JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
ARTICLE
pubs.acs.org/JACS

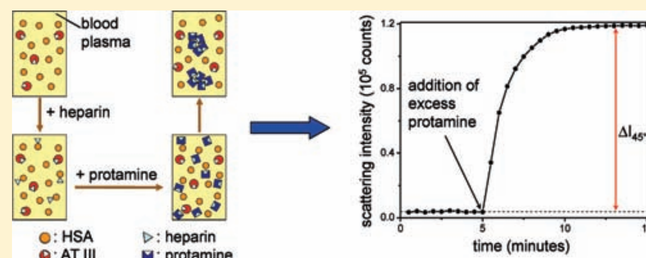
Analysis of the Complex Formation of Heparin with Protamine by Light Scattering and Analytical Ultracentrifugation: Implications for Blood Coagulation Management

Jürgen Maurer, Stephanie Haselbach, Oliver Klein, Doan Baykut, Vitali Vogel, and Werner Mäntele*

Institute of Biophysics, Johann Wolfgang Goethe-University Frankfurt, Max von Laue-Strasse 1, 60438 Frankfurt am Main, Germany

S Supporting Information

ABSTRACT: Heparin, a linear glycosaminoglycan, is used in different forms in anticoagulation treatment. Protamine, a highly positive charged peptide containing about 32 amino acids, acts as an antagonist for heparin to restore normal blood coagulation. The complex formation of protamine with heparin was analyzed by a combination of analytical ultracentrifugation and light scattering. Titration of heparin with protamine in blood plasma preparations results in a drastic increase of turbidity, indicating the formation of nanoscale particles. A similar increase of turbidity was observed in physiological saline solution with or without human serum albumin (HSA). Particle size analysis by analytical ultracentrifugation revealed a particle radius of approximately 30 nm for unfractionated heparin and of approximately 60 nm for low molecular weight heparin upon complexation with excess protamine, in agreement with atomic force microscopy data. In the absence of HSA, larger and more heterogeneous particles were observed. The particles obtained were found to be stable for hours. The particle formation kinetics was analyzed by light scattering at different scattering angles and was found to be complete within several minutes. The time course of particle formation suggests a condensation reaction, with sigmoidal traces for low heparin concentrations and quasi-first-order reaction for high heparin concentrations. Under all conditions, the final scattering intensity reached after several minutes was found to be proportional to the amount of heparin in the blood plasma or buffer solution, provided that excess protamine was available and no multiple scattering occurred. On the basis of a direct relation between particle concentration and the heparin concentration present before protaminization, a light scattering assay was developed which permits the quantitative analysis of the heparin concentration in blood plasma and which could complement or even replace the activated clotting time test, which is currently the most commonly used method for blood coagulation management.



INTRODUCTION

Since the discovery of its anticoagulant properties,[1] heparin has been used in different forms for more than 60 years in anticoagulation treatment.[2] Its unfractionated, high molecular weight form (UFH) with a mean molecular weight (MW) of approximately 10−20 kDa[3] is mostly used for anticoagulation treatment, particularly in cardiovascular surgery, while the fractionated, low molecular weight form (LMWH; MW approximately 3−6 kDa) is mainly used for antithrombotic prophylaxis in out-of-hospital patients. Low molecular weight heparins currently in use contain a substantial fraction (up to 20−30%) of high molecular weight heparin.[4,5]

The typical procedure for anticoagulation treatment in cardiac surgery is the pre- and intraoperative intravenous administration of UFH. The dose required is empirically obtained with 300−400 IU/kg of body mass for any patient, which corresponds to approximately 1.5−2.0 mg/kg, aiming for an activated clotting time (ACT) longer than ∼300−480 s before intervention.[6] Currently, the control for the efficiency of this dose is based on an intermittent measurement of the ACT. Although established since 1966,[7] this method is considered to be less reliable for anticoagulation monitoring since its performance is influenced by temperature, the patient's individual sensitivity to heparin, reduction of clotting factors by hemodilution, interpatient variability of the antithrombin III (AT III) level, and preoperative use of certain drugs.[8−10] In fact, ACT is considered to be the least sensitive hemostatic test for heparin.[11] A more accurate monitoring of the coagulation status can be obtained with more recent systems such as Hepcon HMS PLUS or ITC-Hemochron-Response, which, however, are indirect, time-consuming, and expensive methods.[12] Up to now, there have been several approaches for the determination of heparin.[13−17] Nevertheless, a sufficient and more individual anticoagulation management with exact and frequent monitoring of the actual heparin level is still waiting to be developed.

