In Vitro and In Vivo Evaluation of Oral Heparin–Loaded Polymeric Nanoparticles in Rabbits

Yuyan Jiao, PhD; Nathalie Ubrich, PhD; Monique Marchand-Arvier, PhD; Claude Vigneron, PhD; Maurice Hoffman, PhD; Thomas Lecompte, MD; Philippe Maincent, PhD

Background—Owing to its short half-life and lack of oral absorption, heparin has to be administered by the parenteral route. An oral heparin formulation, however, would avoid the disadvantages of parenteral injections and would consequently be highly desirable for patients. Polymeric nanoparticles (NPs) prepared with biodegradable poly-ε-caprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA) and nonbiodegradable positively charged polymers (Eudragit RS and RL), used alone or in combination, were evaluated in vitro and in vivo after a single oral administration of heparin-loaded NPs in rabbits.

Methods and Results—After oral administration of heparin-loaded NPs in rabbits (600 IU/kg), increases in both anti–factor Xa activity and activated partial thromboplastin time (aPTT) were detected with each formulation. Moreover, the anti-Xa activity was detected for a longer period than when a heparin solution was administered intravenously. A peak concentration of 0.16±0.01 IU/mL and an average aPTT of 24 seconds (2-fold increase) were obtained 7 hours after oral dosing of Eudragit RL/PCL NPs containing heparin, exhibiting an absolute bioavailability of 23%.

Conclusions—The significant increases in anti–factor Xa activity and aPTT confirmed the oral absorption in rabbits of heparin released from polymeric NPs. (Circulation. 2002;105:230-235.)

Key Words: heparin ■ anticoagulants ■ drugs ■ nanoparticles

Polymeric drug delivery systems have been widely developed and provide an attractive alternative for progressive and long-term delivery of therapeutic agents. These polymeric dosage forms offer many advantages: (1) drugs can be delivered to tissues in a sustained and continuous fashion; (2) drugs are well protected; and (3) site-specific delivery can be achieved, and repeated drug administration may not be necessary. Because macromolecules, such as peptides and proteins, are very sensitive in terms of stability, encapsulation allows their protection, especially against enzymes and pH effect, when they are administered by the oral route. Nanoparticles (NPs) are colloidal polymeric drug carriers that hold promise for peroral drug delivery. In addition, their uptake by paracellular, intracellular, or intercellular pathways or via the M cells and Peyer patches and their stability in the gastrointestinal tract indicate that NPs display the potentials of those carriers for the transport of drugs or proteins. Macromolecules, such as hormones, have been entrapped within polymeric particles.

Heparin is a glycosaminoglycan used mainly as an anticoagulant for the prevention of venous thrombosis and pulmonary embolism in patients undergoing surgery. Because heparin has no oral bioavailability, presumably because of its size and its negative charge, it has to be administered by the parenteral route, which requires careful patient monitoring.

Moreover, high doses have to be administered to induce an anticoagulant effect, and bleeding complications may occur. Although low-molecular-weight heparins may be self-administered and do not require intensive monitoring, the oral administration of heparin would still be highly desirable for patients. Several attempts to develop new oral formulations of heparin have been reported but have met with limited success. More recently, a novel absorption enhancer, sodium N-[8(2-hydroxybenzoyl)amino]caprylate (SNAC), coadministered orally with heparin has been shown to improve heparin absorption in humans. Nevertheless, the anticoagulant activity lasted only 4 hours for escalating doses ranging from 30 000 to 150 000 IU.

We now report that heparin-loaded NPs prepared with blends of biodegradable polymers and nonbiodegradable positively charged polymers facilitated the gastrointestinal absorption of heparin in rabbits, with doses similar to those administered by the parenteral route in humans, with both an increase and a prolongation of the anti–factor Xa activity and activated partial thromboplastin time (aPTT).

Methods

Preparation of Polymeric NPs

The polymeric NPs were prepared with biodegradable polymers, poly(ε-caprolactone) (PCL) and poly(l-lactic-co-glycolic acid)
(PLGA) 50/50, and 2 nonbiodegradable positively charged polymethacrylates (Eudragit RS and RL) used alone or in combination (ratio 1/1).

