dependency of the pH triggering the dissociation between tinzaparin/acacia and gelatin. Cell binding experiments on Caco-2 led to slightly increased adhesion of gelatin A microparticles compared to gelatin B (A: $3.5 \pm 0.3\%$; B: $2.5 \pm 0.3\%$; solution: $1.9 \pm 0.1\%$), while drug transport did not differ from free tinzaparin solution. In-vivo results demonstrated an oral bioavailability of about $4.2 \pm 2.9\%$ with gelatin B particles while gelatin A led to no absorption of tinzaparin. In conclusion, tinzaparin microparticles exhibited excellent particle properties in vitro and demonstrate potential for a formulation increasing the oral bioavailability of low molecular weight heparins.


*Keywords:* Microparticles; Complex coacervation; Low molecular weight heparin; Tinzaparin; Oral absorption; Gelatin

## 1. Introduction

Low molecular weight heparin (LMWH) has been shown to be a potent anticoagulant in the prevention and the treatment of deep vein thrombosis [1] and pulmonary embolism [2]. Although various new pharmacological molecules have been developed, heparins still remain the classical treatment in venous thromboembolism [3]. However, the parenteral administration of LMWH by intravenous or subcutaneous routes and repetitive injections are the main disadvantages which demand for an improvement of the administration strategy.

The oral route represents the most convenient administration pathway highly accepted by all patients. However, since LMWH are not absorbed from the gastrointestinal tract (presumably owing to their molecule size and their strong anionic charge provoking ionic repulsions from negatively charged mucus and epithelial tissue) [4], recent works have been performed by combining heparin with carriers. One approach to oral heparin delivery was based on the design of peptide-like compounds that promoted the gastrointestinal absorption of macromolecules like heparin [5] without intestinal damage. This technology resulted from the discovery that microspheres (MS) formed from thermally condensed α-amino acid mixtures could be used to favor oral drug delivery [6].

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Different studies were based on non-naturally occurring cationic polymers as starting materials for the condensation reaction that demonstrated the gastrointestinal absorption of heparin in animals [7] and in humans [8]. Besides, other cationic polymers have been proposed for the microencapsulation of heparins by using a multiple emulsion method [9,10].

Alternatively, different microencapsulation techniques are possible, among them the complex coacervation of polyelectrolytes which is a common encapsulation method. Coacervation is the separation of an aqueous polymeric solution into two immiscible liquid phases: a dense coacervate phase, which is relatively concentrated in the polymers and a dilute equilibrium phase [11]. To form microcapsules, the coacervate phase is dispersed in the equilibrium phase, and the resulting coacervate droplets are stabilized applying different solidification methods [12]. An initial in-vitro study on complex coacervation reported the use of gelatin to be coacervated with heparin [13]. A major advantage is that gelatin is a biocompatible and biodegradable polymer formed by hydrolysis of collagen. It is zwitterionic, carrying both carboxyl and amino groups while heparin is charged negatively composed of iduronic acid, iduronic acid sulfate, 2-deoxy-2-sulfamino-D-glucose 6-sulfate, and glucuronic acid units. Thus, the heparin/gelatin complex coacervate can be formed as a result of electrostatic interaction between oppositely charged heparin and gelatin when gelatin's charge turns cationic.

In order to test this very simple and low-cost design for oral LMWH delivery we studied the formulation of tinzaparin MS prepared by either gelatin A or B. The physicochemical characterization of the MS properties was followed by their in-vitro testing in Caco-2 cell culture on their adhesive behavior and possible influences on drug transport. Finally, experiments were completed with a preliminary pharmacokinetic study in rabbits.

## 2. Materials and methods

### 2.1. Materials

Low molecular weight heparin tinzaparin (Innohep®) was purchased from Leo (Saint-Quentin-en-Yvelines, France). Gelatin, types A and B (bloom number 200), was obtained from Deutsche Gelatine Fabrik Stoess and Co. (Eberbach, Germany), acacia gum was obtained from Caesar and Lorentz (Hilden, Germany). *N*-(Dimethylaminopropyl)-*N*-ethylcarbodiimide HCl and fluoresceinamine were purchased from Sigma (Deisenhofen, Germany).