Received: October 28, 2010
Published: December 27, 2010

ACS Publications © 2010 American Chemical Society

dx.doi.org/10.1021/ja109699s | J. Am. Chem. Soc. 2011, 133, 1134–1140

LMWH as used in antithrombotic prophylaxis is more predictable in anticoagulation response[18] and exhibits a longer half-time in plasma (2−4 h). UFH exhibits half-times between 60 and 90 min; supplementary doses during operation are thus necessary and are mostly based on ACT testing. Upon termination of surgical intervention, the neutralization of heparin with protamine is initiated. Protamine is a peptide with a high fraction of arginine residues leading to a high positive charge. It contains about 32 amino acids and is used as the standard antagonist for heparin to restore normal blood coagulation. The dosing is essentially empirical, frequently following a simple rule: *1 IU of protamine (sulfate) is needed to neutralize 1 IU of heparin given*. This dose aims to reach ACT values of approximately 120 s measured prior to heparinization, but it does not account for individual heparin sensitivity, for any coagulation disturbance, or for heparin degradation. Protamine, which has a shorter half-life than heparin, can also generate an anticoagulant effect if overdosed.[19] In addition to that, protamine exhibits several toxic effects,[20] including allergy, the generation of protamine antibodies, and interaction with other proteins or factors of the coagulation system. Moreover, an overdose of protamine decreases both the platelet number and function in dogs.[21] In cardiac surgical patients, overdoses of protamine were associated with alteration of ADP-induced platelet aggregation and prolongation of the ACT.[22] Free protamine was reported to precipitate fibrinogen[23] and to reduce the procoagulant effect of thrombin.[24]

The empirical estimation of the protamine dose required thus bears the risk of an over- or underprotaminization for the patient after surgery and may thus result in increasing bleeding tendency, blood clotting, and/or other side effects.

Heparin is a polymeric mucopolysaccharide with repeating units of disaccharides consisting of sulfatated D-glucosamine and L-iduronic acid, with D-glucoronic acid as a minor constituent. At physiological pH, heparin is a polyanion due to the negatively charged sulfate groups. This results in unspecific heparin binding to cellular membranes. The pentasaccharide sequence of heparin (three D-glucosamine and two uronic acid residues) is responsible for its pharmaceutical activity by binding to AT III and thus amplifying its coagulation-inhibiting action.[25] The neutralization of heparin by protamine, a highly positively charged peptide with a mean molecular weight of approximately 4.5 kDa containing about 32 amino acids, is based on complex formation of the positively charged amino groups of protamine with the negatively charged sulfate groups of heparin. This complex formation competes with the binding of heparin with AT III, platelet factor 4, and others. Commonly, a fixed dose of 1.0−1.3 mg of protamine for 1 mg (or 100 IU) of heparin is used for heparin neutralization.[20] In clinical practice, 1 IU of protamine is used for the neutralization of 1 IU of heparin (which means that 1 mg of protamine corresponds to 100 IU of protamine).

In spite of the widespread use of heparin and protamine, little is known about the interaction, complex formation, and complex size of these molecules. In order to investigate the complex formation, we have performed studies on heparin−protamine mixtures under physiological conditions. The turbidity of a heparinized blood plasma solution upon addition of protamine indicates the formation of nanoscale particles, much larger than the complexes expected from the size of heparin and protamine.[26] Here we report on the study of the size and formation kinetics of these particles by analytical ultracentrifugation and by light scattering techniques. On the basis of the latter data, a simple yet precise determination of heparin in blood samples is proposed which could complement and partially replace ACT determination in cardiac surgery and interventions with extracorporeal circulation and lead to a more rational dosing of heparin and protamine.

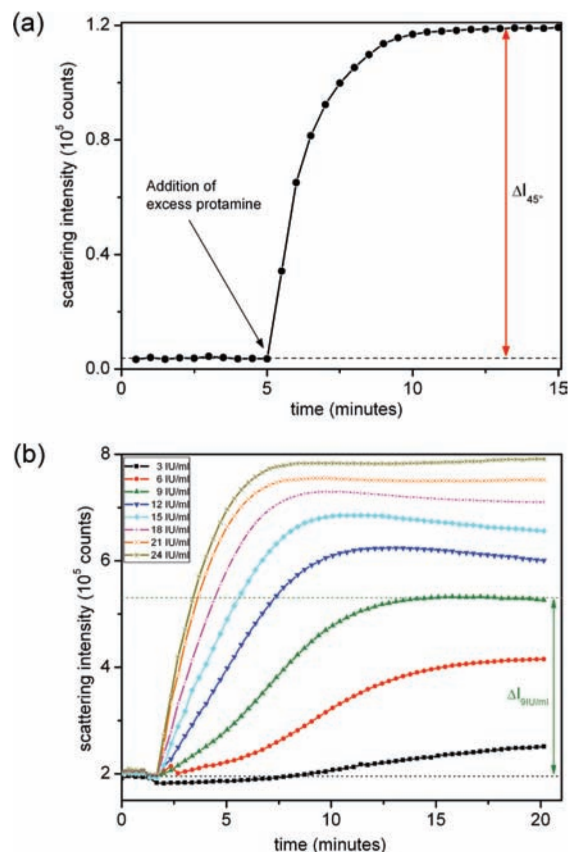


Figure 1. (a) Time course of light scattering for blood plasma from heparinized blood after addition of protamine. (b) Scattering for plasma samples with different heparinizations.

