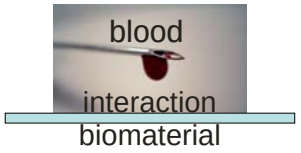
Hemocompatibility Assessment

Determination of the interaction of blood with biomaterial surfaces



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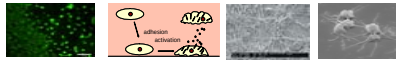
Outline

- **Topics / goals of our work**
- **Interaction of materials and blood – overview of reactions (short repetition)**
- **Experimental set-up of contact between material + blood: Incubation tools**
- **Parameters to identify / determine interaction of material and blood**

Topics of our group



Blood activation processes

Hemocompatible coatings

Hemocompatible Interfaces

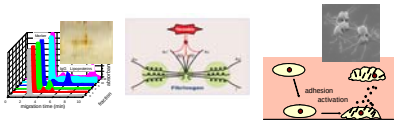
Standardized and reliable testing methods

Blood activation processes

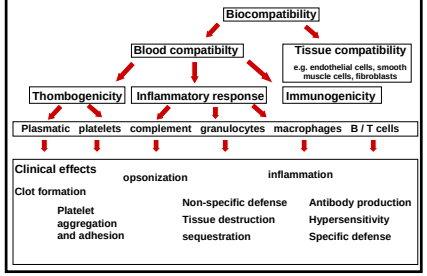
- Surface functionalities impact - protein adsorption
- Adsorbed proteins impact - activation of coagulation - cell adhesion / activation
- initiation of blood coagulation: - contact activation ? - tissue factor ?

time

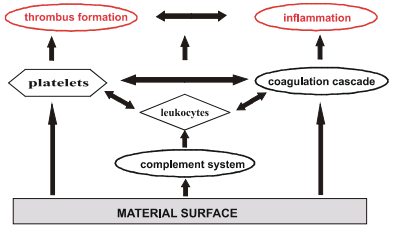
proteins enzymes cells



Hemocompatibility assessment



Interactions in blood activation



Interactions in blood activation

Use of platelet rich plasma and of washed platelets enables research on platelet interactions

Use of plasma and of purified proteins enables research on coagulation enzyme reactions

Use of isolated cells enables research on cell – surface interactions

Only whole blood with minimized anticoagulation enables us to understand the complex interactions happening after blood – material contact

Blood incubation – blood collection

Fresh venous human blood

Blood from blood bags (blood bank)

Animal blood

Blood treatment: anticoagulation / stabilization

Blood incubation – basic requirements

- **First question:**
  - **Relevance of test system for specific materials**
  - **Relevance of parameters for material use**
- **Fresh human blood**
- **No blood – air contact during incubation**
- **Maximal contact between test surface and blood**
- **Minimal contact between other (foreign) surfaces and blood**
- **Construction materials have to be hemocompatible**
- **Shear stress / blood flow / prevention of cell sedimentation**
- **Versatility for different sample geometries**
- **Sterilizability of construction materials**
- **Relevant control materials (internal reference)**
- **Incubation at 37°C**
- **Duration of incubation: in accordance to designated use, blood treatment**

Blood incubation models - static

Static blood incubation

- first experiments by W. Hewson 1864
- relatively simple
- high amount of samples possible
- almost no other materials / influences
- negative: no physiologic blood flow
- Examples:
  - incubation in open systems
  - incubation in tubes
  - incubation systems with parallel plates


Isolated vein


bowl

Hewsons results:

Blood stays liquid for a longer time if placed inside an isolated vein than if placed inside a bowl ⟶ surrounding of blood influences reactions inside of blood volumen

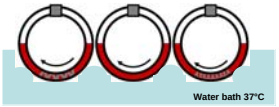
Blood incubation models - static

Static blood incubation – open systems

- very simple
- sedimentation of cells
- strong air contact
- surface / blood contact not well defined

Blood incubation models - static

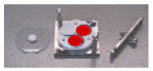
Chandler loop (developed 1958 by A.B. Chandler)



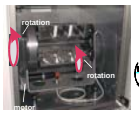
- Tube is coated or small materials like stents are inserted
- Blood fill in with air bubble to allow blood „mixture" – air contact!
- No foreign materials, good relation of material to blood volume
- Only possible for coatable materials
- New development: no air but movement realized using pump

