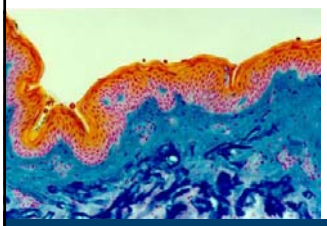
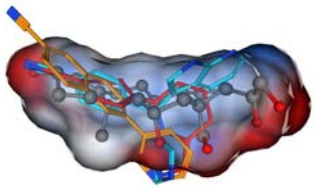
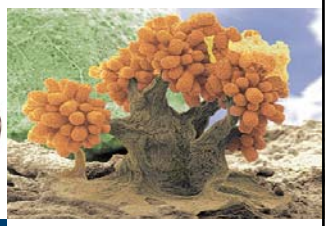
# Inhalation Nanomedicines – Opportunities and Challenges

**“Les traitments nebulisees en Pneumologie”**
**Geneve, 13. January 2010**

Prof. Dr. Claus-Michael Lehr

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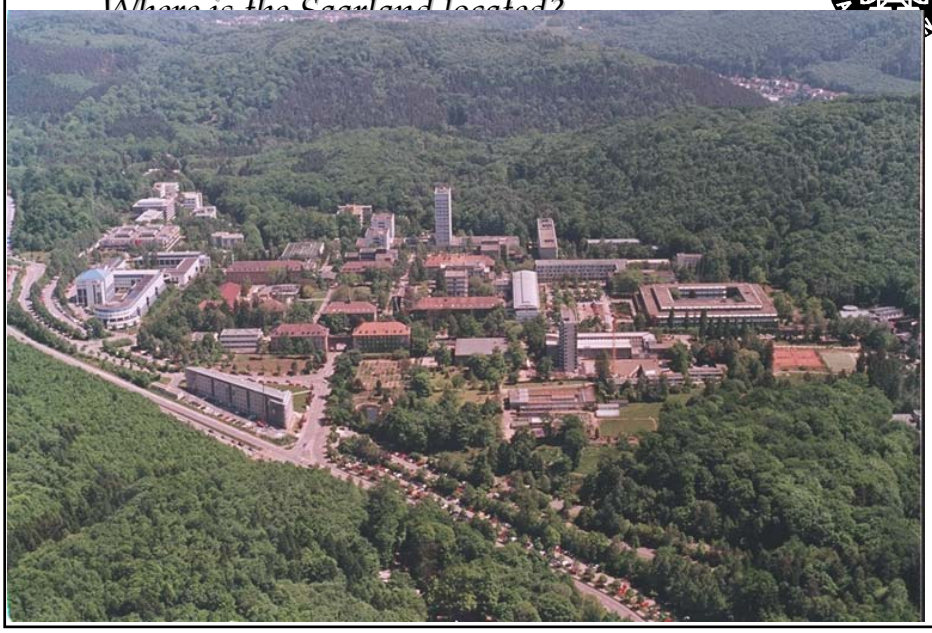
HELMHOLTZ ZENTRUM FÜR INFEKTIONSFORSCHUNG

UNIVERSITÄT DES SAARLANDES

*Where is the Saarland located?*



**"Drug Delivery Research"**

## HIPS Dept. of Drug Delivery (DDEL)

### Central Research Theme:

### „*Biological Barriers*"

Cell & tissue based in-vitro models

- intestinal mucosa
- skin
- lung

Drug delivery technologies

=> Explore the potential of (nano-sized) carriers to OVERCOME these barriers

## *Inhalation Nanopharmaceuticals – What are potential advantages?*

First Thought:

Improved lung deposition of nanosized aerosols (10-20nm) compared to „classical" pharmaceutical aerosols (2-5µm)

BUT: - Making drug/carrier particles *that* small in not easy…
- Still need to deliver a significant dose…
means (too) many particles!

**=> Probably of limited potential**

## *Inhalation Nanopharmaceuticals – What are potential advantages?*

Second Thought:
Controlling the disposition of drugs/particles AFTER deposition:

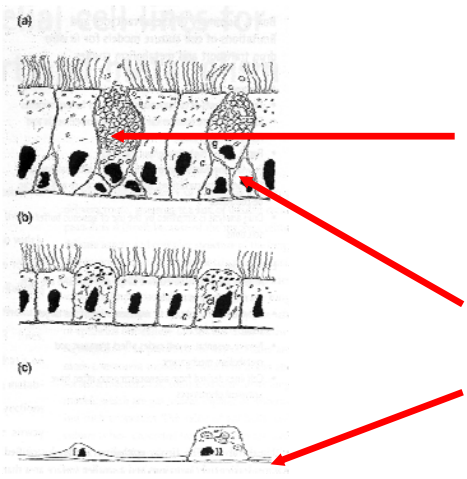
- Improve ABSORPTION across the „air-blood-barrier" (e.g. to deliver macromolecular biopharmaceuticals)
- Control/avoid pulmonary CLEARANCE (e.g. to create a platform for inhalable controled release systems)
- Cell-specific TARGETING ( e.g. for vaccines, gene therapy, anti-cancer drugs)

**= > appears as the major potential**

*Outline*

- Deposition of (nano) particles on mucosal epithelial cells and their effects on drug absorption
- Pulmonary clearance of (Nano-)particles
  - mucociliary clearance
  - macrophage clearance
- Cellular delivery of telomerase inhibitors by polymeric nanocarriers for the treatment of lung cancer

## *Epithelia - The place of landing*



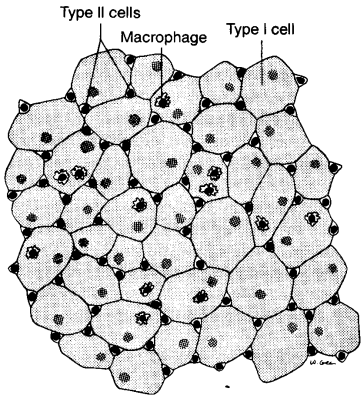

Examples for available *in vitro* models:

Calu-3 (human cancer cell line)
Foster *et al.* 2000 *Int J Pharm*
Florea *et al.* 2003 *J Control Rel*

16HBE14o- (human immortalised cell line)
Ehrhardt *et al.* 2002 *Cell Tissue Res*
Ehrhardt *et al.* 2003 *Pharm Res*

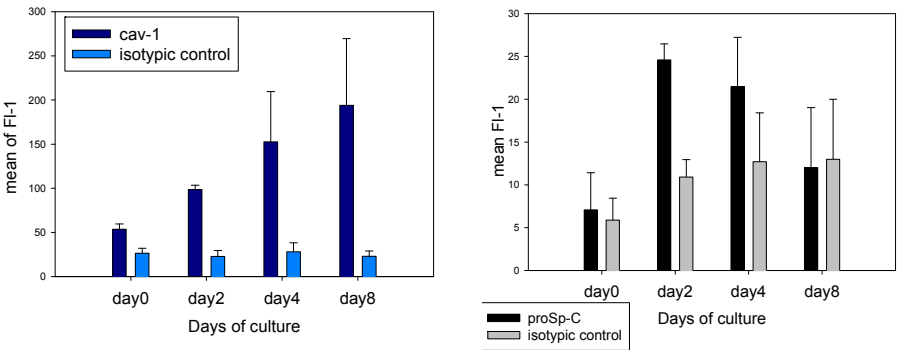
HAEpC (human cells in primary culture)
Elbert *et al.* 1999 *Pharm Res*
Fuchs *et al.* 2003 *Cell Tissue Res*

*The alveolar epithelium:*
*Cellular morphology of a single alveolus*

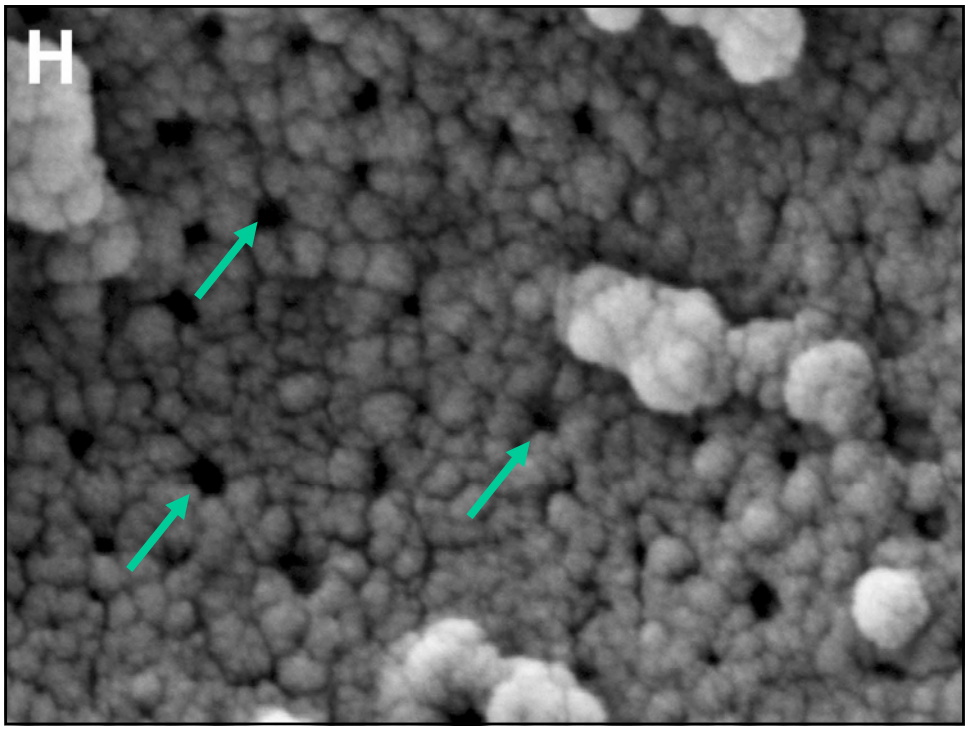



J.S. Patton, Advanced Drug Delivery Reviews 19 (1996) 3-36.