■ RESULTS AND DISCUSSION

A clear solution of blood plasma from heparinized blood turns visibly turbid if protamine is added. This indicates formation of nanoscale particles from complexes formed either by heparin−protamine interaction or by interaction with plasma proteins. The latter can be excluded since turbidity is also observed for the same heparin:protamine ratio in a phosphate-buffered physiological saline solution (PBS) with or without human serum albumin (HSA). In contrast, no turbidity is obtained after protamine is added to plasma samples containing no heparin.

Turbidity measurements with conventional spectrophotometers suffer from badly defined scattering angles in the forward direction as well as from an unknown fraction of multiple scattering. We have thus analyzed the formation of complexes between heparin and protamine by light scattering at well-defined angles.

Figure 1a shows the time course of the light intensity at a scattering angle of 45° for plasma from heparinized blood after addition of protamine. The scattering intensity is given in arbitrary units (counts). The initial nonzero scattering intensity before addition of excess protamine, termed background scattering, arises from residual cell debris and macromolecular aggregates which are present in blood plasma in spite of the centrifugation process. This background scattering can be further reduced by a second centrifugation procedure at higher *g* values (data not shown). As an

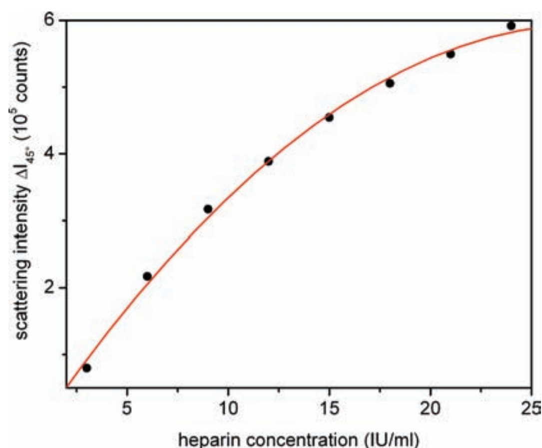


Figure 2. Light scattering intensity ($\Delta I_{45°}$) as a function of the heparin concentration.

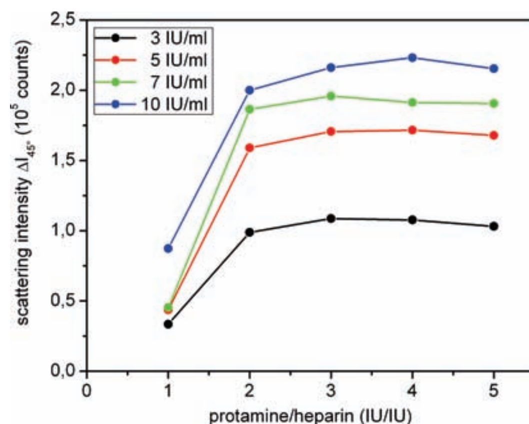


Figure 3. Titration of heparin (Clexane) model solutions in PBS/HSA with protamine. The scattering intensity at 45° was taken after 20 min.

alternative to centrifugation, cell separation techniques based on filtration can be used.

After addition of protamine, a rapid increase of scattering light intensity is observed which reaches the final amplitude after approximately 5−10 min. The difference between the initial and final scattering intensities, $\Delta I_{45°}$, is related to the nanoparticles formed from heparin and protamine in the plasma sample. The time course of the increase of scattering intensity appears simple and would at first sight suggest a treatment as first-order kinetics. In that case, the half-time would be on the order of 1−2 min.

Figure 1b shows time courses for the light scattering intensity at 45° for plasma samples with different heparinizations between 3 and 24 IU/mL. These values represent heparin concentrations typical for cardiac surgical interventions, for example, in coronary bypass surgery with or without extracorporeal circulation. More complex time courses are observed for lower heparin content, rather resembling nucleation reactions. In general, they exhibit a lag time followed by an increase that becomes faster with higher heparin concentration. Very similar time courses were obtained for the analysis of turbidity upon self-assembly of microtubili and non-native protein aggregation.[27,28]

The difference between initial and final scattering, $\Delta I_{45°}$, as a function of heparin present in the plasma sample is shown in Figure 2. For low heparin concentrations, a linear relation between heparin content and scattering change is obtained, which is expected for particles of similar size condensed from heparin and protamine. At higher heparin concentrations, a deviation from linearity toward a saturation behavior is observed. A plausible explanation for this phenomenon is the multiple scattering of light where the probability of a scattered photon to undergo a second scattering process increases with increasing particle concentration. Indeed, a linear relation over the entire concentration range can be restored if the path length in the scattering cuvette is reduced or the heparinized plasma is diluted before scattering analysis (data not shown).

Comparable time courses are obtained for plasma heparinized with LMWH and UFH, and calibration curves have been obtained for both heparin types. Moreover, similar time courses are obtained if physiological saline solutions, containing heparin with or without HSA, are used instead of plasma (data not shown).