The preparation of the NPs was carried out by the multiple emulsion technique previously described by Lamprecht et al.15 The method was based on the use of a homogenizer in a 2-step emulsification process. Briefly, 1 mL of an aqueous heparin solution (5000 IU) was first emulsified in methylene chloride (10 mL) containing the polymer(s) (0.25 g) by sonication for 1 minute at 60 °C. The resulting water-in-oil emulsion was thereafter poured into 200 mL of a polyvinyl alcohol (PVA) aqueous solution (0.1%) and homogenized at high shear for 3 minutes, involving the formation of the second, water-in-oil-in-water emulsion. After evaporation of methylene chloride, the polymer precipitated, and the NPs were isolated by centrifugation. The NPs were washed 3 times with deionized water before freeze-drying. Drug-free NPs were formulated in the same way.

Heparin Encapsulation Efficiency

The amount of heparin entrapped within polymeric NPs was determined with a modified Azure II colorimetric method16 by measuring the amount of nonentrapped drug in the external aqueous solution recovered after centrifugation and washing of the NPs. Typically, aliquots (500 μL) of aqueous samples were reacted with 4.5 mL of the Azure II solution (0.01 mg/mL) and assayed in triplicate at 330 nm by UV spectroscopy. The drug entrapment efficiency was expressed as the percentage of heparin entrapped with respect to the theoretical value, and the drug loading was presented as the amount of heparin (IU) entrapped per gram of polymer.

Mean Diameter Analysis

The NPs were analyzed for their mean diameter by photon correlation spectroscopy (Zetamaster II, Malvern Instruments).

In Vitro Release Experiments

Freeze-dried NPs (10 mg) were suspended in 10 mL of PBS (0.011 mol/L, NaCl 0.15 mol/L, pH 7.4) in a flask containing Tween 80 (0.1%) and incubated in a water bath at 37 °C under gentle magnetic stirring (150 rpm). At various time intervals, 2-mL samples were withdrawn and centrifuged for 30 minutes at 40 000g. The supernatant was removed and assayed for heparin according to the colorimetric method previously described.

A second series of experiments was done by adding esterases (25 mg, 50 U/mL) to the release medium. To compensate for the loss of enzyme activity at 37 °C, an additional 25 mg of esterase was added to the release medium every 6 hours.

Biological Activity of Heparin

Anti–factor Xa activity was determined with a chromogenic substrate by use of a standard kit (Stachrom Heparin) from Diagnostica Stago. The assay has a coefficient of variation of <7% at a limit of detection of 0.02 IU/mL.

The clotting time was measured by the aPTT with a standard kit (C.K. Prest) from Diagnostica Stago.

In Vivo Study

Male New Zealand rabbits with a mean body weight of 2300 ± 300 g were used for the experiments. Lyophilized polymeric NPs containing 2000 IU of heparin were resuspended in water (10 mL) and immediately administered by oral gavage through a cannula to rabbits that had been fasted overnight. A solution of heparin (2000 IU) administered both intravenously and orally and unloaded NPs also administered by oral gavage were used as controls. The anti–factor Xa activity as well as the clotting time were determined in blood plasma before and 1, 2, 3, 4, 5, 6, 7, 8, 10, and 24 hours after administration of each dosage form. Blood samples (500 μL) were previously withdrawn from the ear vein into a vial containing sodium citrate (50 μL), which was gently mixed before centrifugation for 8 minutes at 5000g.