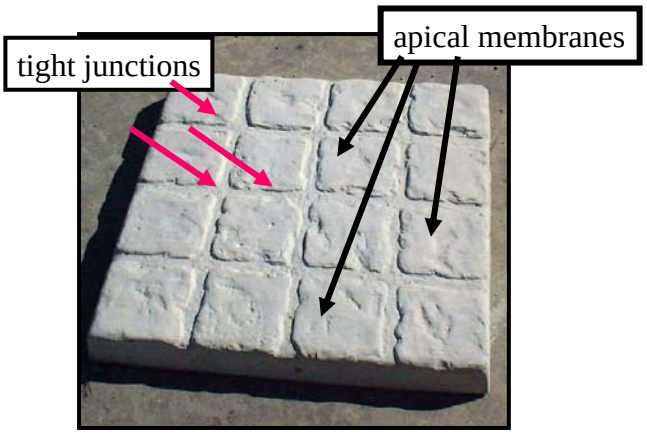
**Expression of Cav-1 and Sp-C in time course (FACS)**




Fuchs et al., Cell & Tissue Res., 311 (2003), 31-45




## NEEDED: Barrier Properties!

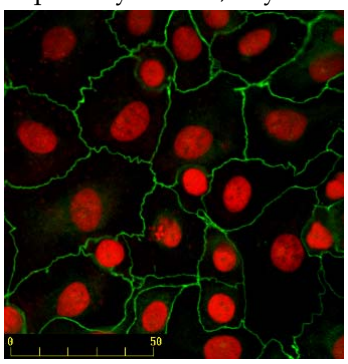



A functional epithelium is more than just an assembly of cells!

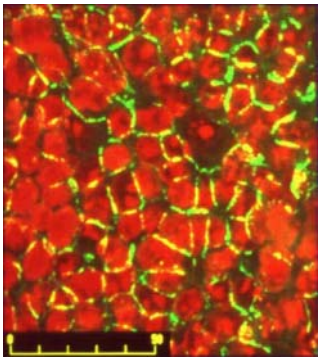
# Human lung epithelial cell culture systems - Development of tight junctions:

Visualization of tight junctional protein ZO-1 by specific antibody, nuclei counterstained by propidium iodide, observed by CLSM

human alveolar epithelial cells in primary culture, day 8

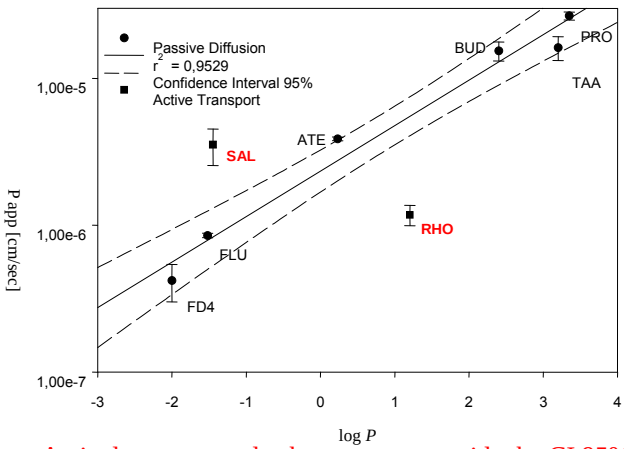


A549 cell line, day 8



Elbert et al, Pharm. Res. 16 (5) (1999) 601-608.

# Active vs. Passive Transport




Apical to basolateral absorption of

Salbutamol (SAL)
FITC dextran 4k (FD4)
Fluorescein-Na (FLU)
Atenolol (ATE)
Rhodamine 123 (RHO)
Budesonide (BUD)
Triamcinolon-acetonide (TAA)
Propranolol (PRO)

across 16HBE14o- bronchial epithelial cell layers

Actively transported substances are outside the CI 95% Ehrhardt et al, 2003

*What needs to be considered when studing the interaction of aerosolized (nano)PARTICLES*

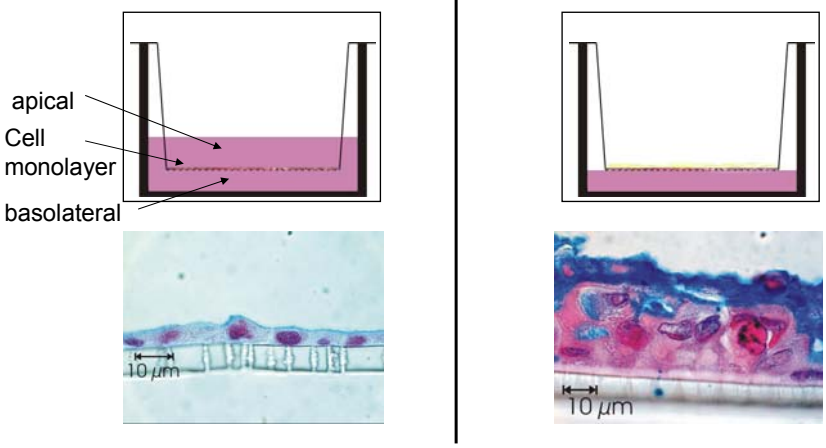
*- rather than SOLUTES –*

*with pulmonary epithelial barriers ?*

*KNOWN:*
*Effect of Cell Culture conditions:*
*Liquid versus Air interface culture*

Liquid interface culture

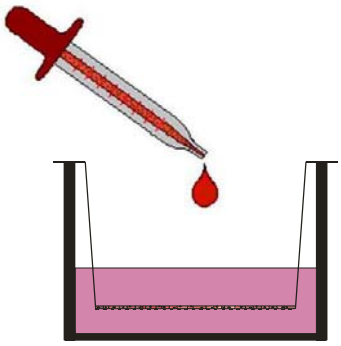
Air interface culture



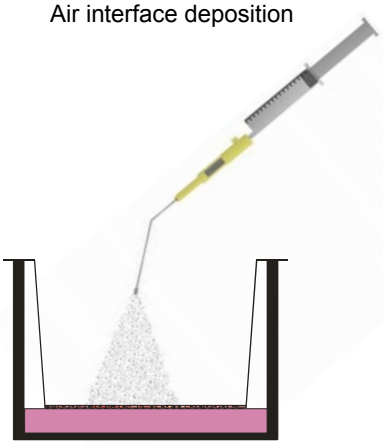

→ Significant consequences for cellular differentiation and proliferaftion!

*NOT SO MUCH KNOWN:*
*Effects of deposition conditions:*
*Liquid versus Air interface deposition*

Liquid interface deposition

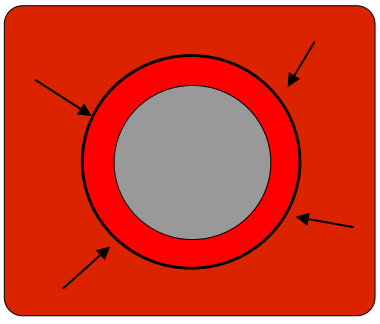


Air interface deposition



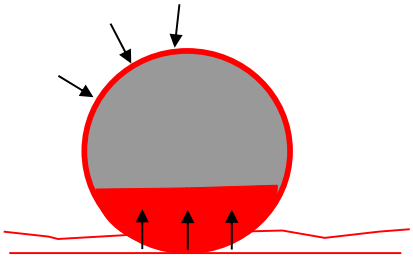
→ Significant consequences for transport kinetics!

# Particle-Water Interactions



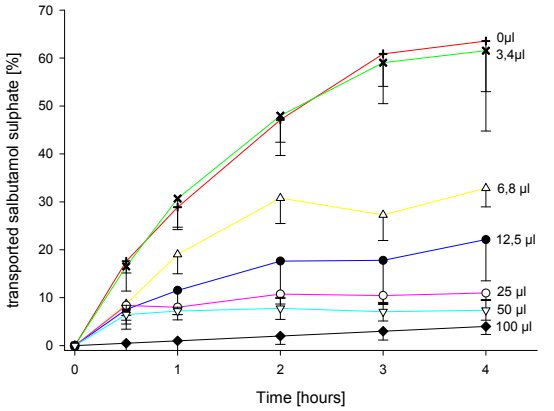
water uptake from bulk fluid

Absorption of water from humidified environment

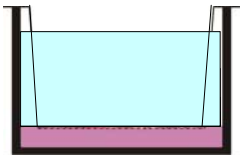


water uptake from thin fluid layer covering lung epithelial cells

## *The volume of the liquid donor phase influences drug transport across AIC cell monolayers*




Transport of salbutamol sulfate (Easyhaler®) across hAEpC cell monolayers; (data present mean ± standard deviation; n = 4)



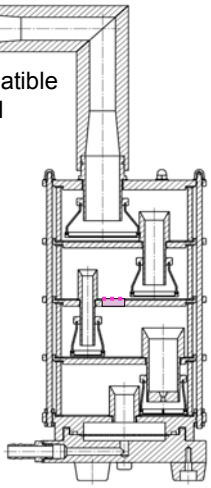
→ Concentration gradient controls absorption rate!

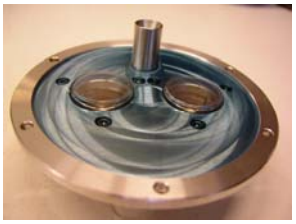
## How to reproducibly deposit aerosol particles on cell monolayers?

PennCentury DP4 Insufflator

Aerosol deposition without size classification

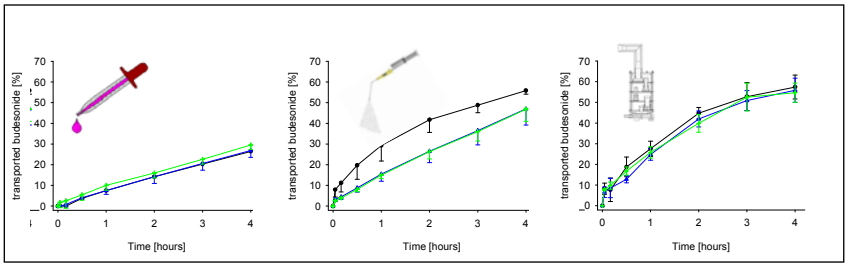
Cell compatible MSLI





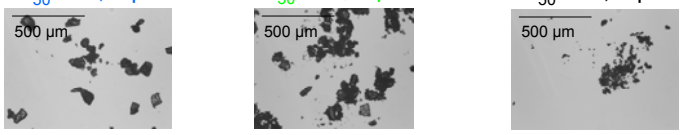
Aerosol deposition with size classification

# *Influence of particle size - budesonide*




Transport of budesonide (Δ Cyclocaps®; • Autoinhaler®; ♦ Easyhaler® in all cases applied 30 µM) across air interface cultivated hAEpC.