### 2.2. Methods

#### 2.2.1. Preparation of microparticles

A sample containing 250 mg of gelatin was dissolved in 10 ml water at 40 °C. Then, 2000 IU (≈20 mg) tinzaparin was added to the gelatin solution and mixed for 5 min under magnetic stirring. Ten milliliters of an acacia gum solution (1.5%) was added and stirred for another 5 min. Finally, 30 ml of water was added, the pH was lowered with hydrochloric acid (1 N) to $3.0 \pm 0.3$ and the system was cooled to 5 °C under continuous magnetic stirring. After separating the coacervates either by simple sedimentation or centrifugation (1000*g* for 5 min), the supernatant was decanted and the particles were hardened by adding 20 ml of 2-propanol to the sediment. Finally, the MS were filtered and dried under vacuum overnight until constant weight.

#### 2.2.2. Analysis of morphology and particle size

The external morphology of MS was analyzed by transmission light microscopy and scanning electron microscopy (SEM). MS were observed as simple dry powder with the light microscope (Zeiss Axiovert 100 microscope, Carl Zeiss, Oberkochen, Germany), while they were fixed on supports with carbon-glue, and coated with gold using a gold sputter module in a high-vacuum evaporator for SEM images. Samples were then observed with the scanning electron microscope (JEOL JSM-T330A scanning microscope, Tokyo, Japan) at 10 kV. All MS batches were analyzed for their size distribution using a Malvern Mastersizer X (Malvern Instruments Ltd., Worcestershire, UK).

#### 2.2.3. Drug loading and in-vitro drug release

The encapsulated tinzaparin amount within MS was determined by nephelometry [14] by measuring the amount of non-entrapped drug in the supernatant after the sedimentation step. Aliquots of 0.5 ml of each sample were incubated with 0.5 ml acetic buffer (pH 4.4) and 2 ml of a 0.1% cetylpyridinium chloride in NaCl (0.94%) for 1 h at 37 °C and analyzed at 500 nm by spectrophotometry (Uvikon 922, Kontron, Eching, Germany).

For in-vitro release experiments, 20 mg of drug-loaded MS was suspended in 20 ml phosphate buffer systems of different pH. The dissolution medium was kept under stirring at 100 rpm. All the experiments were carried out at 37 °C for 4 h. Aliquots of the dissolution medium (1 ml) were withdrawn at predetermined time intervals and replaced by fresh buffer. Samples were filtered with a 0.22 μm Millipore® filter and assayed with the nephelometric method.

#### 2.2.4. Fluorescence labeling of tinzaparin and confocal laser scanning microscopy

The labeling protocol was adapted to a method described earlier [15]: tinzaparin was incubated with carbodiimide and fluoresceinamine overnight at room temperature and thereafter free marker and linker were removed by dialysis (Spectrapor® 7, Spectrum Ltd., USA; membrane pore size: 1000 Da) against distilled water until no fluorescence was detected in the external phase.

A Biorad MRC 1024 Laser Scanning Confocal Imaging System (Hemel Hempstead, UK), equipped with an argon ion laser (American Laser Corp., Salt Lake City, USA) and

a Zeiss axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany), was used to investigate the structure and morphology of the microcapsules. All confocal fluorescence pictures were taken with a 40× objective (oil immersion, numeric aperture 1.30).

*2.2.5. Coacervation studies*

Predetermined amounts of gelatin A or B (0–100 mg) were dissolved in 4 ml water at 40 °C. 2000 IU (100 μl) tinzaparin was diluted in 900 μl distilled water and added to the gelatin solution. Coacervation was induced by pH reduction to pH 3.0 with hydrochloric acid (1 N). Turbidity was measured by spectrophotometry at 420 nm directly after the coacervation. Thereafter, samples were centrifuged for 10 min at 20,000*g* and free tinzaparin was quantified in the supernatant.

*2.2.6. Tinzaparin stability*

Dried MS were stored at room temperature under subdued light. After 1, 2, or 3 month samples were assayed for anti-Xa activity of the entrapped tinzaparin. The biological activity of tinzaparin incorporated in MS was evaluated by measuring the anti-Xa activity after complete particle dissolution with a commercial kit according to the supplier's instructions (Diagnostica Stago, Asnières-sur-Seine, France).

