Activity of maleimide functionalized unfractionated and low molecular weight heparins

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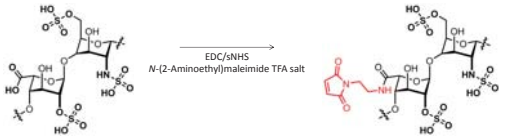
Motivation and Concept

- **background:** heparin is an established drug for anti-coagulant surface coatings on biomaterials
- **motivation:** various heparin coating technologies are established, but they are limited in precise orthogonal and modular immobilization
- **concept:** use of maleimide functionalization of a single COOH-group on heparin to enable modular binding to SH-presenting molecules and surfaces
- **aim of research:** Does maleimide functionalization affect the anti-coagulant property of heparin or lead to inflammation?



A reactive maleimide moeity was introduced to heparin by binding N-(2-Aminoethylmaleimide) to heparin carboxyl groups, after activating them by EDC/sNHS chemistry. Reaction conditions were adjusted to result in a functionlization degree of one reactive maleimide group per heparin [1, 2].

Chemical heparin characterization

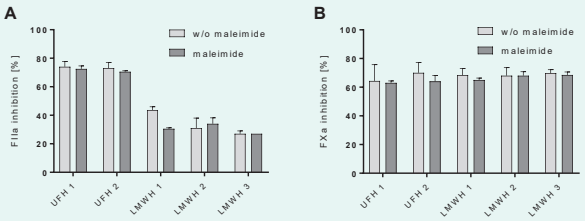
- determination of disaccharide (DS) units by elemental analysis (EA)
- quantification of functionalized reactive maleimide groups by HPSEC analysis of TNB^{2-} and model peptide conjugation

Chemical characterization of the unfractionated (UFH) and low molecular weight (LMWH) heparin molecules used in this study.

code	heparin	company	est. M_W [kDa]	DS units [EA]	#maleimide [TNB^{2-}]	#maleimide [peptide]
UFH 1	UFH Merck	Merck	14.0	18.9	0.84	1.10
UFH 2	UFH ratiopharm	ratiopharm	14.0	20.5	0.98	1.07
LMWH 1	LMWH Tinzaparin	LEO Pharma	6.3	9.4	0.90	1.06
LMWH 2	LMWH Certoparin	Aspen Pharma	5.6	7.8	1.06	1.13
LMWH 3	LMWH Enoxaparin	Sanofi-Aventis	4.5	6.2	0.85	1.09

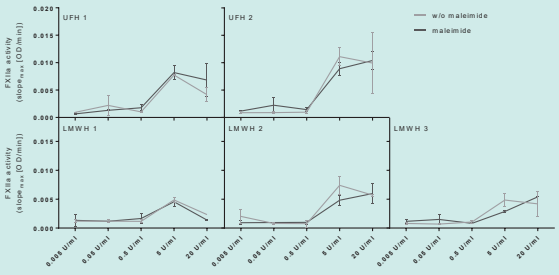
Performance of functionalized heparin

Inhibitory activity of heparin in buffer system

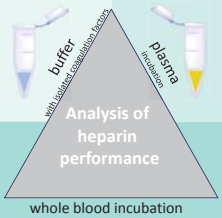


Anti-FIIa (A) and anti-FXa activity (B) of nominally 1U/ml pure heparin and maleimide functionalized heparin were determined in chromogenic assays and compared to a control without inhibitor.
→ **Inhibitor activity not affected by maleimide functionalization.**

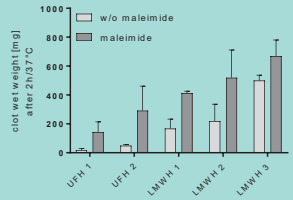
Heparin contact phase activation in plasma



Modification of heparin may lead to contact phase activation and inflammatory kinin activation. Heparins at different concentrations were checked for contact phase activation by a chromogenic assay [3].
→ **Maleimide functionalization did not enhance contact phase activation of any of the heparins.**

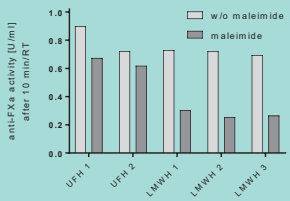


Anti-coagulant heparin activity in whole blood



The influence of maleimide functionalization on heparin anti-coagulant activity was measured as clot formation after incubation of whole blood with 1U/ml heparin under activating conditions.
→ **Molecular weight dependent blood clotting with increase for maleimide functionalized molecules.**

Maintenance of heparin activity in whole blood



To evaluate the maintanance of heparin activity in the test system, whole blood with 1U/ml heparin was incubated under non-activating conditions and checked for anti-FXa activity by a chromogenic assay.
→ **Rapid loss of activity for maleimide functionalized heparins in whole blood.**

Conclusion and Outlook

- inhibitory activity to isolated coagulation factors comparable for unmodified and functionalized compounds
- inhibition of blood coagulation significantly decreased upon maleimide binding
- rapid activity loss of functionalized heparins in whole blood, esp. for LMWH → reason for decreased anti-coagulant effect?

next steps

- quenching of free maleimide groups to evaluate their effect on activity loss
- surface immobilization of functionalized heparin molecules

Funding & References

Funding

Federal Ministry of Education and Research, Project Materialinnovationen für gesundes Leben: ProMatLeben – Polymere: *Low Thrombogenic Blood Circuit*

References

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Standards Institute guidelines using residual anonymized samples.