For an estimation of the size of these particles from light scattering, the precise angular dependence of the scattering intensity is required. In a semiquantitative analysis (data not shown), we have obtained satisfying estimations of particle sizes by analyzing the ratio of scattering intensities at 45° and 135°. On the basis of an estimation by using latex model particles of 182 and 246 nm diameter, it appears that the time-dependent increase of turbidity or of the scattering intensity at 45° is related to the formation of nanoscale particles in the range of 20−100 nm.

Since particle formation is also observed in the absence of HSA, although at different sizes and kinetics of formation, there are good reasons to assume that they represent heparin−protamine hetero-oligomers. As a further control, scattering experiments with HSA in PBS buffer were performed. Neither the addition of heparin to an HSA solution nor the addition of protamine to an HSA solution induces an increase of light scattering. Electrostatic binding of heparin and protamine to HSA is expected under these conditions, but will be limited because of high salt concentration. Nevertheless, no large hetero-oligomeric complexes are formed under these conditions, thus excluding the participation of HSA in the complex formation between heparin and protamine.

To investigate the stoichiometry of these hetero-oligomers, we have titrated heparin model solutions (heparin in PBS/HSA) with different LMWH concentrations (Clexane) with protamine (Figure 3). Complex formation was allowed to proceed for 20 min. Light scattering at 45° for red light (λ = 635 nm) indicates that particle formation moderately starts at a heparin:protamine ratio of 1:1 but is strongly enhanced as soon as a heparin:protamine ratio of 1:2 is reached. No further scattering increase is observed upon an increase of protamine excess beyond this value. We take this as evidence that neutralization of low molecular weight heparin, the molecular weight of which is similar to that of protamine, requires a 2-fold excess of protamine and that the hetero-oligomeric complex may be written as (heparin−protamine$_2$)$_n$, with n exceeding 2000, which corresponds to an MW of more than 40 MDa (supposing an MW of the heparin−protamine complex of ∼20 kDa and a partial specific volume ($\tilde{v}$) of the complex of ∼0.6 mL/g (assuming that for heparin is 0.45 mL/g[29])).

To determine more precisely the size and size distribution of these particles, we have performed an analysis of sedimentation velocity by analytical ultracentrifugation. A sedimentation velocity experiment is the commonly used technique for the determination of sedimentation profiles as well as for size distributions of nanoparticle solutions.[30] Recently, analytical ultracentrifugation (AUC) has been applied to protamine−oligonucleotide nanoparticles to characterize the size distribution and stability of the nanoparticles.[31] Figure 4 shows particle size distributions of the particles formed from protamine and UFH and LMWH,

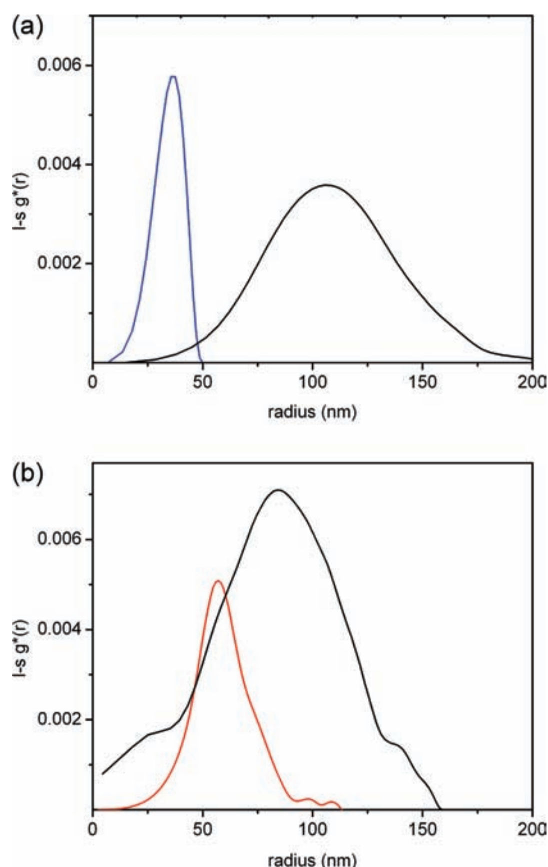


Figure 4. Size distribution of heparin−protamine complexes as obtained by analytical ultracentrifugation (in the framework model ls-$g^*(s)$ using the program sedfit of P. Schuck[32]): (a) UFH, 3 IU/mL; heparin:protamine = 1:2 (IU/IU); blue trace, with 50 mg/mL HSA; black trace, no HSA; (b) LMWH, 3 IU/mL; heparin:protamine = 1:2 (IU/IU); red trace, with 50 mg/mL HSA; black trace, no HSA.