*2.2.7. Caco-2 cell binding*

Caco-2 cells were obtained from ATCC (Manassas, VA, USA). Approximately 10,000 cells per well were seeded in 96-well plates and grown in Dulbecco's Modified Eagle's Medium until confluency. Thereafter, cells were incubated for 1 h with the different fluorescently labeled tinzaparin formulations ($n = 4$) or tinzaparin alone. After carefully removing the supernatant and repeated washings the cell binding was determined using a CytoFluore II Microplate Fluorescence Reader from PerSeptive Biosystems (Wiesbaden, Germany) with the excitation/emission wavelengths at 485/530 nm. Cell binding was expressed as percentage of total administered tinzaparin. Cell viability was tested after the experiments with the MTT test.

*2.2.8. In-vivo experiments*

Experiments were carried out in compliance with the French legislation on animal experiments under the personal experimentation authorization No. 54–68. Male New Zealand rabbits with a mean body weight of $3000 \pm 300$ g were used. tinzaparin loaded MS were filled into gelatin hard capsules at a dose of 600 IU/kg body weight and administered to overnight fasted rabbits by oral route. Blood samples were withdrawn before and 2, 4, 6, and 8 h after administration of each dosage form from the ear vein. Blood (1.5 ml) was gently mixed with sodium citrate (125 μl) before plasma was separated by centrifugation for 10 min at 4000*g*. The biological activity of tinzaparin was evaluated by measuring the anti-Xa activity in rabbit plasma with the standard kit as described above.

*2.2.9. Statistical analysis*

The results were expressed as mean values ± SD. For the analysis of statistical significance the Kruskal–Wallis test was applied followed by Dunn's test for all pairwise comparisons, except when normality and equal variance were passed it was followed by the Tukey test. In all cases, $P < 0.05$ was considered to be significant.

## 3. Results and discussion

The microencapsulation by complex coacervation is widely known due to its simplicity, low costs of excipients and preparation procedure which still nowadays appears to be attractive for studies in this field. Precedent research was carried out on the caocervation of gelatin with unfractionated heparin alone. The subsequent addition of polyvinylpyrrolidon stabilized the system for successful spray drying [13]. The here employed tinzaparin is a "short chain" heparin compared to unfractionated heparins with a mean molecular weight of about 4500 Da only [16]. Subsequently, changes in the behavior of gelatin/heparin coacervates may be possible.

In initial studies, varying tinzaparin amounts were encapsulated at a constant total concentration of colloids of 1% with a 1:1 ratio of gelatin and acacia. The observed decrease of encapsulation rate with increased theoretic drug loading was based on the competitive displacement of acacia with tinzaparin and vice versa, both negatively charged components in the coacervate "concurring" for the interaction with gelatin (data not shown). Moreover, a general deterioration of the particle quality including reduced yield and fluidity was found. This might be explained by the excess of negatively charged components leading to an imbalance between positively and negatively charged coacervates.

Subsequently, an optimal formulation was hypothesized to be the coacervate with equivalent cationic and anionic charges including an optimal mixture of tinzaparin and acacia in order to avoid their competitive displacement. Various amounts of gelatin A and B were coacervated with a fix amount of tinzaparin in the absence of acacia and led to an optimal ratio of gelatin to tinzaparin (Fig. 1). While turbidity increased towards an optimal gelatin/tinzaparin ratio, free (non-coacervated) tinzaparin amount was highest with imbalanced gelatin/tinzaparin mixtures.

Gelatin A and B are known to have different isoelectric points due to their different (acidic or alkaline) preparation methods. However, differences in the isoelectric point do not change the absolute number of cationic groups in both gelatin types, only the number of anionic amino acids is increased in the case of alkaline collagen degradation method. Thus, it might be plausible that the optimal ratio for coacervation is similar with both types of gelatin.

Although the optimal coacervation ratio can be found easily, a distinct lack of physicochemical stability of the built suspension is observed soon after the phase separation. In a precedent study stabilization was ensured by

