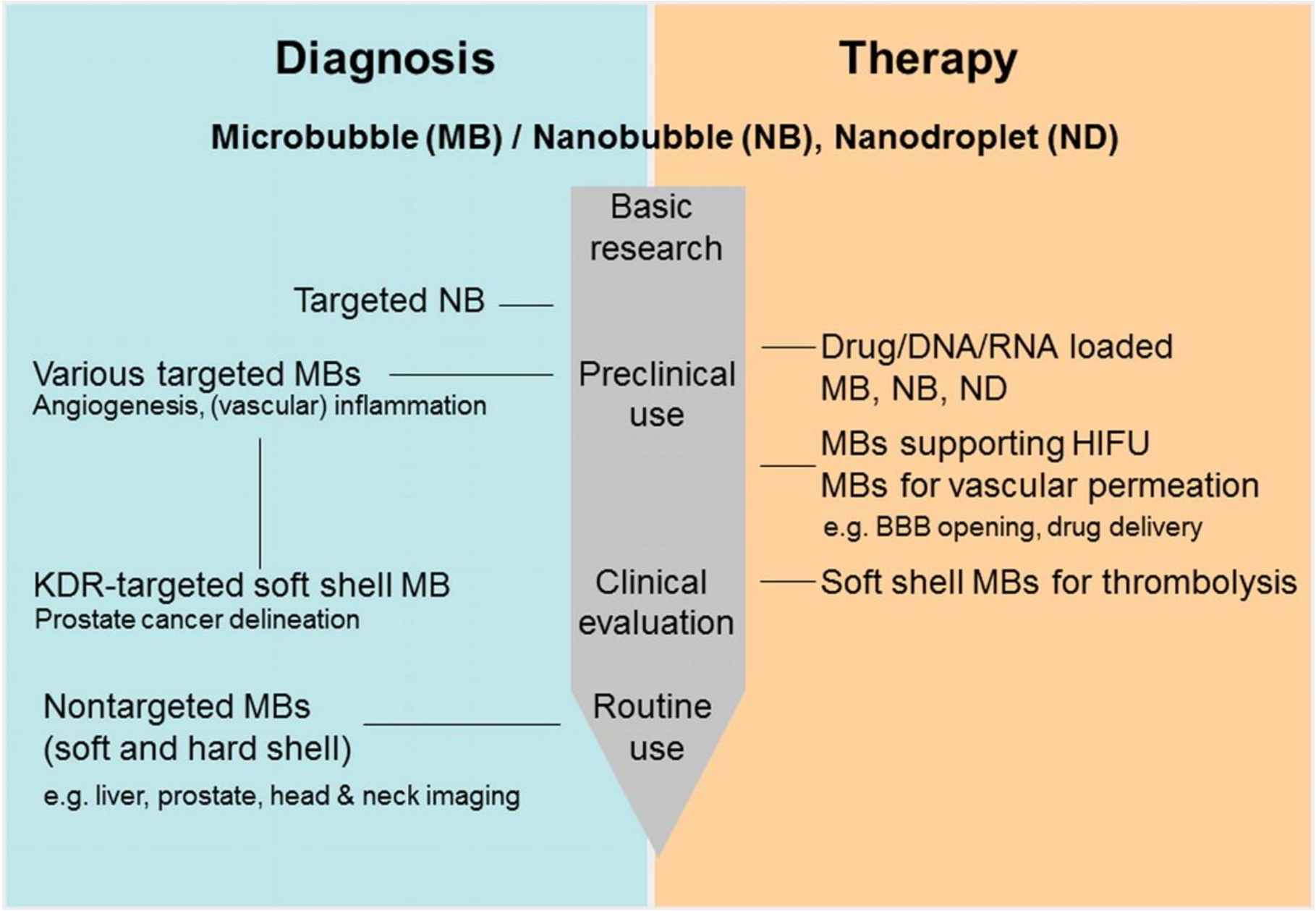
# Functionalized Microbubbles and Their Application in Ultrasonography

# Functional Molecular Imaging

- Describes a heterogeneous family of non-invasive techniques, suitable **both** for **basic science and clinical settings**
- Requires **molecular specific contrast agents** to obtain real-time images
- Provides a **cheap, portable and minimally invasive system**