### Table 1. Physicochemical Characteristics of Heparin-Loaded Nanoparticles

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Drug Loading, IU/g polymer</th>
<th>Entrapment Efficiency, %</th>
<th>Mean Diameter, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS</td>
<td>11 825 ± 140</td>
<td>60 ± 3</td>
<td>269 ± 16</td>
</tr>
<tr>
<td>PLGA</td>
<td>2792 ± 801</td>
<td>60 ± 4</td>
<td>267 ± 4</td>
</tr>
<tr>
<td>PCL</td>
<td>1673 ± 209</td>
<td>8 ± 1</td>
<td>285 ± 10</td>
</tr>
<tr>
<td>RL</td>
<td>19 477 ± 490</td>
<td>97 ± 2</td>
<td>266 ± 8</td>
</tr>
<tr>
<td>RL/PCL</td>
<td>10 663 ± 321</td>
<td>53 ± 2</td>
<td>306 ± 3</td>
</tr>
<tr>
<td>RS/RL/PLGA</td>
<td>7498 ± 138</td>
<td>38 ± 1</td>
<td>275 ± 3</td>
</tr>
<tr>
<td>RS/PLGA</td>
<td>7101 ± 431</td>
<td>36 ± 2</td>
<td>273 ± 7</td>
</tr>
</tbody>
</table>

Data are mean ± SD. n=4.

Results

Characterization of Heparin-Loaded NPs

As reported in Table 1, the mean diameter of heparin-loaded NPs prepared with each polymer used alone or in combination ranged from 260 to 300 nm.

The entrapment efficiency within the polymeric NPs was significantly affected by the nature of the polymers. It ranged from 8% to 97% and reached the highest values when positively charged polymethacrylates were used for the NP preparation. The highest encapsulation efficiency was obtained with Eudragit RL (97%), which has more quaternary ammonium groups (8.8% to 12%) than Eudragit RS (4.5% to 6.8%). In addition, when Eudragit RS and RL were blended with PCL and PLGA, heparin entrapment was higher than when PCL and PLGA were used alone. Figure 1 illustrates the in vitro release profiles of heparin in PBS at 37°C and pH 7.4 from NPs prepared from a single polymer or from mixtures of Eudragit with PCL and PLGA, respectively. Both a low and a biphasic release pattern were observed for each dosage form. Indeed, after an initial burst stage during which heparin was rapidly released over 1 hour, the drug release profiles displayed a plateau characterized by a very slow and incomplete subsequent release for an extended period of time. Although high entrapment efficiencies were obtained with Eudragit (particularly Eudragit RL), very low amounts of heparin were released from NPs prepared with these 2 polymers. NPs prepared with PLGA, alone or in combination with Eudragit, exhibited higher drug release than those prepared with PCL. The combination of Eudragit RS and RL with PCL or PLGA did not influence the drug release compared with these polymers used alone. The addition of esterases to the dissolution medium, however, changed the release profiles of heparin markedly (Figure 2). Indeed, the burst was not immediate, and the release of heparin was increased (by 10-fold for NPs of RL/PCL at 24 hours). Because the encapsulation process involves steps of both shear and evaporation, as well as interface contact with the organic solvent, it was important to verify whether or not heparin retained its biological activity. The results showed that heparin was unaltered by the double emulsion and solvent evaporation process; indeed, a good correlation was found between the amount of heparin released from heparin-loaded NPs suspended for 24 hours into the dissolution...
medium containing esterases, as determined by the colorimetric method with Azure II, and that determined by the biological method based on the anti–factor Xa activity (Figure 3).

**Absorption of Heparin After a Single Oral Dose of Heparin-Loaded NPs in Rabbits**

To display a therapeutic effect, significant amounts of the entrapped drug must be released from the polymeric carriers.

Figure 1. Release profiles of heparin from NPs prepared (a) with a single biodegradable or nonbiodegradable polymer: Eudragit RS (†), RL (‣), PLGA (△); and (b) with blends of biodegradable and nonbiodegradable polymers: RL/PCL (●), RS/RL/PLGA (♦), RS/PLGA (▪). Experiments were performed in phosphate buffer at 37°C and pH 7.4. Data are mean±SD (n=3).

Figure 2. Release profiles of heparin from NPs prepared with blends of biodegradable and nonbiodegradable polymers: RL/PCL (●), RS/RL/PLGA (♦), RS/PLGA (▪), and a single polymer, Eudragit RL (♦). Experiments were performed in phosphate buffer containing esterases (50 U/mL added every 6 hours) at 37°C and pH 7.4. Data are mean±SD (n=3).

Figure 3. Comparison of amount of heparin released after 24 hours from polymeric NPs determined by colorimetric method with azure II (solid bars) and by anti–factor Xa activity with a chromogenic substrate (open bars). Data are mean±SD (n=3).