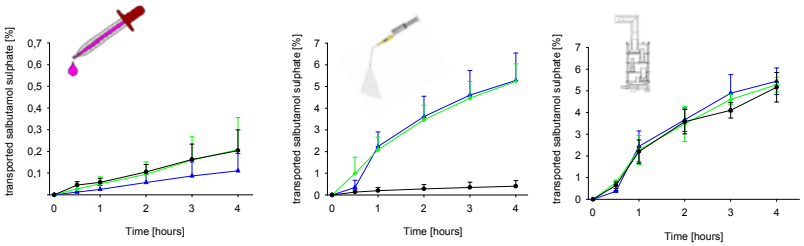
| Cyclocaps | Easyhaler | Autoinhaler |
|---|---|---|
| $x_{50}$= 55,64µm | $x_{50}$= 49,56µm | $x_{50}$= 18,00µm |




→ Small soluble particles yield higher absorption rates

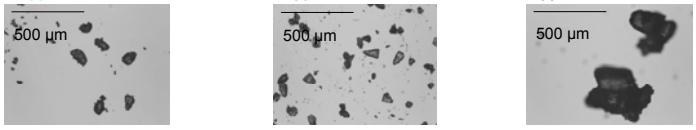
Bur et al., in press

# *Influence of particle size - salbutamol*




Transport of salbutamol sulphate (Δ Cyclocaps®; • Ventilastin® Novolizer®; ♦ Easyhaler®, in all cases 1000 µM donor concentration) across air interface cultivated hAEpC monolayers.

| Cyclocaps | Easyhaler | Novolizer |
|---|---|---|
| $x_{50}$= 45,15µm | $x_{50}$= 58,33µm | $x_{50}$= 217,58µm |




→ large soluble particles yields lower absorption rates

Bur et al., in press

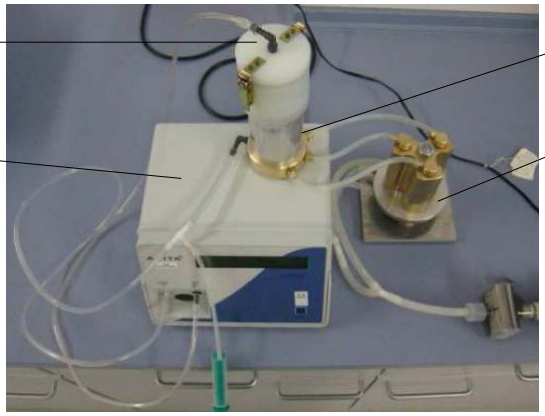
BfR
Risiken erkennen – Gesundheit schützen

*"A new **P**harmaceutical **A**erosol **D**eposition **D**evice **o**n **C**ell **C**ultures (PADDOCC) as alternative method for biocompatibility and ADME screening"*

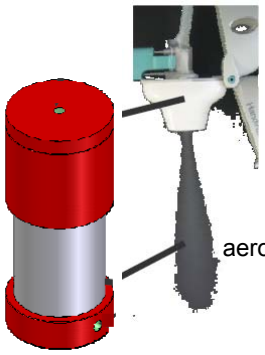
*in collaboration with*

ACTIVAERO

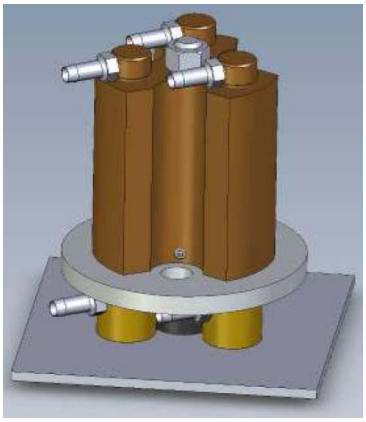
## *Experimental setup*



## *Main components*

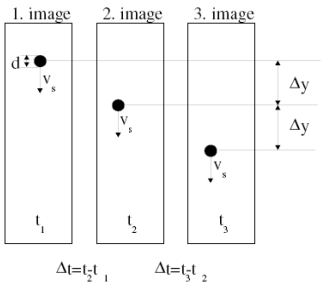



Aerosol generation



Aerosol deposition

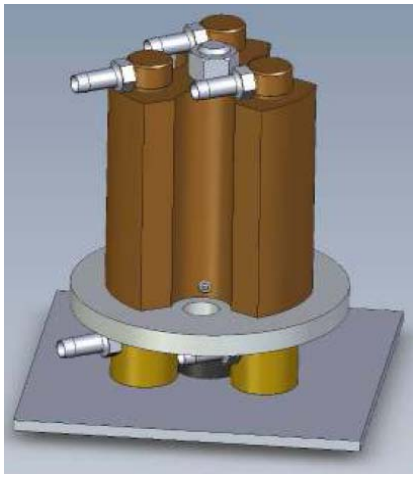
## *Principle of sedimentation chamber*



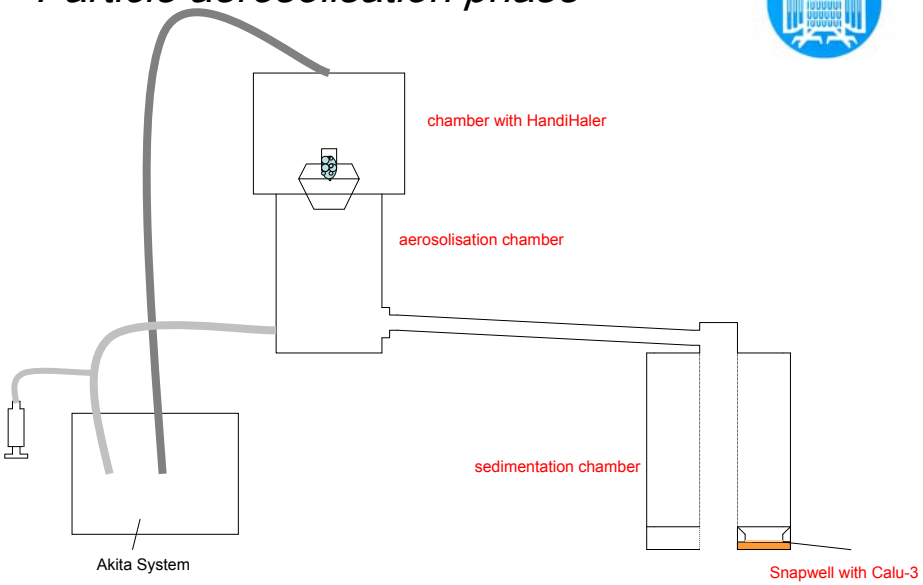

$$v_s = \frac{\Delta y}{\Delta t}$$

$$d_{ae} = \sqrt{\frac{18\eta_L v_s}{g\, C_C}}$$

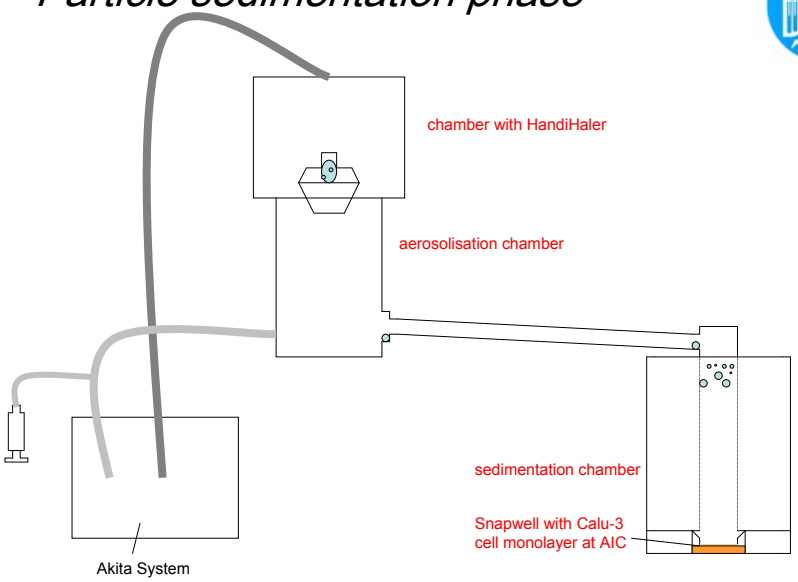
$v_s$: settling velocity
$d_{ae}$: aerodynamic diameter of particle
$\eta_L$: dynamic viscosity of air
g: gravitational constant
$C_C$: slip correction factor (Cunningham)



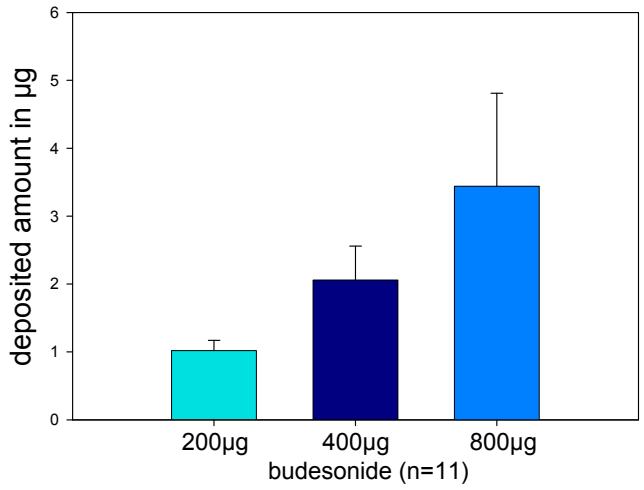
# Particle aerosolisation phase



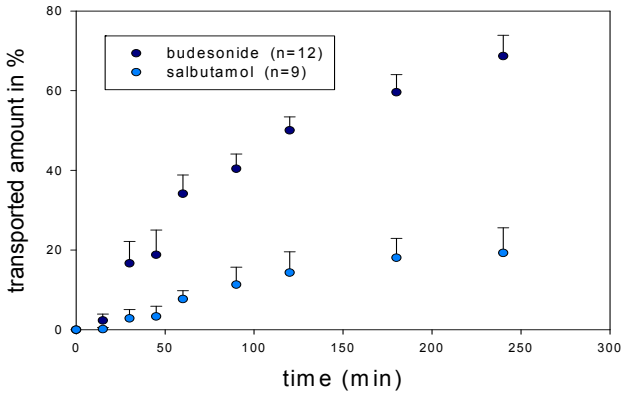

# Particle sedimentation phase



## Reproducible deposition
### same drug - different concentrations




## First transport experiments




air interface deposition in PADDOCC and subsequent air interface transport

*Outline*

- Deposition of (nano) particles on mucosal epithelial cells and their effects on drug absorption
- Pulmonary clearance of (Nano-)Particles
  - mucociliary clearance
  - macrophage clearance
- Cellular delivery of telomerase inhibitors by polymeric nanocarriers for the treatment of lung cancer

# NanoInhale

Bundesministerium für Bildung und Forschung

„*Nanotechnology based formulations for sustained-release pulmonary drug delivery*“

Boehringer Ingelheim

UNIVERSITÄT DES SAARLANDES

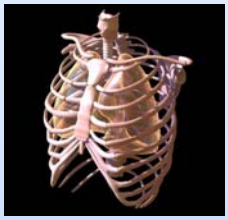
DWI an der RWTH Aachen e.V.