Blood incubation models - static

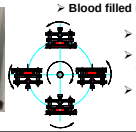
Special incubation chamber with defined characteristics



- ease of handling, many samples possible
- No air contact, good relation of sample surface and blood volume
- spacer material: PTFE
- Blood filled in directly from syringe



Epicyclic gear

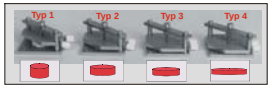


- Appr. 21 parallel samples
- After incubation blood taken out with pipettes
- No directed blood flow only rotation to prevent cell sedimentation

Blood incubation models - static

Opimisation of chamber geometry concerning samples surface size

Blood volume: similar in all chambers



Type of chamber	1	2	3	4
sample [cm²]	8,6	17,7	28,2	39,9
sample [cm²] / wall [cm²]	1	3	6	10
sample [cm²] / blood [ml]	1,8	3,6	5,7	8

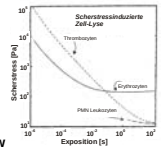
Blood incubation with shear forces

Rheologic considerations for blood incubation

Shear force influences cell-surface interactions

Cell lysis should be avoided

Laminar and well defined flow conditions desirable

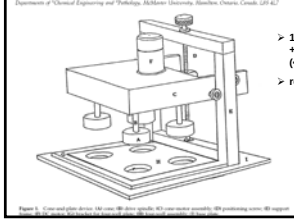


Physiologic conditions of blood flow

	Diameter [mm]	volume / s [ml/s]	velocity
aorta	23	364	876
artery	1	0.034	44
vein	2	0.066	21
capillary	$6*10^{-3}$	$7.5*10^{-9}$	0.27

Model: not possible to picture all physiologic conditions

Blood incubation models – shear forces

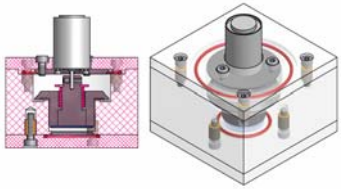

A cone-and-plate device for the investigation of platelet biomaterial interactions
Journal of Biomedical Materials Research, Vol. 34, 427-438 (1997)

- 1 plate with test material + cone in small distance (< 1 mm)
- rotation of cone

Hemocompatibility Assessment

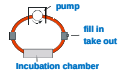
Plate – plate incubation system

- 2 plates with test material in small distance (< 1 mm)
- rotation of top plate

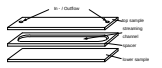


Blood incubation models

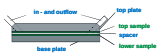
Perfusion chamber



- Streaming channel with well defined geometry enables laminar flow and adjustable shear force (variation of channel height)
- Tubes made from hemocompatible silicon
- (only) 8 parallel chambers possible



- Slight hemolysis (destruction of red blood cells) even with lowest pumping rate
- Building / foreign materials relatively big surface

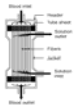


Blood incubation models

Bead columns

- High material surface possible
- Possible incubation for coatable substances
- Flow conditions not well defined, turbulent and laminar conditions possible, no shear force can be defined
- Pumping and tubing needs to be considered – big surface with other materials

For dialysis materials: mini-modules



- Good correlation with end-use-product
- High contacting surface
- Pumping and tubing necessary

Hemocompatibility Assessment

Blood donation

1 Anticoagulation of blood

Analysis of blood before incubation

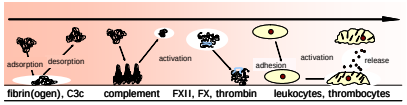
Blood into chambers

2 Incubation at 37 °C

3 Analysis of blood and materials surface

Parameters for hemocompatibility

What do we want to know?

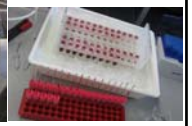


fibrin(ogen), C3c	complement	FXII, FX, thrombin	leukocytes, thrombocytes
→ released or generated proteins free in plasma (ELISA) → Adsorbed (to the surface) proteins (immunologic detection)	→ enzymatic activity (chromogenic or fluorogenic assay)		→ cell numbers in blood (Coulter counter) → cell activity (flow cytometry + release of proteins) → cell adhesion (SEM, fluorescence microscopy)
changes of proteins	enzyme activation		cell activation

Analysis of blood and surface

After the incubation: blood is taken from the incubation set-up

Blood and surface will be analyzed

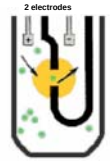


Determination of blood cell numbers using Coulter Counter AcTdiff.