Findings: Trueness was evaluated by normal and pathological Siemens controls and values were well within the recommended acceptable ranges. Within run precision provided a CV of 0.4% in the normal and 0.5% in the pathological sample pool and day to day precisions were 0.8% and 0.6% respectively for vWF antigen measurements and similar results were obtained for the vWF activity values. The limit of detection was 0.7% for vWF antigen and 4% for vWF activity. The limit of quantitation for both assays were 5% and a single calibration provided vWF results in the range 5-160%. The Deming regression analyses carried out on >120 samples showed good agreement between vWF antigen assay on the Cobas and CS2500 systems (Pearson's r = 0.996) and similar results were obtained with vWF activity (Pearson's r = 0.998). On Bland-Altman plots a systemic deviation could be observed in the lower ranges (below 60% in case of antigen and 40% in case of of activity) where higher values were obtained by the Cobas t511 when compared to the Sysmex CS2500 analyser by using the same reagents. The differences in the lower concentration range may be explained by the difference in calibration procedures.

Conclusion: The CDC is an outstanding possibility to apply 3rd party reagenst ono the Cobas coagulation analyser. Based on the excellent analytical performance and good agreement with relevant comparator method, the method is useful for routine diagnostic use in core laboratories.

P10-6. Activity of Maleimide Functionalized Unfractionated and Low Molecular Weight Heparins

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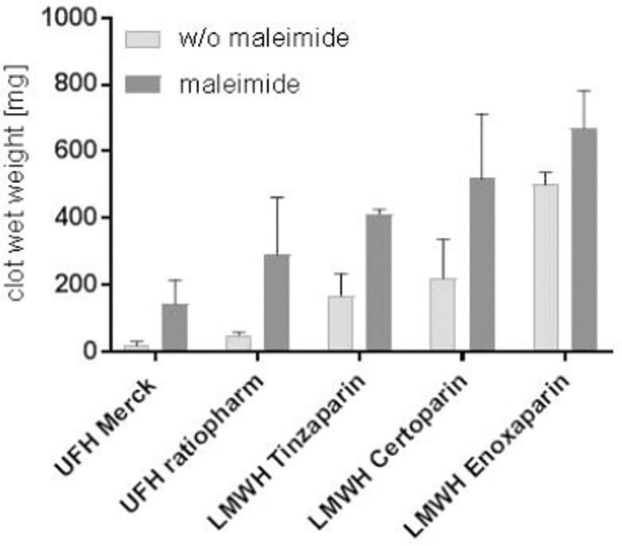
The development of modern anti-coagulant high performance surface coatings can require the immobilization of heparin in a modular orthogonal fashion. The precision of current commercial technologies as the Carmeda® BioActive Surface coating for this application is limited. Here we present the use of click chemistry as a potential approach for the immobilization of maleimide functionalized heparin molecules to thiol decorated surfaces. Thereby the influence of maleimide functionalization on the anti-coagulant activity was investigated and compared for different heparins of varying molecular size.

Three different LMWH (enoxaparin, certoparin and tinzaparin) as well as two UFH (Merck, ratiopharm) were functionalized with N-(2-aminoethyl)maleimide TFA to a final degree of one maleimide group/heparin molecule. Functionalization degree was analyzed by HPSEC and NMR analysis. To determine reactivity of the maleimide moieties binding of TNB^{2-} as well as an RGDSP peptide was measured by substrate consumption. Inhibitory activities of the unmodified and functionalized heparin molecules for FIIa and FXa were measured as chromogenic assay in a buffer system. Besides heparins were incubated with whole blood together with coagulation stimulating or non-stimulating surfaces and analyzed for their inhibitory potential and hemocompatible properties.

For all five tested heparin molecules, a functionalization degree of one reactive maleimide per heparin molecule was achieved. The dependence of anti-FIIa activity on the heparin molecule size was confirmed, but anti-FXa and anti-FIIa activity were not affected by the maleimide functionalization. Upon exposure of blood heparinized with comparable anti-FXa units under coagulant or non-coagulant conditions, a heparin size dependent clot formation was detected. Maleimide functionalized compounds did not induce increased cell activation or inflammatory response. However, the maleimide moiety triggered an immediate loss of heparin activity upon its addition to whole blood and an increased clot formation.



Heparin molecule size dependent clot formation of heparinized whole blood under coagulant conditions

The functionalization of heparins with a reactive maleimide moiety offers a potential strategy for surface immobilization of both UFH and LMWH. The approach does not affect the heparins potential to inhibit the coagulation factors FIIa and FXa, but an immediate loss of activity was detected for the functionalized compounds in whole blood.

P10-7. New Normal Ranges Measuring APC Resistance on a New Coagulation Analyzer (COAG360)

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Background: The well-established activated protein C resistance (APC-R) clotting assay, Pefakit APC-R Factor V Leiden (DSM Nutritional Products AG, Branch Pentapharm, Basel, Switzerland), is used to detect an increased resistance of factor V against inactivation by the presence of activated protein C. This resistance is caused by the presence of functional active factor V mutations (e.g factor V Leiden, factor V Cambridge, i.a.). So far control material may give APC-R results between 3.6 and 6.8 for control samples without active factor V polymorphisms (wild-type or non-mutated). This assay fitted to a new generation of coagulation analyzers needs re-evaluation of the expected results of the commercially provided control material (i.e. new reference ranges for the normal control material).

Material and Methods: Heterozygous and wild-type samples for the factor V Leiden (F5 R506Q) polymorphism were tested with Pefakit APC-R Factor V Leiden (DSM Nutritional Products AG, Branch Pentapharm, Basel, Switzerland) on 40 consecutive on two Atellica COAG 360 Systems (Siemens Healthineers, Marburg, Germany).