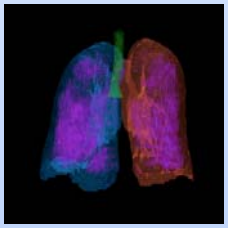
Philipps Universität Marburg

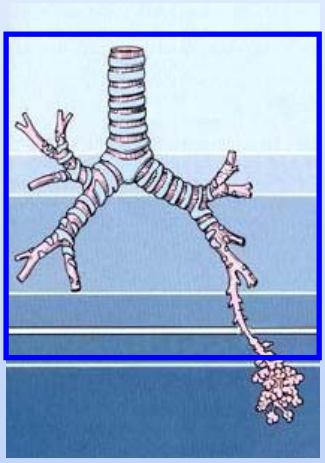
ACTIVAERO

J.V. Liebig Univ. Giessen

## Mechanisms of particle clearance from the lungs



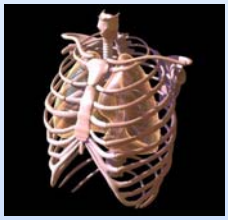


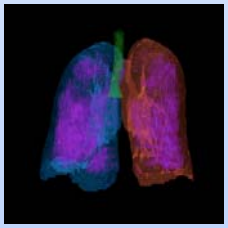


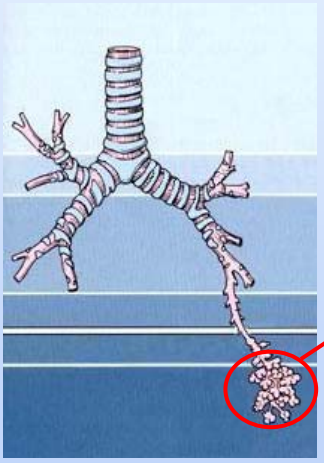
**Airways:**

**Particles cleared by Mucociliary Clearance**

## Mechanisms of particle clearance from the lungs







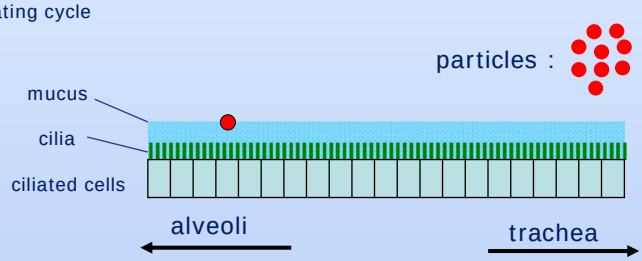
**Peripheral lung:**

**particles cleared by *macrophages***

# Mucociliary Clearance

**Goal:** Removal of particulate matter from the airways by coordinated transport of mucus

1. metachronal: propagation of the wave in t and x
2. 200-300 cilia per ciliated cell (*Reinhardt*, 2001)
3. beating-frequence 10-20 Hz (*Iravani* et al., 1975)
4. complex beating cycle



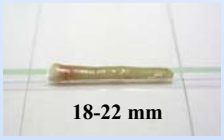




# ECT – Embryonic Chicken Trachea

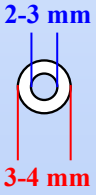
**Controlled breeding**

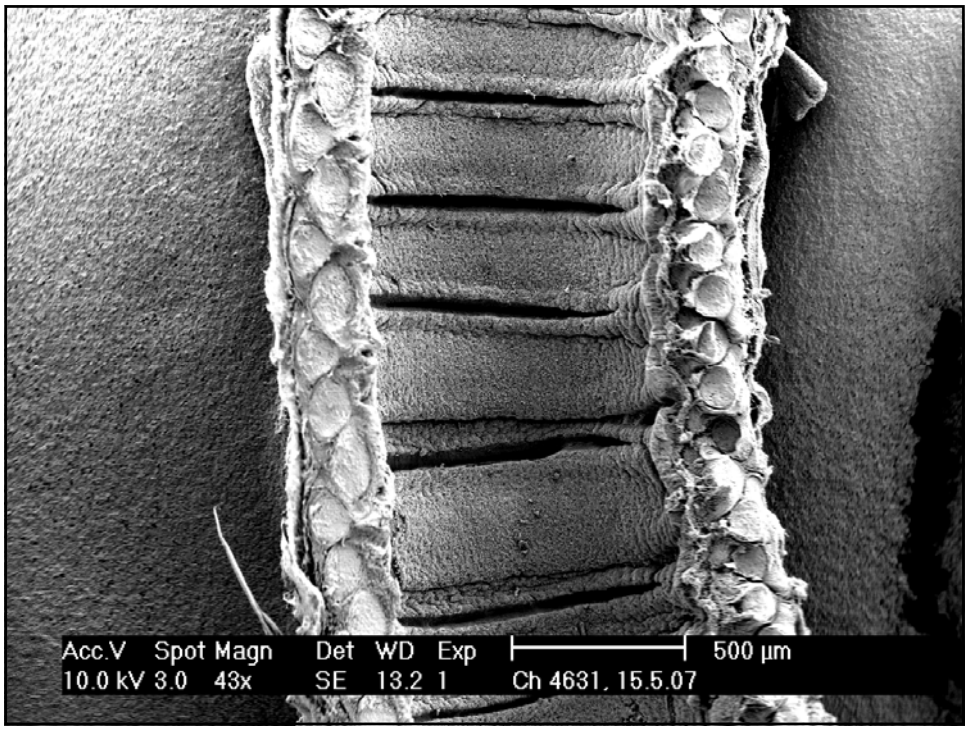
**Trachea isolation**



**Particle deposition**

**Oblong cut**



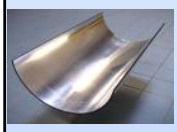


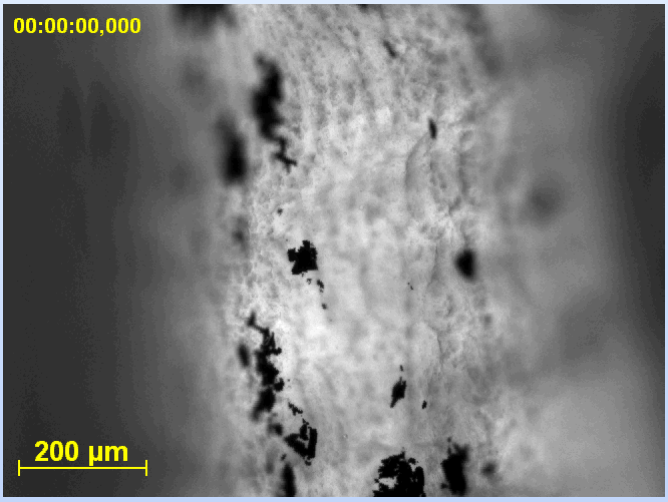

## Ex-vivo experiments
## Embryonic Chicken Trachea

***Ludin* chamber**
temperature (33° C) & humidity (99%) controllable

**particle tracking videos**
transmission light & fluorescence light

## Clearance Velocity





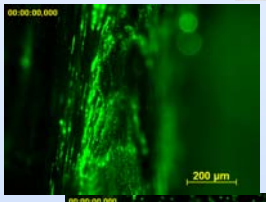

**Carbon particles, ~ 5-10µm**

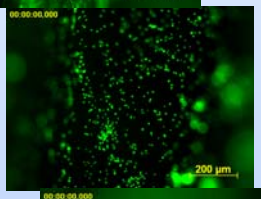
## Is the particle size influencing clearance rate ?

**Polystyrene particles** / FluoresBrite™

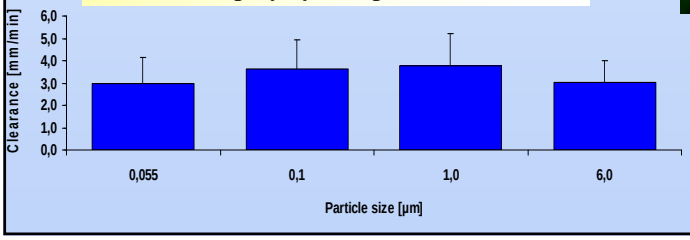
→ **Fluorescently labeled** (YG - ex/em 441/486)