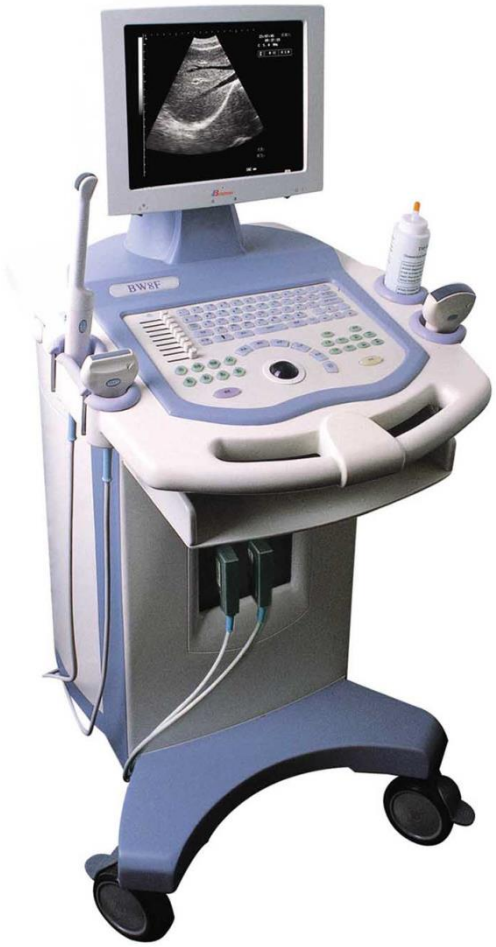
# Application of MBs



# Contrast-Enhanced Ultrasound (CEUS)

- Primarily applied in clinical use
  - in **echocardiography** ("echo contrast agents")
  - in hepatology for **liver cancer diagnostics**, additional to the MRI, CT
  - for blood-pool **radiology** (plaque vulnerability)
  - destruction of **tumor vascular endothelium**
- Only small volumes of contrast agents required for intravenous injection

# Ultrasound (US) I



- Most often used in terms of molecular imaging
- ADVANTAGES:
  - real time
  - able to visualize motion (convenient for needle biopsies and tissue ablations)
  - safe, portable, commercially available

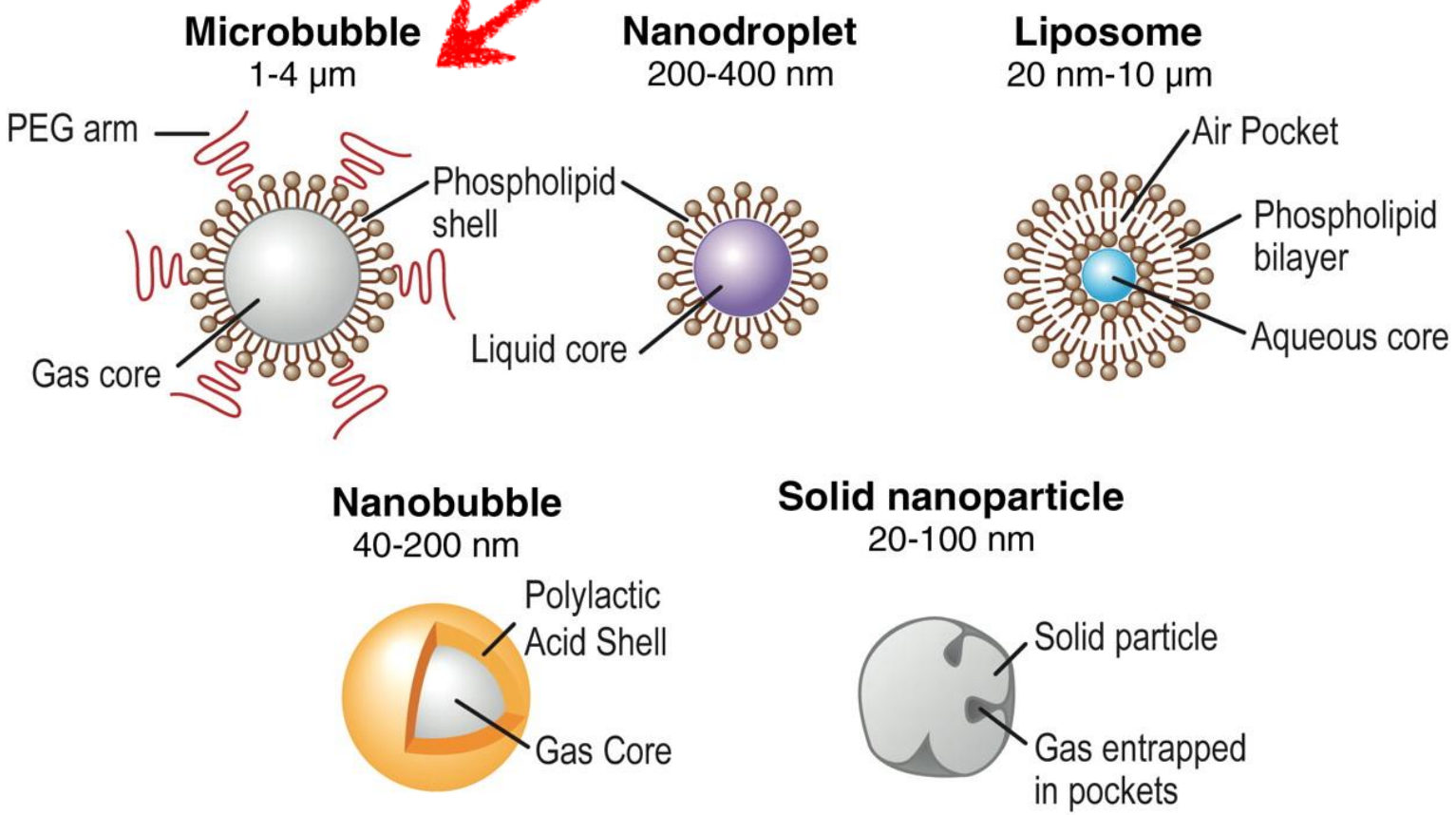
# Ultrasound (US) II

- DISADVANTAGES:
  - US propagation attenuated by tissues (bone, fat)
  - practical experience and sufficient training of sonographer is required to reveal adequate results
  - rather small field of view (20-30cm)
  - two-dimensional images only