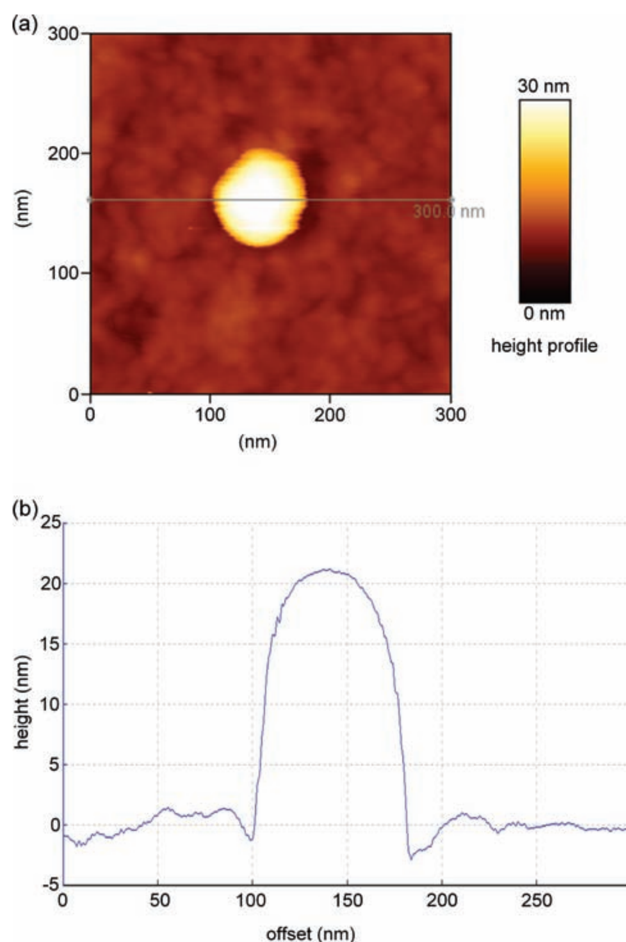


Figure 5. AFM picture of the heparin−protamine nanoparticles formed in blood plasma (15 IU/mL Clexane, 45 IU/mL protamine): (a) $x-y$ scanning map, (b) height profile of the particle shown in (a).

respectively, in the absence and presence of HSA. In all cases, an excess of protamine over heparin of 2:1 (IU/IU) and heparin concentrations of 3 IU/mL were used, and sufficient time was given for stable particle formation (cf. Figures 1 and 2). The analysis of sedimentation velocities yielded an average particle radius of approximately 30 and 60 nm for UFH and LMWH in the presence of HSA, respectively. In the absence of HSA, complexes formed from heparin and protamine exhibit an average particle radius of approximately 100 and 80 nm for UFH and LMWH, respectively. Size distributions are much narrower for UFH and LMWH in the presence of HSA. A particle size of 30 nm (UFH) corresponds to a molecular mass of up to 100 MDa.

The size of the nanoparticles determined by AUC is in good agreement with particle sizes determined by atomic force microscopy (AFM). Particles formed in blood plasma solution containing 15 IU/mL Clexane and 45 IU/mL protamine were transferred to glass slides (cf. the Experimental Section given in the Supporting Information). Round objects were observed with approximately 50−80 nm diameter after correction with the cantilever radius (Figure 5a). The height profile (Figure 5b) shows substantially lower values, suggesting that the particles might have collapsed upon exposure to air or due to the sticking at the glass slide.

Heparin−Protamine Particles: An Essential Step for the Antagonistic Action of Protamine? Since nanoparticle formation between heparin and protamine is observed not only in blood plasma, but also under conditions where HSA is not present, we conclude that these particles also form in full blood upon antagonization of heparin. Earlier, protamine−heparin nanoparticles were observed in water and after intravenous injection of protamine and heparin in rabbits.[33] The diameters of these particles were between 50 and 500 nm. Figure 6 represents a simplified scheme for the presence of heparin and protamine in blood plasma or full blood.

In normal human blood plasma, AT III is present in a concentration of approximately 2.3 μM.[34,35] In contrast, HSA is available at very much higher concentration, approximately 1 mM (A). Addition of heparin leads to tight binding of heparin to AT III (B), which amplifies its action and thus leads to reduced blood coagulation. Heparin also binds to HSA, although less tightly. Nevertheless, much of the heparin is bound to HSA simply because of its high concentration. If protamine is added, it tightly binds all heparins, the ones previously bound to AT III as well as the ones previously bound to HSA (C). AT III is thus deregulated again, and blood coagulation is restored to its normal value. Finally, the heparin−protamine complexes condense, by some yet unknown mechanism, to form the nanoparticles that have been observed (D).

The time courses observed for nanoparticle formation can be understood on the basis of a simple kinetic scheme (Figure 7) correlated with the graphical representation shown in Figure 6.