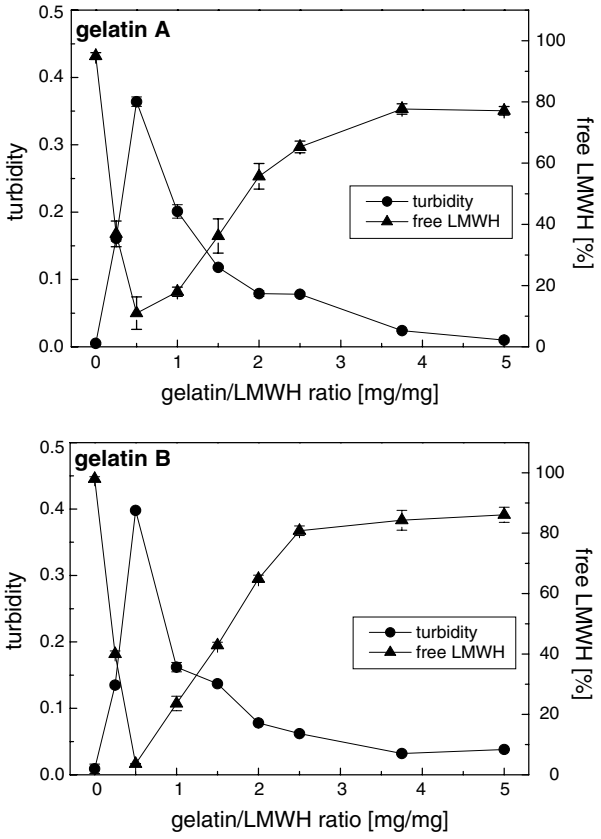


Fig. 1. Coacervation of varying amounts of either gelatin A or B with a constant quantity of tinzaparin. Turbidity of the obtained suspension and non-coacervated amount of tinzaparin are given for the different gelatin/tinzaparin ratios ($n = 3$). Data are shown as means ± SD.

the addition of polyvinylpyrrolidone and subsequent particle hardening by a spray-drying step which avoids the use of toxic chemical cross-linking agents [13]. Here, tinzaparin was replaced partially by amounts of acacia with respect to the initial total charge equivalent of acacia or tinzaparin to gelatin in blank MS. Such particles had extraordinarily good powder properties. However it was observed that the tinzaparin/acacia quotient had to be lower than 0.3 in order to maintain this high powder quality (data not shown).

Optimized MS prepared with gelatin A or B were of spherical shape, monodisperse, and had a relatively smooth surface (Fig. 2A–D). The MS prepared with gelatin B were smaller while process yield and encapsulation rates were comparable for particles prepared by gelatin A or B (Table 1). The analysis of internal particle structure demonstrated a homogeneous distribution of the fluorescently labeled tinzaparin due to the charge interaction with the cationic gelatin which is similar to observations in earlier coacervation studies (Fig. 2E and F). The particle wall formation takes place based on electrostatic interaction between the polymers and leads to a statistical distribution of the polymers throughout the wall material owing to the necessary charge equivalence of the two counterions during coacervation [17].

The simultaneous presence of tinzaparin and acacia did not alter their homogeneous distribution throughout the particle matrix which has been also reported for complex coacervation with other polymer mixes [18]. The gelatin type did not influence its distribution inside the particle which is in line with the expectations due to the equivalence between gelatin A and B in the percentage of basic amino acids. Different to the ordinary microencapsulation where the drug is entrapped inside the particle matrix, tinzaparin with its negative charge becomes here also a matrix forming component and is not only encapsulated in the standard sense.

Generally, pH-sensitive release profiles were observed which is in line with the hypothesis that a dissociation of the coacervates can occur in aqueous media at pHs where the overall charge of gelatin turns negative (Fig. 3). Subsequently, no sustained release was provided but either efficient retention of tinzaparin inside the particle matrix at pHs below 4 or fast and complete release within 15 min was obtained. Slight differences between release profiles from gelatin A or B were observed as drug release occurred near the isoelectric point of each gelatin type. Although only cationic groups are the basis for the electrostatic binding between tinzaparin and gelatin, the isoelectric point indicating the total charge of the zwitterionic gelatin takes into account negative charges being responsible for possible repulsion forces between tinzaparin and gelatin.

While encapsulation rates were determined by nephelometry, a test on remaining anti-Xa activity of the tinzaparin demonstrated sufficient stability of the entrapped drug after the preparation process as well as during storage (Fig. 4). Tinzaparin released from MS was found to retain ability to bind to and inactivate factor-Xa by maintaining around 90% of its biological activity during the encapsulation process. Thus, nearly no deteriorating influences by the low pH or the 2-propanol water extraction step were observed. Moreover, tinzaparin trapped inside MS was stable for at least a period of 3 months. The charge interaction between gelatin and tinzaparin remained reversible when pH increased and no deterioration was observed for the drug release.