→ **Size: 6 µm / 1 µm / 0.1 µm / 0.05 µm**

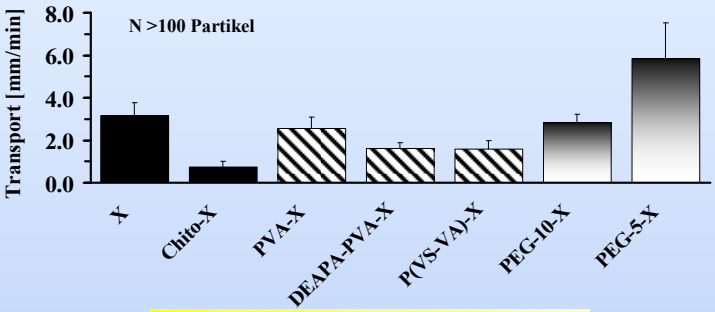



**No significant influence of particle size for polystyrene particles!**



## Effect of Polymer-Material

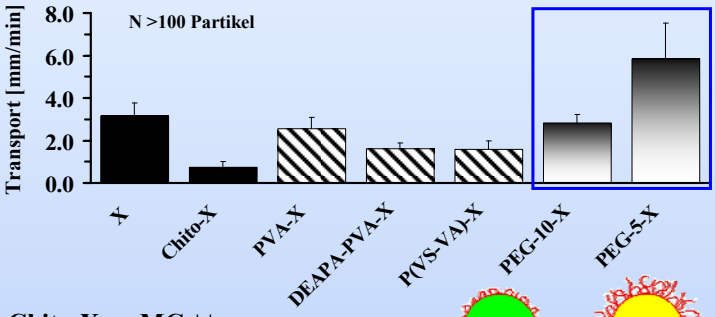



→ Particlesize: 83 nm < d < 389 nm

→ Zeta-Potential: -42 mV < ζ < 47 mV

**Signifcant differences in Clearance rates, but NOT due to differences in size or shape!**

## Effect of Polymer-Material

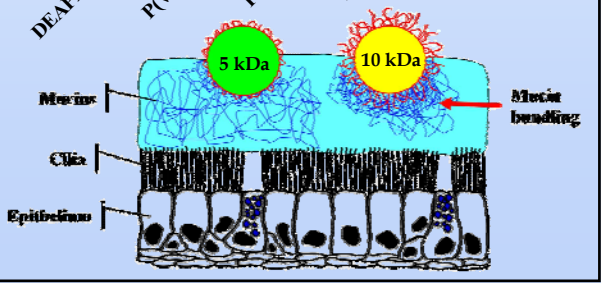



1) Chito-X → MC ↓↓

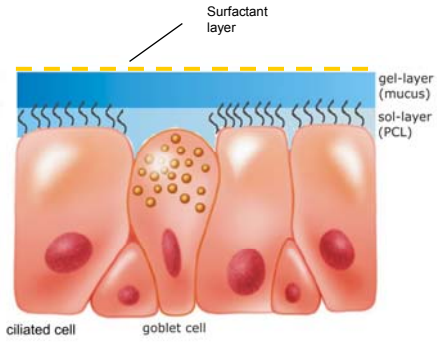
2) DEAPA → MC ↓

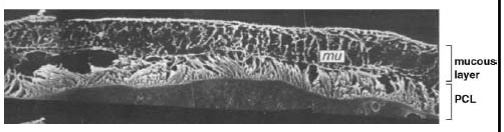
3) VS → MC ↓

4) PEG-5kDa → MC ↑↑



# Epithelium and Mucus




*Sanderson* et al., 1981

**two layers:**

„**gel**"-**layer**: viskoelastic, non-newtonian gel, usually „mucus"

and

„**sol**"-**layer** (*Periciliary Layer, PCL*): watery, newtonian liquid



# Mucociliary Clearance Embryonic Chicken Trachea

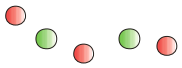
**interpretation of results:**

**hypothesis 1** („passive", inert particles)

nanoparticles with different $v_{clearance}$ if applied alone

=slowly cleared

=quickly cleared



flow-velocity

# Mucociliary Clearance
# Embryonic Chicken Trachea

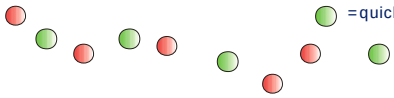
**interpretation of results:**

**hypothesis 2** („active“/ „reactive“ particles)

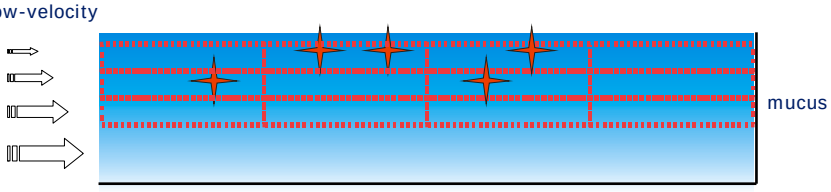
nanoparticles with different $v_{clearance}$ if applied alone

=slowly cleared

=quickly cleared



flow-velocity



# Mucociliary Clearance
# Embryonic Chicken Trachea

**interpretation of results:**

**hypothesis 2** („active“/ „reactive“ particles)

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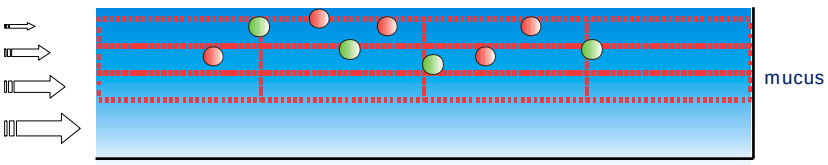
=quickly cleared

flow-velocity



# Ex-vivo experiments
# Embryonic Chicken Trachea

**Discrimination:**

→„multi-tracking“: direct comparison by parallel tracking of different particles on one trachea

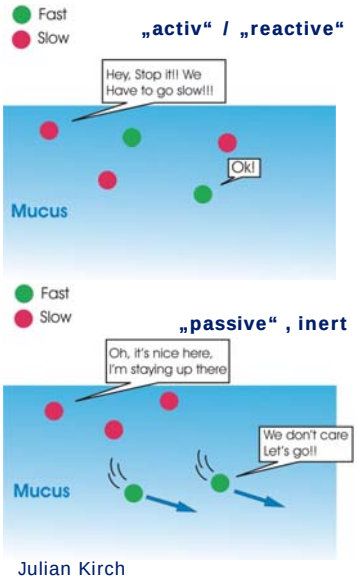
advantage:

interindividual and batch-dependent variability can be avoided

disadvantage:

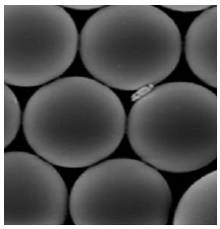
no difference in clearance visible if **all** particles avoid clearance (more or less) „actively“ by altering mucus/clearance properites

→ rheological measurements!



# Mucociliary Clearance
# Embryonic Chicken Trachea

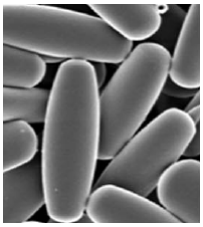
Influence of particle shape on $v_{clearance}$



Spheres:

Ø 0.5 µm Polystyrene,

labelled with fluorescend dye



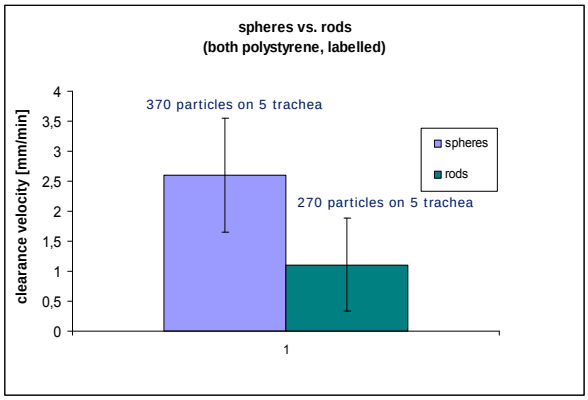
Rods:

made of streched Ø 0.5 µm Polystyrene spheres

particles donated by *Mitragotri-group*, UCSB, Santa Barbara, USA

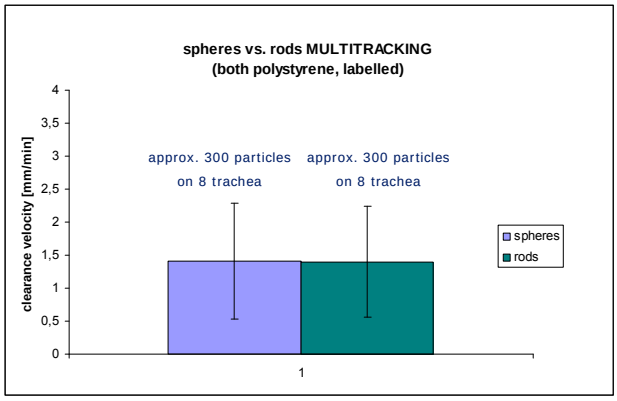
# Mucociliary Clearance
# Embryonic Chicken Trachea

influence of particle shape on $v_{clearance}$ : **single tracking**

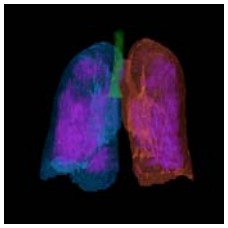


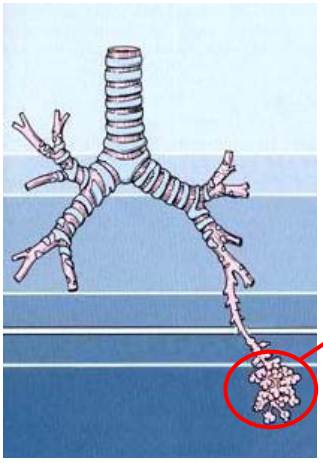
# Mucociliary Clearance
# Embryonic Chicken Trachea

influence of particle shape on $v_{clearance}$ : **multitracking**



# Macrophage Clearances of Nanopharmaceuticals

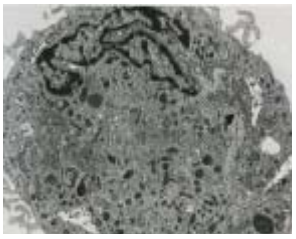


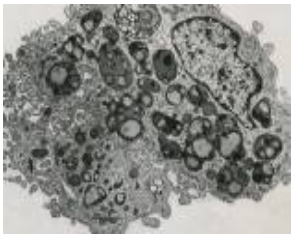


**Peripheral lung:**

**particles cleared by *macrophages***

# *Species differences relevant to alveolar clearance*



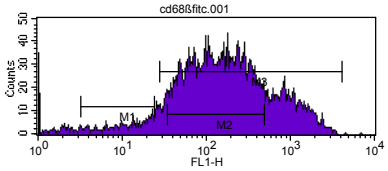


Macrophages from Non-Smoker and Smoker

| Parameter | Mouse | Rat | Dog | Human | Unit |
|---|---|---|---|---|---|
| Alveolar diameter | 47 | 70 | 126 | 219 | µm |
| Number of alveoli | 18 | 43 | 1040 | 950 | x 10 to 6 |
| Macrophages/alveolus | 0.037 | 0.11 | 3.7 | 6.8 | |
| Area patrolled by each macrophage | 190000 | 140000 | 13400 | 13400 | µm x µm |

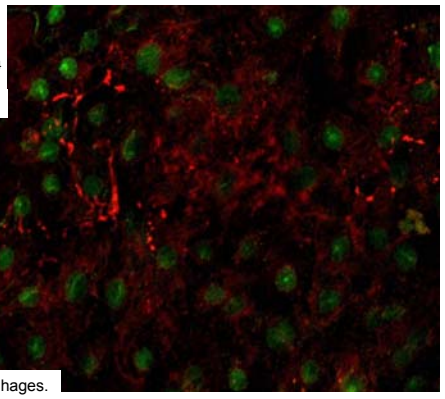
Adapted from *Valberg and Blanchard (1991).*

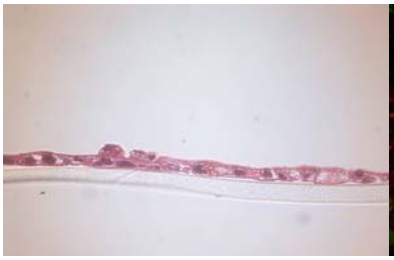
*The ideal model for alveolar clearance (?):*
*„Human alveolar macrophages in primary co-culture with autologus epithelial cell monolayers“*




FACS analysis of isolated CD 68 marked macrophages.