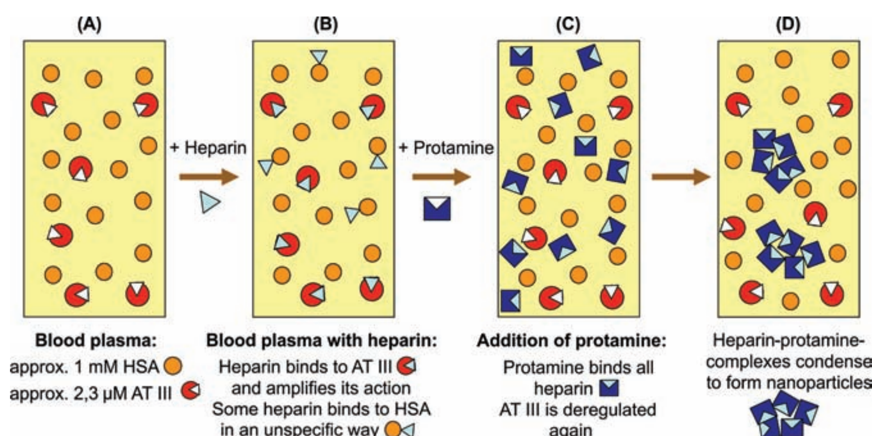


Figure 6. Simplified scheme for heparin and protamine action in blood plasma.

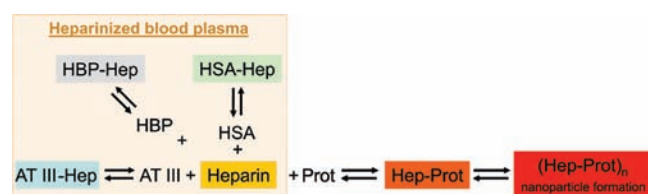


Figure 7. Kinetic scheme for heparin binding, protamine action, and nanoparticle formation.

The equilibrium (horizontal) between AT III and heparin (Hep) and their bound version, AT III−Hep, is essentially on the left side. In contrast, the equilibrium (vertical) between Hep and HSA and their bound form, HSA−Hep, represents a high on−off rate for heparin. Furthermore, heparin can bind to many other heparin-binding proteins (HBPs) in blood with different affinities. As soon as protamine is added, the binding of heparin and protamine leads to a tight complex and draws heparin from the equilibria with AT III as well as with HSA and other HBP. HSA, in this scheme, can act as a huge buffer for heparin molecules and thus regulates the forward reaction constant for Hep−protamine (Prot) formation. Finally, Hep−Prot complexes condense to $(\text{Hep}-\text{Prot})_n$ nanoparticles, which further reduce the reverse rate from Hep−Prot to Hep + Prot.

This kinetic scheme can explain the rate at which the nanoparticles are formed and which was directly observed by light scattering measurements. It could further explain the differences in particle size and size distribution in the absence or presence of HSA (Figure 4), since condensation of a heparin−protamine hetero-oligomer should depend on the rate of availability of one of the partners.

Heparin and Protamine Binding to Blood Proteins. Because of its negative charge, heparin is bound unspecifically by many different molecules. About 50 heparin-binding proteins were identified in whole plasma.[36] Most of them are present only in micromolar concentrations and exhibit dissociation constants in the range of 10−100 nM. Heparin binding to these proteins could potentially reduce the apparent activity of therapeutically administered heparin and thus modulate its biological activity.[36] Protamine is also physiologically active and can release some cell surface proteins to plasma if present at a high dose. An involvement of protamine in inflammatory reactions has also been reported.[37] The structural similarity of heparin to the cell surface and matrix glycan heparan sulfate gives rise to the anti-inflammatory activity of heparin. The routine use of heparin antagonists such as protamine is thus questionable; at least its dosing should be based on a precise determination of the heparin level.

Heparin Monitoring. At present, clinical administration of heparin follows empirical rules. The hemostatic status of the patient is typically monitored with the ACT test. The ACT test can be performed on full blood and is considered as a point-of-care procedure. Other tests, such as the activated partial thromboplastin time test (aPTT), are rather laboratory tests to assess the overall competence of the patient's coagulation system.

Patients undergoing surgical intervention that requires an extracorporeal circuit with the heart−lung machine (HLM) receive the highest heparin doses. An initial dose (typically 30000−50000 IU) is given in addition to the heparin required to maintain the extracorporeal circuit, and ACT is expected to rise to 600−1000 s. Once the desired coagulation state is reached, the heparin level has to be kept constant over hours by additional heparin dosing. Finally, at the end of the intervention, an adequate dose of protamine is required to restore normal coagulation. It is general practice in clinics that an ACT determination is made before initial heparinization, and a second one a few minutes after that. However, additional heparin dosing in the course of the surgery is frequently given without information on the actual heparin level, and protamine dosing is frequently estimated by a rule of thumb, for example, an amount sufficient to antagonize the initially given amount of heparin at the beginning of intervention. Different clinics report variations in these rules.