Cell adhesion experiments showed an only very limited binding tendency of MS on Caco-2 cells compared to the simple drug solution mainly independent from the gelatin type (Fig. 5). With decreasing particle concentrations differences between MS formulations and tinzaparin in solution became statistically significant. However, the gradual increase of cell binding at pH 4 might be simply based on the integrity of MS as gelatin and tinzaparin are completely dissociated at pH 7 where MS adhesion was equivalent to tinzaparin solution. It might be concluded that the "charge covering" of gelatin influenced the binding on cells only slightly. A sufficient adhesion may require an excess of positive charge of the carrier as it is reported for other types of heparin carriers. Furthermore, transport studies did not result in significant differences between gelatin MS compared to control groups with tinzaparin solution alone (data not shown).

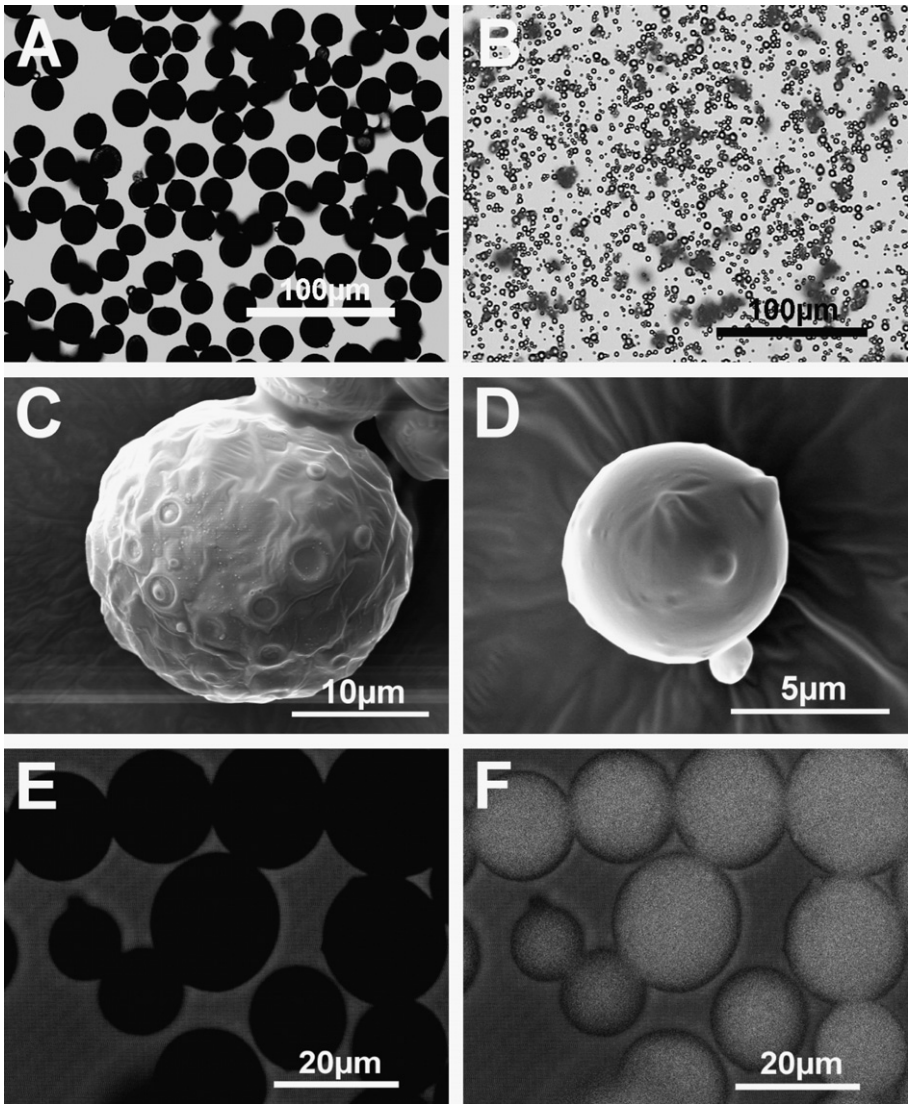


Fig. 2. Optical transmission and scanning electron microscopic images of MS prepared by complex coacervation of gelatin A (A and C) or B (B and D) with the optimized ratio of acacia and tinzaparin. Confocal laser scanning microscopy demonstrated the homogeneous distribution of fluorescently labeled tinzaparin within the MS matrix, here exemplarily shown for gelatin A MS (transmission light image (E) versus green fluorescence light (F)).