The RL/PCL, RS/PLGA, and RS/RL/PLGA formulations affording both a suitable drug entrapment efficiency and the highest drug releases were selected for the in vivo study. Owing to the high heparin entrapment efficiency within NPs prepared with Eudragit RL, however, this formulation was tested in vivo as well. Figure 4 illustrates (A) the amount of anti–factor Xa activity and (B) the mean clotting time.

Figure 4. Mean prolongation of (A) anti–factor Xa activity and (B) aPTT over 24 hours after oral administration of RL/PCL (●), RS/RL/PLGA (♦), RS/PLGA (▪), and Eudragit RL (‡) NPs loaded with heparin (2000 IU). Inset, Mean prolongation of anti–factor Xa activity after administration of an aqueous solution of heparin (2000 IU IV) in rabbits. Data are mean of 4 rabbits. ‡, Anti-Xa activity beyond limit of detection. SDs not given for clarity.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>RL/PCL</th>
<th>RS/RL/PLGA*</th>
<th>RS/PLGA*</th>
<th>RL</th>
<th>Heparin in solution (IV route)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{max}, IU/mL</td>
<td>0.16±0.01</td>
<td>0.12±0.05</td>
<td>0.13±0.06</td>
<td>0.12±0.04</td>
<td>Not determined</td>
</tr>
<tr>
<td>t_{max}, h</td>
<td>6–8</td>
<td>3–5</td>
<td>6–8</td>
<td>5–6</td>
<td>Not determined</td>
</tr>
<tr>
<td>AUC_{0–24 h}, IU·h·L^{-1}·kg^{-1}</td>
<td>0.74±0.24†</td>
<td>0.39±0.08</td>
<td>0.29±0.03</td>
<td>0.32±0.07</td>
<td>3.30±0.59</td>
</tr>
<tr>
<td>Absolute bioavailability, %</td>
<td>22.73±5.46†</td>
<td>12.10±2.41</td>
<td>9.07±0.81</td>
<td>9.87±2.06</td>
<td>100</td>
</tr>
</tbody>
</table>

AUC indicates area under the curve. Data are mean±SEM, n=4.

*Statistically different from RS/RL/PLGA, RS/PLGA, or RL nanoparticles at P<0.05 (Student’s t test).

†Statistically different from RS/RL/PLGA, RS/PLGA, or RL nanoparticles at P<0.05 (Student’s t test).

The anticoagulant effect of heparin is related to its absorption after a single oral dose of heparin-loaded NPs in rabbits. As shown in Figure 4A and Table 2, anti-factor Xa activity was detected after oral administration of each formulation containing 2000 IU of heparin. The highest anti-factor Xa response (peak concentration of 0.16±0.01 IU/mL 7 hours after dosing) was observed in rabbits receiving RL/PCL NPs loaded with heparin. Lower peak concentrations (0.12±0.05, 0.12±0.04, and 0.13±0.06 IU/mL) were observed 3, 6, and 7 hours, respectively, after dosing with RS/RL/PLGA, Eudragit RL, and RS/PLGA NPs loaded with heparin. The anti–factor Xa activity was detected for a longer period when heparin-loaded NPs were administered orally than administered as an aqueous solution by the intravenous route. Indeed, a detectable prolongation of anti–factor Xa activity (0.04 IU/mL) was measured up to 7 to 10 hours after oral administration of each formulation. Compared with heparin administered intravenously, a decrease in both the maximal plasma concentration (C_{max}) and the area under the curve was observed for all the polymer carrier formulations (Table 2). The best absolute bioavailability (23%) was observed with RL/PCL NPs, whereas lower figures were obtained for the other formulations. As shown in Figure 4B, the normal aPTT in rabbits is ∼12 to 14 seconds. There is a good temporal relationship between Figure 4A and 4B. Indeed, from 2 hours after dosing of each formulation, an increase in anticoagulant activity was detected for all formulations (Figure 4B), with a maximal aPTT value of 24 seconds (corresponding to an ∼2-fold increase) 6 and 8 hours after oral administration of RL/PCL and RS/PLGA NPs, respectively.