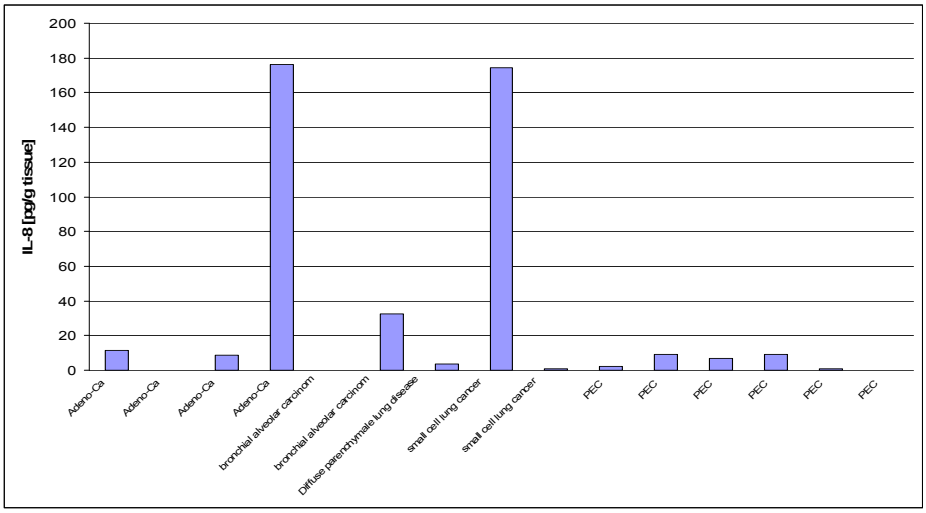
Coculture of human alveolar epithelial cells and and human alveolar macrophages from the same individuum. Red: RCA stained epithelial cells, green: CD68 FITC stained macrophages.



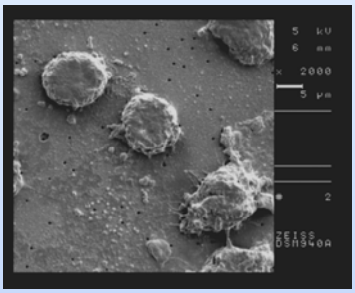


Coculture of human alveolar epithelial cells and macrophages.

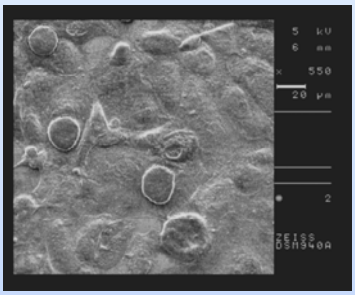
High variability of the „quality“



## Immortalized mouse alveolar macrophages (MHS) as model for alveolar clearance

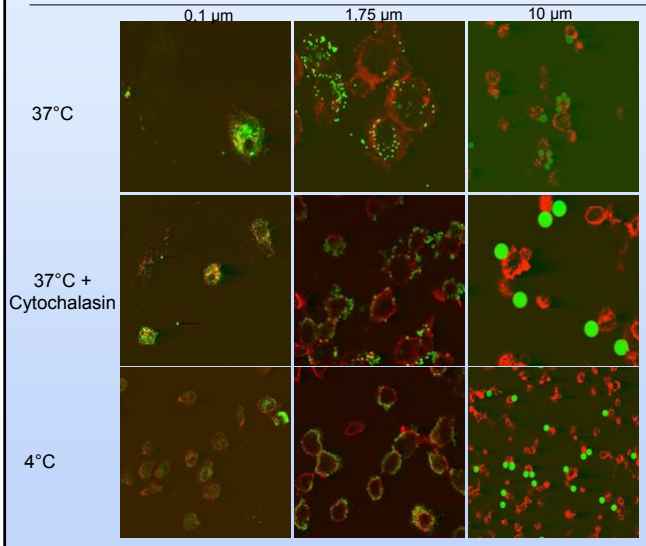


Single culture



Coculture with human alveolar epithelial cells

## Alveolar Clearance of Polystyrene particles




0,1µm Polystyrene particles cross the cell membran also without active uptake.

1,75µm Particles are phagocytosed actively (37°C).

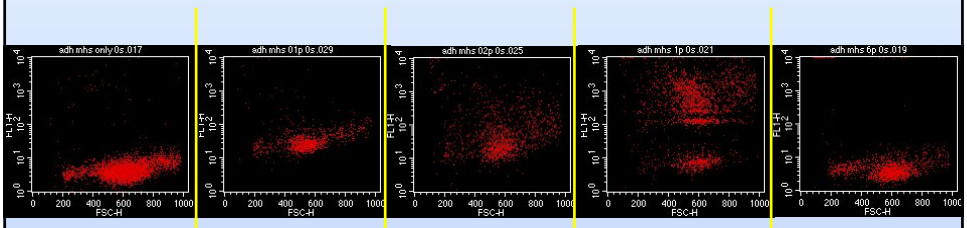
10µm Particles circumvent alveolar macrophages clearance

Red: RCA stained macrophages
Green: Polystyren particles

→ But quantitative Interpretation ???

**Uptake of particles by MHS cells and the mechanisms involved appear to be size dependent!**

# Macrophage particle interactions – size dependency -



MHS only

0,1µm → 100% cells are interacting

0,2µm → 100% cells are interacting

1µm → 73,12% cells are interacting

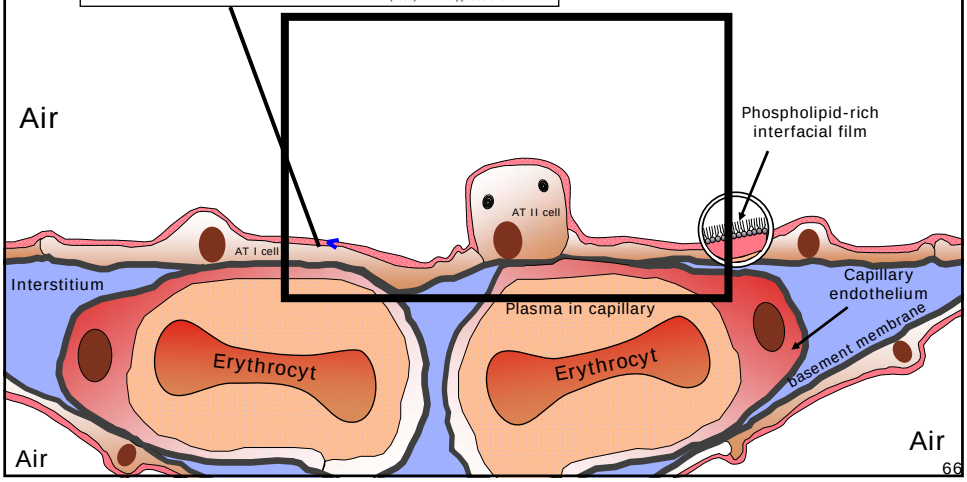
6µm → 0% cells are interacting

# The Air - Blood - Barrier

- Not only an epithelial barrier... -

Alveolar lining fluid: 90-140 nm *
~ 90% lipids (mainly phospholipids)
~ 10% proteins

* Green et al. The Role of Surfactant in Disease Associated with Particle Exposure. Lung Biology in Health and Disease (2000) vol. 143 pp. 533-576

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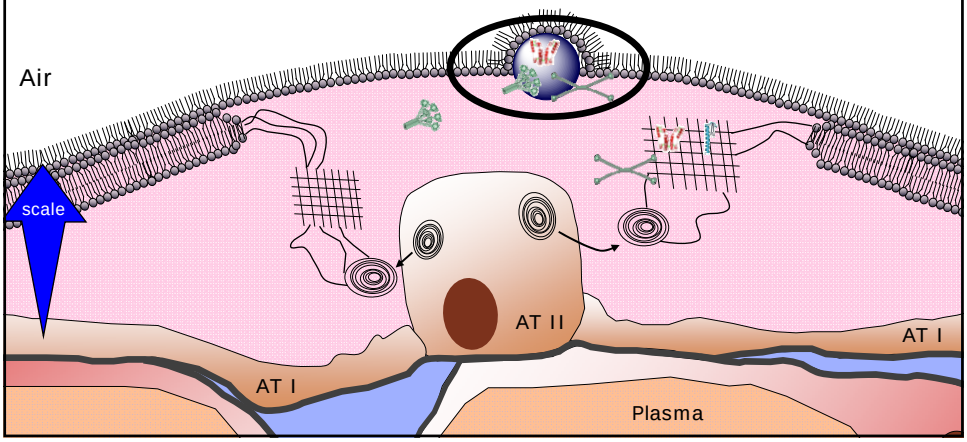
Particle deposition

„What happens after landing?“

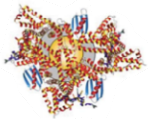
SP adsorption on particles?

Role of Surfactant Proteins ?



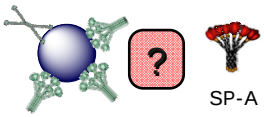

# Role of Surfactant Proteins

- Protein Corona Theory
  (Dawson et al.)