Table 1
In-vitro characteristics of the tinzaparin microparticles prepared with gelatin A or B ($n = 3$)

| Parameter | Coac A | Coac B |
|---|---|---|
| Mean particle diameter [µm] | $35.5 \pm 0.5$ | $5.6 \pm 0.3$ |
| Process yield [%] | $75.3 \pm 2.5$ | $68.3 \pm 1.9$ |
| Encapsulation rate [%] | $97.1 \pm 0.5$ | $98.4 \pm 0.5$ |

Table 2
Pharmacokinetic parameters of gelatin MS after oral administration to rabbits compared to subcutaneous administration of tinzaparin ($n = 4$)

| | AUC (mg/ml) | AUC/kg (mg/ml/kg) | $F$ [%] |
|---|---|---|---|
| Subcutaneous | $4.45 \pm 0.42$ | $1.23 \pm 0.10$ | 100 |
| Coac A | $0.00 \pm 0.00$ | $0.00 \pm 0.00$ | $0.0 \pm 0.0$ |
| Coac B | $0.59 \pm 0.44$ | $0.16 \pm 0.12$ | $4.2 \pm 2.9$[a] |

[a] Significantly different from subcutaneous administration and coac A formulations.

A preliminary in-vivo study was performed in rabbits after the oral administration of MS formulations made from gelatin A or B. MS prepared with gelatin B exhibited an oral bioavailability of 4.2% while absorption from gelatin A was negligible (Fig. 6; Table 2). Usually no anticoagulant activity is observed after the oral administration of aqueous heparin solution as the drug is rapidly altered by removal of sulfates under acidic conditions and enzymatic degradation in the gastrointestinal tract [19].

It was previously reported that small particles (<10 µm) can be absorbed through the intestinal wall to some extent. The various proposed uptake mechanisms include a paracellular pathway [20], transcellular transport via the epithelial cells of the intestinal mucosa [21], and lymphatic uptake via Peyer's patches [22]. As particle uptake is limited with larger particles, paracellular transport and intracellular uptake may be excluded here. An uptake via gut associated lymphatic tissue might be postulated however

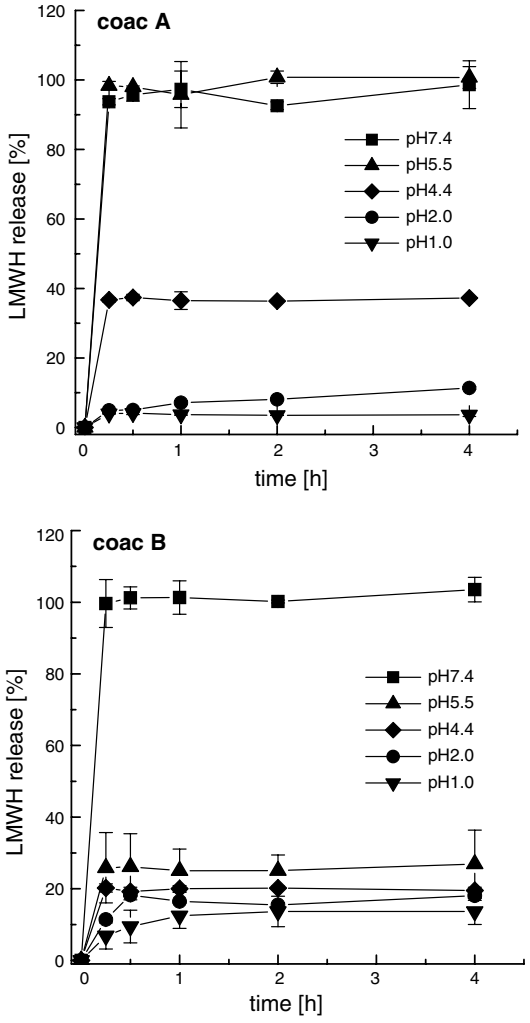


Fig. 3. Cumulated drug release experiments with MS prepared from gelatin A (coac A) or B (coac B) at various pHs ($n = 3$). Data are shown as means ± SD.