Discussion

Preparation of NPs

The choice of the method of encapsulation is actually determined by the solubility characteristics of the drug. Because it is usually used for water-soluble drugs, the double-emulsion and solvent-evaporation process was adopted. The double-emulsion technique involves 2 major steps: the formation of the droplets in the first emulsion and the subsequent removal of solvent from the droplets of the second emulsion; the second step involves the precipitation of the polymer and consequently, the solidification of the core of the NPs. The mean diameter of the resulting particles depends mainly on the mixing velocity of the preparation process. The more vigorous the mixing, the smaller the particles. In our study, the use of a high-pressure homogenization process reduced the mean particle diameter. In addition, the mean diameter is related to a great extent to the emulsion stability obtained by the addition of surfactants such as polyvinyl alcohol to hamper the fast coalescence of the droplets.

In Vitro Characterization of NPs

Encapsulation efficiency and drug release depend on the nature of the polymers, because significant differences were observed between each dosage form. Indeed, owing to the polyanionic nature of heparin and the opposite polycationic nature of Eudragit, the highest encapsulation efficiencies were obtained with Eudragit RL or RS. This can be explained by electrostatic bindings between the drug and the quaternary ammonium groups of the 2 polymers. This hypothesis is strengthened by the fact that a higher drug loading was observed with Eudragit RL, which carries more quaternary ammonium groups than Eudragit RS. Moreover, when Eudragit RS and RL were blended with PCL and PLGA, heparin entrapment was higher than when PCL and PLGA were used alone. However, though the highest encapsulation efficiencies were obtained when NPs were prepared with Eudragit used alone or in combination with PCL and PLGA, the drug release was very low, probably owing to the strong ionic interactions between Eudragit RS or RL and heparin: such interactions are difficult to disrupt in our experimental conditions. The higher drug release observed with PLGA used alone or in combination with Eudragit, compared with PCL, may be explained by the high hydrophobicity of PCL that reduced the wettability of the NPs as well as the diffusion of the drug to the dissolution medium. When esterases were added to the dissolution medium, however, the drug release profiles changed and the amounts of heparin released were higher, probably as a result of the faster biodegradation of PCL and PLGA as well as a possible hydrolysis of the lateral ester bonds present in the Eudragit polymers. In addition, the water uptake was probably more important in PLGA-based NPs than in PCL ones, owing to the less hydrophobic character of PLGA compared with PCL. Because the hydration is expected to favor more hydrolysis associated with a faster fragmentation of the parent particles, it was not surprising that higher heparin release was obtained from NPs prepared with PLGA.

Evaluation of NPs In Vivo

The anticoagulant effect of heparin is related to its absorption after its release from NPs after oral administration. The low
$C_{\text{max}}$ values of all the polymeric NPs can be explained by the slow diffusion of the drug from the polymeric carriers, as is generally observed.\textsuperscript{18} It was also confirmed that no anticoagulant activity occurred after the oral administration of heparin as an aqueous solution, because heparin is rapidly altered by removal of sulfates in the acidic conditions and/or by the proteolytic enzymes of the gastrointestinal tract.\textsuperscript{19} Consequently, the detection and the prolongation of the anti–factor Xa activity result from the protection of heparin, which was then released in an unaltered form from the polymeric particles. Furthermore, these results are also confirmed by the chronological evolution of clotting time. Indeed, an increase in aPTT was observed between 2 and 8 hours for all formulations, with a 1.8- to 2.0-fold aPTT at the peak time. The results presented definitely show the potential absorption of heparin from multiparticular systems and open up discussion of the absorption mechanisms. It was previously demonstrated that particles with a diameter $<10 \text{ mm}$ may be absorbed through the intestinal wall. Three possible uptake mechanisms have been suggested for oral absorption of NPs: (1) uptake via a paracellular pathway,\textsuperscript{4} (2) intracellular uptake absorbed through the intestinal wall. Three possible uptake the drug gradient concentration toward the blood.\textsuperscript{20} Although it is difficult to predict which is the mechanism of heparin absorption in our study, the influence of the polycationic polymer seems important. Indeed, it is well known that the mucous layer is negatively charged at physiological pH. Some residual and noncomplexed cationic charges, unmasked during heparin release, may indeed still increase the residence time of NPs next to the absorption surface of the gastrointestinal tract. This mechanism would reinforce the heparin gradient because of the natural gastrointestinal tract coating of NPs.