  - Dynamic layer of proteins adsorbed to nanoparticle surfaces immediately upon contact with living systems
  - Primary surface which is in contact with cells *

- Open questions after alveolar deposition of particles:




  - **Which proteins adsorb on particles in dependence on particle material?**
    - **Identify** and **quantify** adsorbed surfactant proteins (especially SP-A)
    - Investigate binding affinities and binding forces
  - **How do adsorbed SP influence the biological response** in terms of
    - Clearance by alveolar macrophages
    - Epithelial translocation

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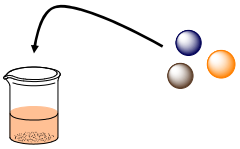
* Lynch. Protein-nanoparticle interactions. Nano Today (2008) vol. 3 (1-2) pp. 40-47

# Methodological approach

Idea: incubation of model particles with a model alveolar lining fluid

**Protein component**

- bBALF

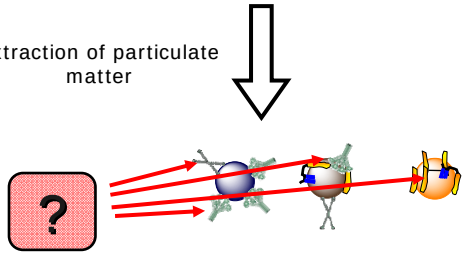


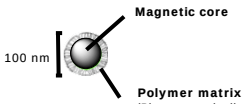
Surfactant protein containing incubation media

**Particle component**

- Material
- Size & shape
- Zeta potential
- Hydrophobicity

Extraction of particulate matter

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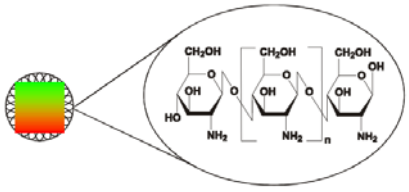
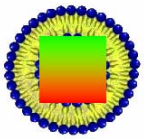
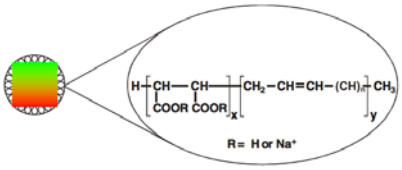
# Used magnetic nanoparticles



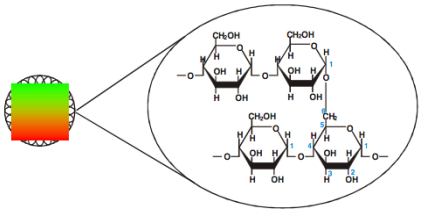
Chitosan



Phospholipid

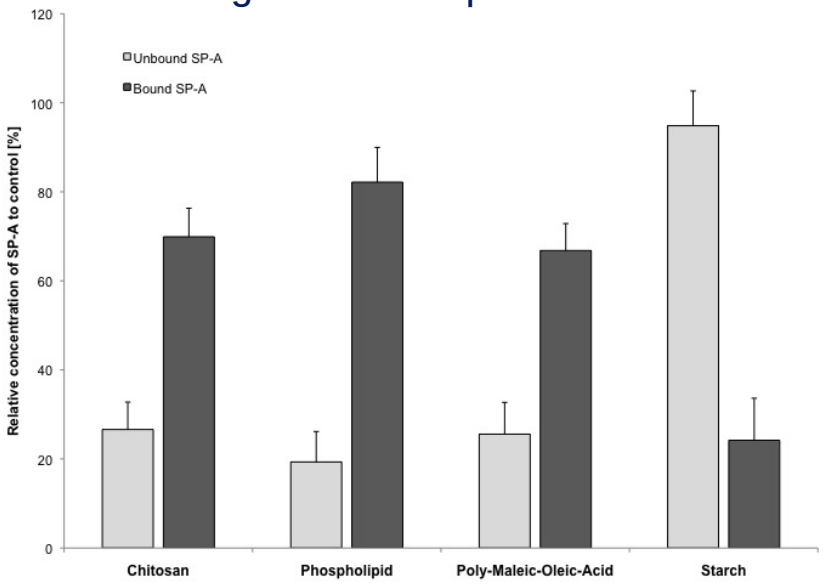



PMO



Starch

# SP-A adsorption on 200 nm magnetic nanoparticles




Data shown as mean ± S.E.M. (n=3)

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# Particle properties determine SP-A adsorption

| Surface modification | Zeta-Potential [mV] | Hydrophobicity [mL/µm²] | SP-A [%] (related to control) |
|---|---|---|---|
| Chitosan | 44,0 ± 2,4 | $8*10^{-10}$ | 69,9 |
| Phospholipid | -40,9 ± 5,3 | $6*10^{-11}$ | 82,17 |
| Poly-Maleic-Oleic Acid | -21,4 ±,02 | $1*10^{-13}$ | 66,81 |
| Oleic Acid | | $3*10^{-12}$ | |
| Starch | -1,1 ±0,4 | $7*10^{-13}$ | 24,2 |

Hydrophobic interactions

Electrostatic interactions

Chitosan Phospholipid PMO Starch

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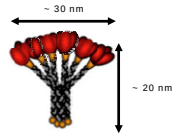
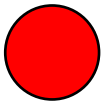
# SP-A adsorption relative to nanoparticle surface (I)




~630 kD

Haagsman und Diemel. Comp Biochem Physiol, Part A Mol Integr Physiol (2001) vol. 129 (1) pp. 91-108

=>  d=30nm => A ≃ 706 nm²

Surface area per particle (d=200 nm) ≃ 0.1256 µm²

=> Theoretical max. number of SP-A molecules per particle (monolayer):
**~178 SP-A molecules / particle**

1.2 mg/mL particles (d=200 nm) ≃ $2.29*10^{11}$ particle / mL

=> Total surface area of particles $A_{tot}$ ≃ $28.8*10^{9}$ µm²/mL

=> Theoretical mass concentration / mL of SP-A for complete coverage of all particles:
**0.0426 mg SP-A**

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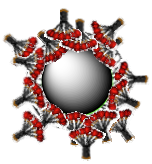
# SP-A adsorption relative to nanoparticle surface (II)

=> Theoretical mass concentration / mL of SP-A for complete coverage of all particles:
**0.0426 mg SP-A**

| Surface modification | Absolute bound amount of SP-A [mg/mL] | „Coverage factor“ |
|---|---|---|
| Chitosan | 0.186±0.033 | 4,36 |
| Phospholipid | 0.232±0.069 | 5,45 |
| Poly-Maleic-Oleic Acid | 0.193±0.066 | 4,53 |
| Starch | 0.118±0.019 | 2,77 |

**=> Multiple layers of SP-A on each particle for NP-to-protein ratio of 2:1 ??**



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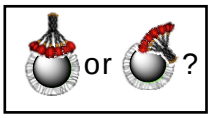
# Summary & Outlook

- Determination of adsorbed SP-A via densitometry is possible (at least on a semiquantitative level)
- There are differences in amount of adsorbed SP-A among the tested particles
- Protein adsorption is strongly dependent on particle material (e.g. Starch vs. Chitosan)

**Future tasks:**

•Adsorptionisothermes (preliminary experiments performed)

•Role of $Ca^{2+}$ on SP-A adsorption („mode of binding“)




•Influence of adsorbed SP-A on clearance by alveolar macrophages (MH-S)

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## *Outline*

- Deposition of (nano) particles on mucosal epithelial cells and their effects on drug absorption
- Pulmonary clearance of (Nano-)Particles
  - mucociliary clearance
  - macrophage clearance
- Cellular delivery of telomerase inhibitors by polymeric nanocarriers for the treatment of lung cancer

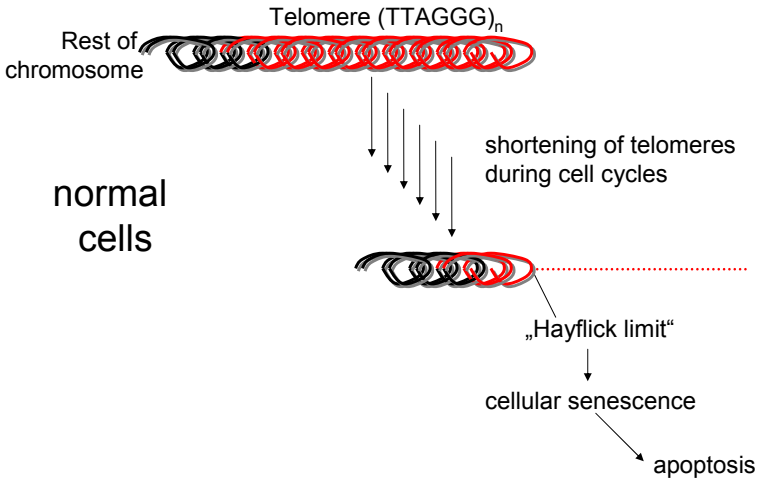
# Cationic chitosan/PLGA nanoparticles enhance the uptake of the antisense telomerase inhibitor 2'-O-Methyl-RNA into A549 lung cancer cells

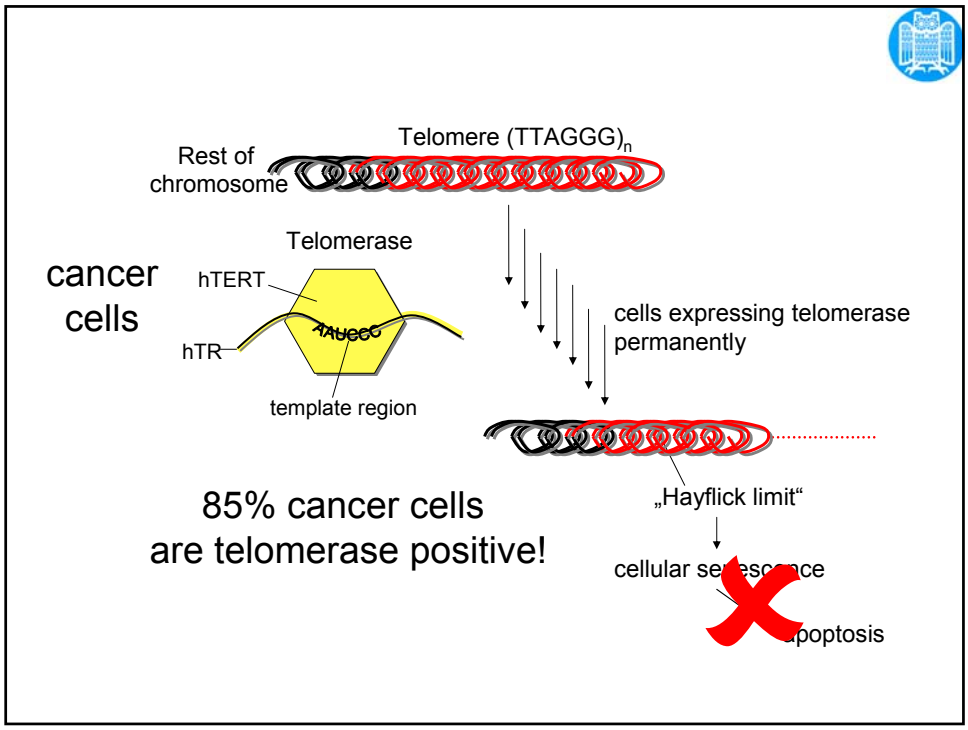
Sebastian Taetz[1], Noha Nafee[1], Christiane Baldes[1],
Kamilla Piotrowska[2], Julia Beisner[2], Thomas Mürdter[2],
Ulrich F. Schaefer[1], Ulrich Klotz[2], Claus-Michael Lehr[1]