Both procedures, additional heparin dosing and protamine dosing, bear the risk of pathological thrombus formation or serious hemorrhagic complications. Underdosing of additional heparin in the course of an intervention may result in a coagulation state that can lead to blood clotting, while overdosing can result in bleeding. With protamine dosing, it is even more complex. Underdosing of protamine can lead to bleeding, because a substantial level of active heparin is still present. Overdosing of protamine also leads to a risk of bleeding, since protamine alone at higher concentrations exhibits anticoagulant activity. Ideally, dosing of heparin and protamine both should hit a small therapeutic window.

Heparin−Protamine Nanoparticle Formation as the Basis for a Quantitative Heparin Determination. At present, no procedure is available to allow a direct and quantitative measurement of heparin concentration in blood as a point-of-care test.

Chromatographic determination of heparin is possible, but it is time-consuming and labor intensive and thus only feasible in the central laboratory of a hospital. We propose to use the formation of heparin−protamine nanoparticles reported here as a quick and easy test for the measurement of the actual heparin level in blood.[38] This test requires a blood volume of 1−3 mL, which is compatible with the requirements during routine surgical interventions, even in children. The blood can be taken from the basilic vein or directly from the heart−lung machine. The centrifugation process to obtain a clear plasma sample from full blood accounts for less than 5 min, and can most probably be replaced by a filtration/separation technique. Finally, a test kit consists of a clear plastic tube that serves as a cuvette for the light scattering measurement and already contains the excess protamine, freeze-dried, in solution, or as a coating at the inner wall. The point-of-care test can thus be performed in a few minutes, which comfortably matches the time for an ACT test. Furthermore, heparin testing could be even faster for high concentrations than for very low concentrations, because formation of the nanoscale particles proceeds much faster (cf. Figure 1b). The performance of this test was analyzed through a calibration and validation series of heparinized blood (data not shown). The sensitivity, accuracy, and reproducibility were found to be more than sufficient for clinical application.

We have already performed a clinical study of the applicability of this heparin level test during open heart surgery with 50 patients at the University Clinics of Frankfurt, Germany, using a simplified version of the light scattering device with a collimated red light-emitting diode (LED) light source centered at 635 nm and photodiodes at two scattering angles at 45° and 135°, respectively. The detailed results of this test will be reported elsewhere. Briefly, the test shows that for a high fraction of the patients (∼2/3) both the rules of thumb for dosing of heparin and protamine as well as the control by ACT work reasonably well. However, for a substantial fraction of the patients, neither does the ACT test yield reliable results nor does the blood coagulation state at the end of the surgery match the optimum values. This is presumably the important fraction of patients that would greatly benefit from a precise determination of the actual heparin level to calculate additional heparin or protamine dosing. With a rational blood coagulation management, the intra- and postoperative risk of bleeding or pathological thrombus formation could be minimized.

■ ASSOCIATED CONTENT

S Supporting Information. Full experimental details and further AFM images of heparin−protamine nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
maentele@biophysik.uni-frankfurt.de

■ ACKNOWLEDGMENT

We thank Cathleen Häse and Lea Schröder for their contribution in the initial phase of this work, Viktor Gesiarz and Christian Seiler (Institute of Biophysics, Johann Wolfgang Goethe-University of Frankfurt) for their contributions in the development of a light scattering prototype for clinical tests of the method, and Helge Grossmann (Institute of Biochemistry, Johann Wolfgang Goethe-University of Frankfurt) for taking the AFM pictures. We would like to thank Prof. Moritz and Dr. Weber of the Dept. of Cardiac Surgery and Anesthesiology, University Clinics Frankfurt, for their cooperation with the clinical test of the method. Funding of the project through Fonds zur Veredlung und Verwertung von Patenten der staatlichen Hochschulen Hessens is gratefully acknowledged.