The bioavailability figures presented in Table 2 confirm that not all the encapsulated heparin is totally absorbed. The best bioavailability was obtained with RL/PCL NPs, with $\approx20\%$ of the encapsulated dose being active. The 3 other types of NPs presented no statistical difference in terms of absolute bioavailability. These results, especially those obtained with RL/PCL NPs, are very promising, considering the low heparin dose administered orally. Indeed, our goal was to show the potential of oral heparin-loaded NPs by using the same dose as that commonly administered by the intravenous route, ie, 600 IU/kg, in treatment. This corresponds to a worst-case protocol, because it is well known that oral bioavailability is always lower than the intravenous one. Our results, however, open up further research possibilities, for instance, on the increase of encapsulated heparin within NPs for future in vivo studies.

Other attempts at the oral administration of heparin have been reported in the literature. It is somehow difficult to compare the published results, because they have been performed with other animal species and different ways of presentation of the in vivo results. It can be stressed, however, that when oral absorption of heparin is claimed, the heparin dose is usually much higher than the one used in our study. For example, Ueno et al\textsuperscript{2} administered orally 150 000 IU of heparin entrapped within liposomes in beagle dogs: the results demonstrated an anticoagulant activity that was surprisingly only 2 to 3 times the activity of a similar dose of heparin in aqueous solution. As has been previously demonstrated many times, we were not able to show an anticoagulant activity after the oral administration of an aqueous solution of heparin in our rabbit model. By oral administration in rats of 100 mg/mL of heparin in proteinoid microspheres, Leone-Bay et al\textsuperscript{13} reported an anticoagulant activity, but this was limited to 1 to 2 hours. Our results clearly display an anticoagulant activity for up to 7 hours. Because the dose of heparin was expressed in mg/kg in the former study, however, the comparison is difficult. To the best of our knowledge, no published article presents results in terms of bioavailability by comparison with parenteral administration, even in more recent publications.\textsuperscript{14} It is also interesting to compare our findings with recent results obtained in humans. Indeed, Baughman et al\textsuperscript{14} reported an anticoagulant activity of oral heparin in humans when it is coadministered with an absorption enhancer called SNAC. The doses of heparin, ranging from 30 000 to 150 000 IU, are much greater than the parenteral doses. With each dose of heparin, a significant and prolonged anti–factor Xa activity and aPTT were observed between 0 and 3 hours after oral administration. Unfortunately, no bioavailability data were presented. Although the interspecies comparisons are difficult, it is noteworthy that a similar anti-Xa activity was observed in humans and rabbits, although the oral dose was less important in our rabbit model. Our results, however, showed a lag time in heparin absorption, because its biological activity is observed at $\approx3$ to 4 hours after oral administration and lasts much longer (5 to 6 hours) than SNAC-heparin. Conversely, the aPTT is lower in our rabbit model, because the basic aPTT is lower as well. Because aPTT reflects primarily anti-IIa activity, its increase and prolongation indicate that the long chains of heparin, endowed with anti-IIa activity, were absorbed. Therefore, the detection of the anti-Xa activity probably corresponds to chains with activity determined by both the aPTT and the anti–factor Xa bioassay. It is also important to notice that regular and well-known polymers were used in our study: PCL and PLGA are polymers used in humans for the parenteral dosage form, and Eudragit polymers are currently used for the coating of tablets. Conversely, the use of SNAC is not yet established in humans and could not be part of a dosage form without extensive toxicity studies.

**Conclusions**

Our findings clearly demonstrated the oral absorption of standard heparin encapsulated within polymeric NPs. The results are very promising, because they have been observed after the administration of a low dose of heparin. Furthermore, the excipients are well accepted worldwide in the
pharmaceutical field. Research is now in progress to evaluate the potential of escalating doses of heparin in our animal model with a view to challenging the proposed NPs in humans.

References
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