Measuring principle: Counts and measures cells

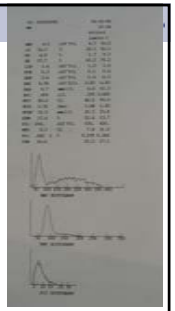
Detects change in electrical conductance (impedance)

Photometric measurement for hemoglobin after cell lysis



2 chambers filled with conductive saline fluid, separated by a small orifice

Blood analysis – Cell counting



Parameters:

Leukocyte numbers (lymphocytes,

monocytes, granulocytes)

Platelet numbers

Average cell sizes

Red blood cells

Hemoglobin

And more……

Blood analysis - ELISA

Parameter determined using ELISA assays (our experimental set-up)

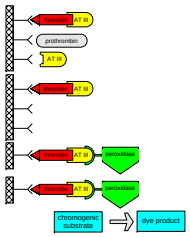
Coagulation activation:	**TAT**
Complement activation:	**C5a, C4d, C5b-9**
Platelet activation:	**PF4, β-TG**
Leukocyte activation:	**Elastase, TNF**

All of them are: proteins or peptides in small quantity to be found in a very complex media

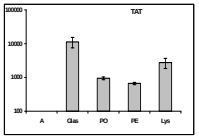
Blood analysis – ELISA

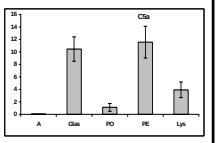
ELISA: enzyme linked immuno sorbent assay

- Antibodies immobilized to surface of microtiterplate well
- Plasma contacts microplate, analyte is bound to antibody
- Rinsing removes non bound substances
- Second antibody is enzyme linked
- Amount of day product reflects surface concentration of bound analyte



Blood analysis - ELISA

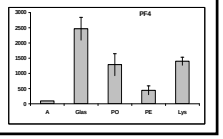




Coagulation activation: **TAT**

Complement activation: **C5a**

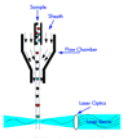
Platelet activation: **PF4**



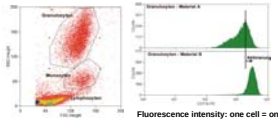
Blood analysis – FACS

Flow cytometry also called FACS: fluorescence assisted cell sorting

- hydrodynamic focussing
- Size and granulation of cells lead to differences concerning light refrection
- Fluorescence used to differentiate cell characteristics



http://biology.berkeley.edu/crl/flow_cytometry_basic.html



Fluorescence intensity: one cell = one count

Blood analysis – enzyme activity

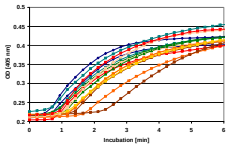
Enzyme activity after blood contact can be tested:

- **Using specific chromogenic or fluorogenic substrates**
- **Using naturals substrates like e.g. fibrinogen for thrombin activity**

thrombin + fibrinogen

fibrin mesh

Optical measurement of fibrin formation



Surface analysis – protein adsorption

Determination of adsorbed proteins using specific antibodies

Dry / frozen surface contacts buffer with specific antibodies

Example: complement fragment C3c or fibrinogen/fibrin

Use of (second) conjugated antibody

Enzymatic (peroxidase)

Surface analysis - SEM

SEM: Scanning electron microscopy

Sample surface rinsing

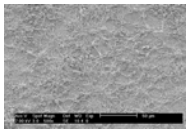
Surface fixation using glutaraldehyde (stabilization of structures)

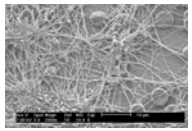
Dehydration using alcohol

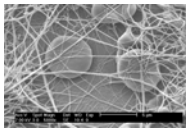
Optional: critical point drying (samples are analyzed in vacuum)