[1]Biopharmaceutics and Pharmaceutical Technology
Saarland University, Saarbrücken
[2]Institute for Clinical Pharmacology,
Dr. Margarete Fischer-Bosch Hospital, Stuttgart

Funding: DEUTSCHE KREBSHILFE

## Why telomerase inhibition for cancer treatment?






# The inhibitor

antisense 2'-O-Methyl-RNA (2OMR)

- 13mer antisense oligonucleotide
- directed against the template region of hTR
- highly selective and effective

Piotrowska et al. 2005, Lab Invest
Pitts et al. 1998, Proc Natl Acad Sci USA

**Disadvantage:**

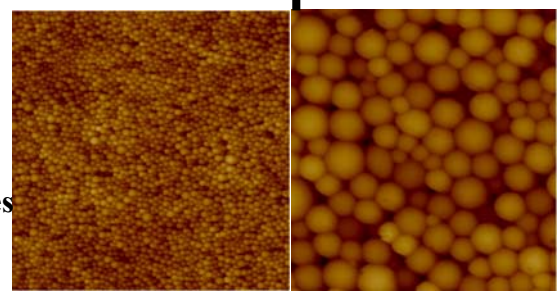
– big molecule

– high negative charge

→ no uptake, i.e. hard to deliver!

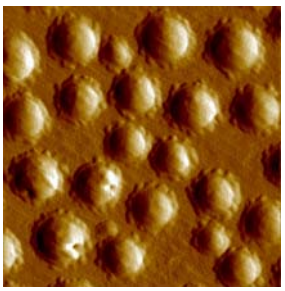
# Chitosan coated PLGA Nanospheres

Nanospheres
< 200 nm



Surface Morphology

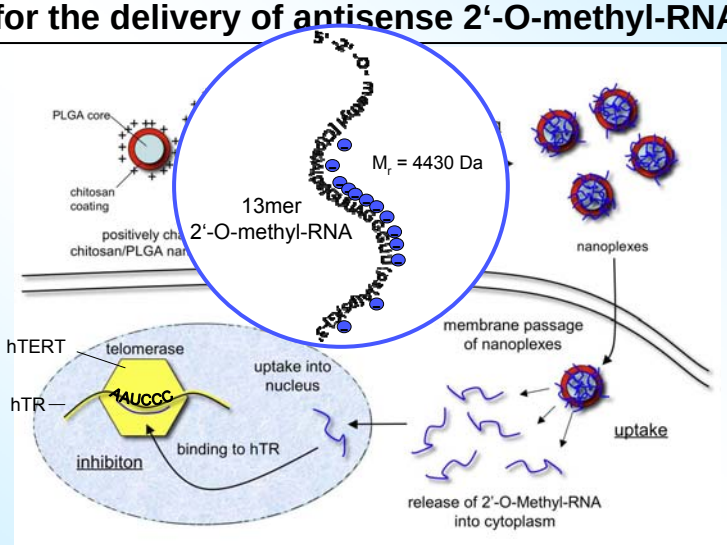
Nanospheres
< 200 nm



**Nanosphere-DNA Complexes**

Kumar et al, Biomaterials 25 (2004) 1771-1777

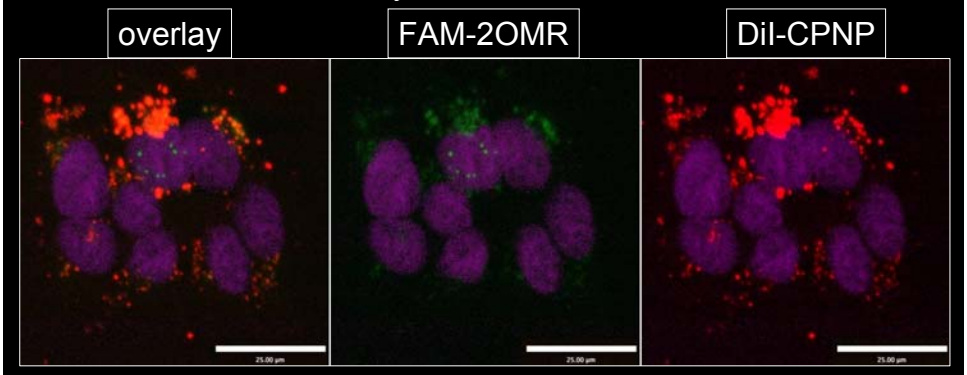
## Cationic chitosan/PLGA nanoparticles for the delivery of antisense 2'-O-methyl-RNA




Taetz, S. et al., 2008, Eur. J. Pharm. Biopharm., in press

## Uptake of nanoplexes into A549 cells

1 day after incubation

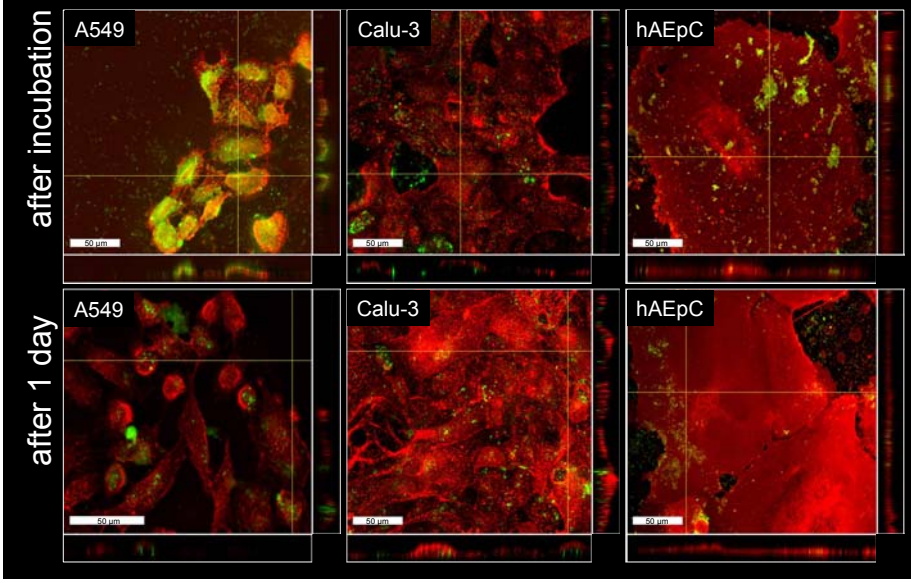



Uptake of 2OMR is mediated via nanoplexes!

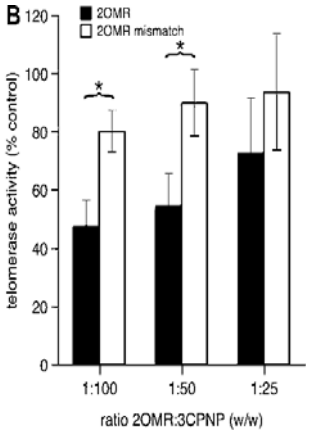
red = DiI-stained chitosan/PLGA nanoparticles (3CPNP)
green = FAM-2OMR
purple = cell nuclei

## Uptake of 2OMR nanoplexes into different cells types

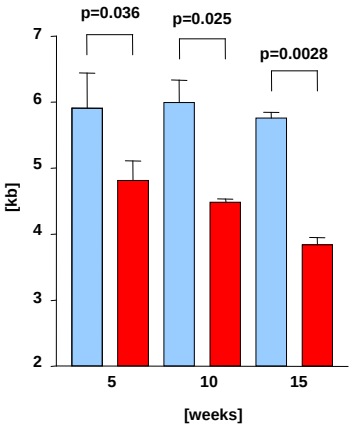
red = cell membrane green = FAM-2OMR



# Influence of 2´-O-Methyl-RNA –nanoplexes on telomerase activity and telomere length




Tätz et al, EJPB 2008




Beisner et al, Lung Cancer 2009

Universitätsklinikum des Saarlandes
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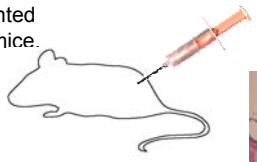
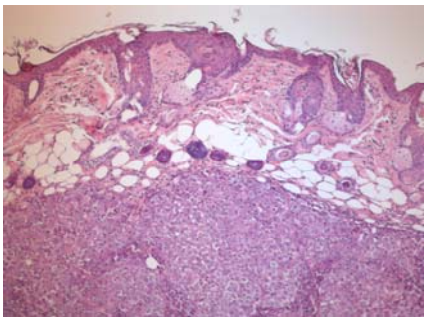
Tumor cells were implanted into the flanks of nude mice.



Tumor size is measured with a calliper every 3 days

The resulting tumors were grown (max tumor size 2 cm)



H&E stained section 100x magnification

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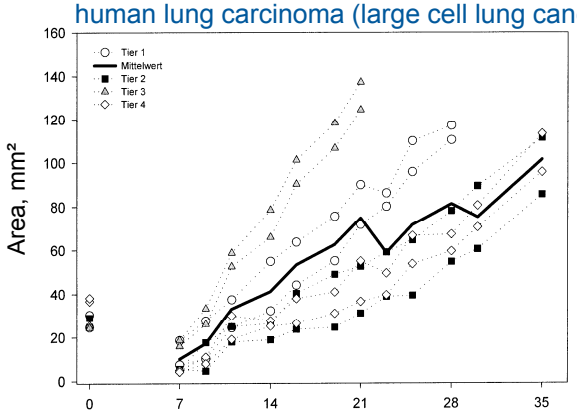
## Towards animal experiments:

Growth curve NCI-H460 (=HTB-177)
human lung carcinoma (large cell lung cancer)




⇒injection of $1x10^6$ cells results in continuos tumor growth in nude mice.

Helmholtz Institute for Pharmaceutical Research Saarland

## Acknowledgements

HELMHOLTZ ZENTRUM FÜR INFEKTIONSFORSCHUNG

UNIVERSITÄT DES SAARLANDES

Deutsche Forschungsgemeinschaft DFG

Bundesministerium für Bildung und Forschung

BfR Risiken erkennen – Gesundheit schützen

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