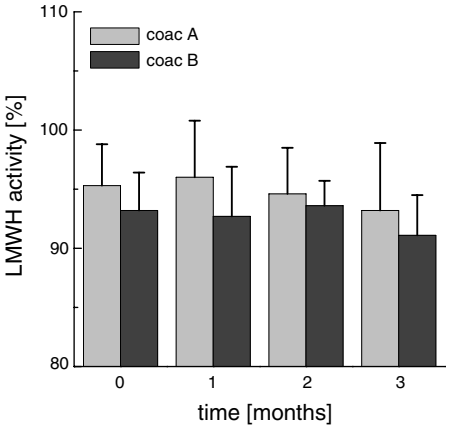


Fig. 4. Tinzaparin stability in gelatin A and B MS (coac A or coac B, respectively) directly after MS preparation and after storage periods of either 1, 2, or 3 months ($n = 3$). Data are shown as means ± SD.

the probability is limited since rabbits have only few Peyer's patches. Moreover, the integrity of gelatin MS is reduced due to their dissociation with increasing pH which

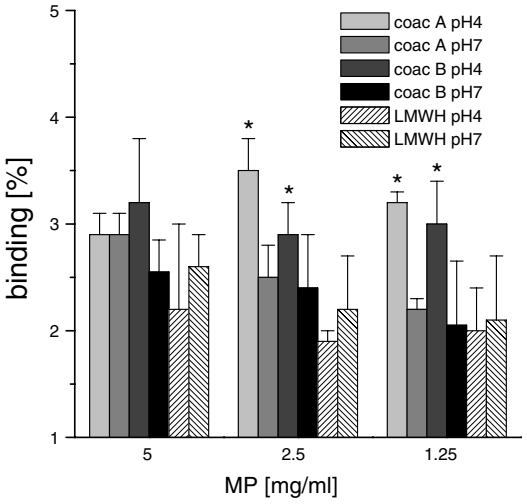


Fig. 5. Cell binding experiments for gelatin A or B MS (coac A or coac B, respectively) at pH 4 or 7.4 in comparison with tinzaparin in solution ($n = 4$). Cell binding was expressed as percentage of total administered tinzaparin. Data are shown as means ± SD. $^*P < 0.05$ compared with cells incubated with tinzaparin solution.

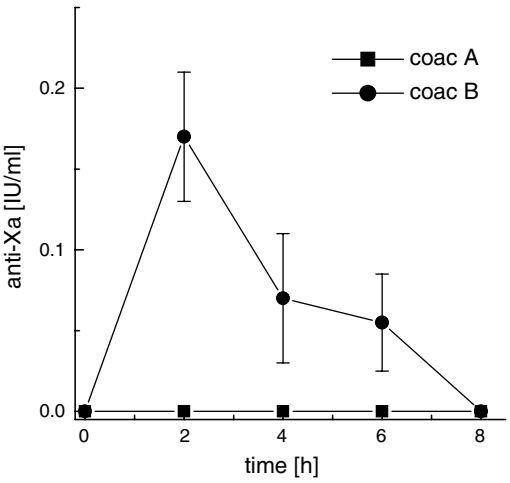


Fig. 6. Preliminary in-vivo study with either gelatin A or B MS (coac A or coac B, respectively) after oral administration in rabbits ($n = 4$). Data are shown as means ± SD.

may lead to a complete dissolution of the carrier in the jejunum or proximal ileum. The higher antithrombotic effect of gelatin B MS is apparently based on the slightly longer persistence of the carrier as pH-dependent release was observed at pHs around neutrality which could be related to a further retention of the LMWH in particle form. This ensures the prolonged protection of tinzaparin against inactivation by low pH or enzymatic degradation.

As complex coacervation is nearly perfectly compensating the negatively charged functional groups of the LMWH this influencing factor is apparently not the only and most important one. Additional effects such as a general positive charge of the carrier may play an important role in terms of adhesiveness to the mucus as described elsewhere [23] and which cannot be provided by gelatin MS.

## 4. Conclusions

Tinzaparin has been successfully entrapped into MS by a complex coacervation method using gelatin A and B. High encapsulation efficiency was achieved without loss of biological activity. An optimized mixture of tinzaparin and acacia gum allowed the preparation of MS with good powder properties. Entrapped tinzaparin was homogeneously distributed throughout the particle matrix which is in line with the theoretical background of this preparation method. The drug release was found to be pH-dependent as the dissociation of the coacervate is triggered by the pH in the aqueous solution. Preliminary in-vivo experiments demonstrated an increased oral bioavailability and made the described MS an interesting approach due to high encapsulation rates, low costs and easy feasibility.

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