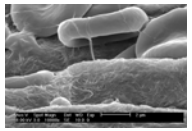
Sputter coating with gold (samples need to be conductive)

Surface analysis - SEM



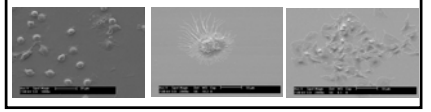




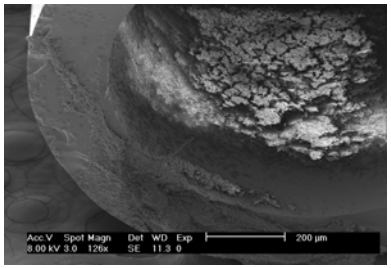


Surface analysis - SEM

Stages of platelet adhesion / activation

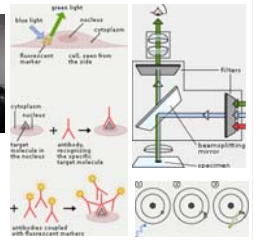


Surface analysis - SEM

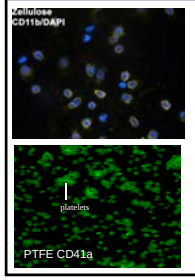


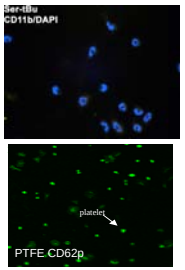
Fluorescence microscopy

Determination of surface cell density AND state of activity possible



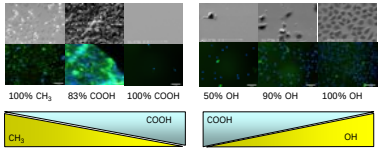
Surface analysis – Fluorescence microscopy





Self assembled monolayers (SAM) as model surfaces to study initial processes of blood coagulation

- alkyl thiols on gold with various functional end groups
- excellent model to study surface chemistry related blood reactions



From research to product

In vitro results from experiments ⟶ promising products

Enhanced *in vitro* experiments in accordance with legal requirements regulated in the EU / Germany through harmonised standards and laws (Medizinproduktegesetz)

ISO 10993, coordination of chosen tests with notified body

Possibly animal experiment

In vivo studies

Release of certified new product

From research to product

ISO 10993 Biological evaluation of medical devices

ISO 10993-1:2003 Part 1: Evaluation and testing
ISO 10993-2:2006 Part 2: Animal welfare requirements
ISO 10993-3:2003 Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity
ISO 10993-4:2002/Amd 1:2006 Part 4: Selection of tests for interactions with blood
ISO 10993-5:1999 Part 5: Tests for in vitro cytotoxicity
ISO 10993-6:2007 Part 6: Tests for local effects after implantation
ISO 10993-7:1995 Part 7: Ethylene oxide sterilization residuals
ISO 10993-9:1999 Part 9: Framework for identification and quantification of potential degradation products
ISO 10993-10:2002/Amd 1:2006 Part 10: Tests for irritation and delayed-type hypersensitivity
ISO 10993-11:2006 Part 11: Tests for systemic toxicity
ISO 10993-12:2007 Part 12: Sample preparation and reference materials (available in English only)
ISO 10993-13:1998 Part 13: Identification and quantification of degradation products from polymeric medical devices
ISO 10993-14:2001 Part 14: Identification and quantification of degradation products from ceramics
ISO 10993-15:2000 Part 15: Identification and quantification of degradation products from metals and alloys
ISO 10993-16:1997 Part 16: Toxicokinetic study design for degradation products and leachables
ISO 10993-17:2002 Part 17: Establishment of allowable limits for leachable substances
ISO 10993-18:2005 Part 18: Chemical characterization of materials
ISO/TS 10993-19:2006 Part 19: Physico-chemical, morphological and topographical characterization of materials
ISO/TS 10993-20:2006 Part 20: Principles and methods for immunotoxicology testing of medical devices

Take home message

Interaction between material and blood is governed by cellular and humoral reactions that may lead to coagulation and inflammation.

Development of enhanced materials with better hemo-compatibility needs sensitive testing with relevant blood incubation and adapted selection of parameters.

Several basic requirements should be respected for planning a blood incubation set-up. The final application should be kept in mind.