■ REFERENCES

(1) McLean, J. *Am. J. Physiol.* **1916,** *41*, 250–257.
(2) Harenberg, J.; Fenyvesi, T. *Hämostaseologie* **2004,** *24*, 261–278.
(3) Mulloy, B.; Gray, E.; Barrowcliffe, T. *Thromb. Haemostasis* **2000,** *84*, 1052–1056.
(4) Fareed, J.; Walenga, J. M.; Hoppensteadt, D.; Racanelli, A.; Coyne, E. *Semin. Thromb. Hemostasis* **1989,** *15*, 440–463.
(5) Linhardt, R. J.; Gunay, N. S. *Semin. Thromb. Hemostasis* **1999,** *25* (Suppl. 3), 5–16.
(6) Bull, B.; Huse, W.; Brauer, F.; Korpman, R. *J Thorac Cardiovasc Surg* **1975,** *69*, 685–689.
(7) Hattersley, P. G. *JAMA, J. Am. Med. Assoc.* **1966,** *196*, 436–440.
(8) Machin, D.; Devine, P. *J. Extra-Corpor. Technol.* **2005,** *37*, 265–271.
(9) Bull, B. S.; Hay, K. L.; Herrmann, P. C. *Blood Cells, Mol. Dis.* **2009,** *43*, 256–259.
(10) Despotis, G.; Levine, V.; Joist, J.; JoinerMaier, D.; Spitznagel, E. *Anesth. Analg. (Baltimore)* **1997,** *85*, 498–506.
(11) Murray, D. J.; Brosnahan, W. J.; Pennell, B.; Kapalanski, D.; Weiler, J. M.; Olson, J. *J. Cardiothorac. Vasc. Anesth.* **1997,** *11*, 24–28.
(12) Ramamurthy, N.; Baliga, N.; Wahr, J. A.; Schaller, U.; Yang, V. C.; Meyerhoff, M. E. *Clin. Chem.* **1998,** *44*, 606–613.
(13) Rodgers, P. J.; Jing, P.; Kim, Y.; Amemiya, S. *J. Am. Chem. Soc.* **2008,** *130*, 7436–7442.
(14) Milovic, N. M.; Behr, J. R.; Godin, M.; Hou, C. J.; Payer, K. R.; Chandrasekaran, A.; Russo, P. R.; Sasisekharan, R.; Manalis, S. R. *Proc. Natl. Acad. Sci. U.S.A* **2006,** *103*, 13374–13379.
(15) Shvarev, A.; Bakker, E. *J. Am. Chem. Soc.* **2003,** *125*, 11192–11193.
(16) Zhong, Z.; Anslyn, E. V. *J. Am. Chem. Soc.* **2002,** *124*, 9014–9015.
(17) van Kerkhof, J. C.; Bergveld, P.; Schasfoort, R. B. *Biosens. Bioelectron.* **1995,** *10*, 269–282.
(18) Weitz, J. I. *N. Engl. J. Med.* **1997,** *337*, 688–698.
(19) Thompson, W. H. *Z. Physiol. Chem.* **1900,** *29*, 1–19.
(20) Park, K. W. *Int. Anesthesiol. Clin.* **2004,** *42*, 135–145.
(21) Velders, A.; Wildevuur, C. R. *Ann. Thorac. Surg.* **1986,** *42*, 168–171.
(22) Mochizuki, T.; Olson, P. J.; Szlam, F.; Ramsay, J. G.; Levy, J. H. *Anesth. Analg. (Baltimore)* **1998,** *87*, 781–785.
(23) Okano, K.; Saito, Y.; Matsushima, A.; Inada, Y. *Biochim. Biophys. Acta* **1981,** *671*, 164–167.
(24) Cobel-Geard, R. J.; Hassouna, H. I. *Am. J. Hematol.* **1983,** *14*, 227–233.
(25) Rosenberg, R. D.; Damus, P. S. *J. Biol. Chem.* **1973,** *248*, 6490–6505.
(26) Vogel, V.; Häse, C.; Baykut, D.; Mäntele, W. *Z. Herz-, Thorax-Gefässchir.* **2007,** *21*, 140–147.
(27) Flyvbjerg, H.; Jobs, E.; Leibler, S. *Proc. Natl. Acad. Sci. U.S.A* **1996,** *93*, 5975–5979.
(28) Wang, K.; Kurganov, B. I. *Biophys. Chem* **2003,** *106*, 97–109.
(29) Lasker, S. E.; Stivala, S. S. *Arch. Biochem. Biophys.* **1966,** *115*, 360–372.
(30) Maechtle, W.; Börger, L. *Analytical Ultracentrifugation of Polymers and Nanoparticles*; Springer: Berlin, 2006.

(31) Vogel, V.; Lochmann, D.; Weyermann, J.; Mayer, G.; Tziatzios, C.; van den Broek, J. A.; Haase, W.; Wouters, D.; Schubert, U. S.; Kreuter, J.; Zimmer, A.; Schubert, D. *J. Controlled Release* **2005,** *103*, 99–111.
(32) Schuck, P.; Rossmanith, P. *Biopolymers* **2000,** *54*, 328–341.
(33) Rossmann, P.; Matousovic, K.; Horácek, V. *Virchows Arch., B* **1982,** *40*, 81–98.
(34) Murano, G.; Williams, L.; Miller-Andersson, M.; Aronson, D. L.; King, C. *Thromb. Res.* **1980,** *18*, 259–262.
(35) Conard, J.; Brosstad, F.; Lie Larsen, M.; Samama, M.; Abildgaard, U. *Haemostasis* **1983,** *13*, 363–368.
(36) Killeen, R.; Wait, R.; Begum, S.; Gray, E.; Mulloy, B. *Int. J. Exp. Pathol.* **2004,** *85*, A69–A69.
(37) Parish, C. R. *Nat. Rev. Immunol.* **2006,** *6*, 633–643.
(38) Mäntele, W.; Vogel, V.; Klein, O.; Schröder, L. Messvorrichtung zur Bestimmung der Grösse, Grössenverteilung und Menge von Partikeln im nanoskopischen Bereich. Patent DE102006005574, Johann Wolfgang Goethe-University Frankfurt, Germany, 2007.