# Ultrasound Contrast Agents

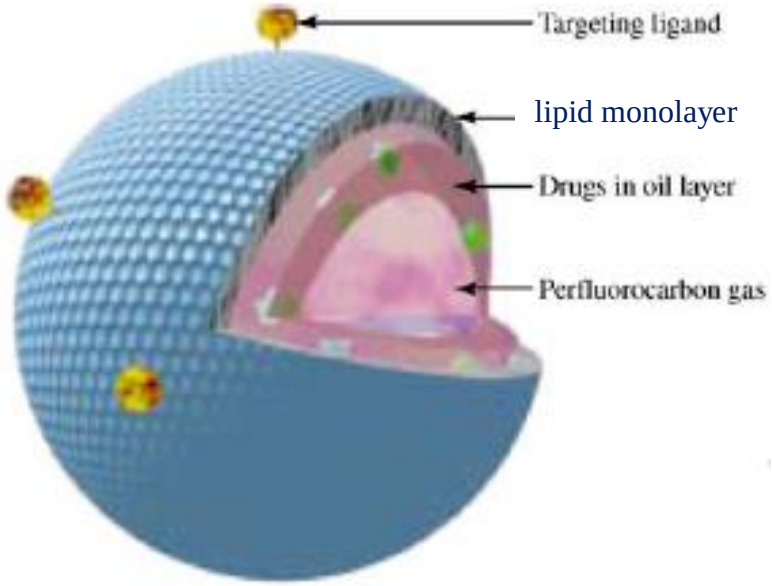
- The “must have”:
  - non-toxic natural or synthetic biodegradable materials
  - easily detected in small amounts
  - stable in the bloodstream
  - cheap, easy to usage, storable

# Types of US Contrast Agents



# What are microbubbles?

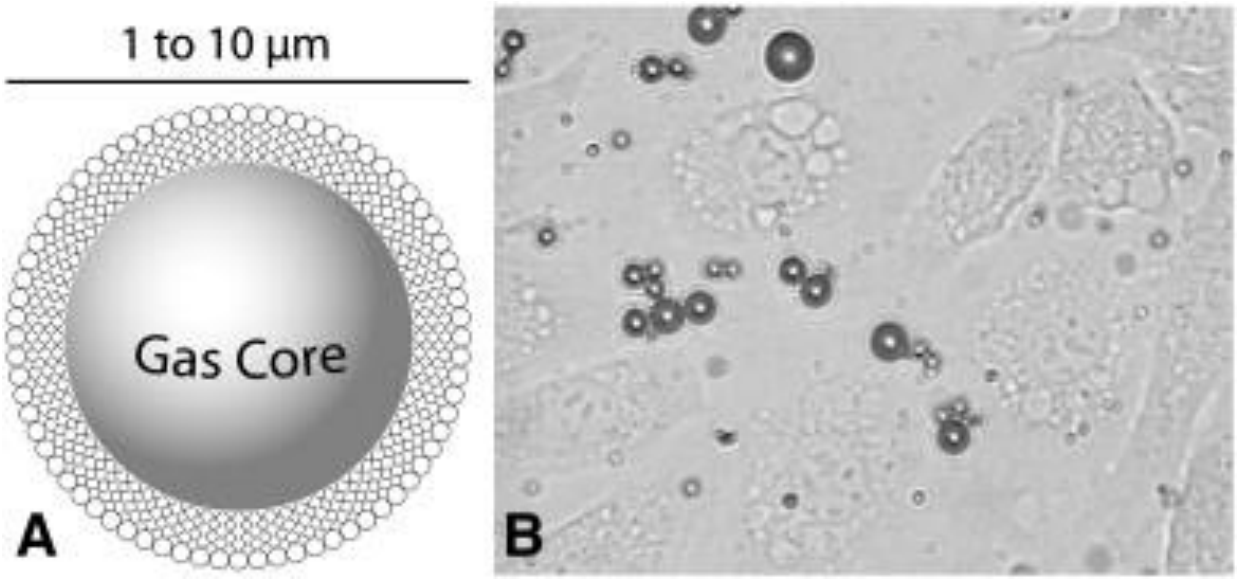
- Basicaly like liposomes **BUT**:
- Lipid monolayer e.g. phosphatidylcholine
- Polar head – water solution
- Non-polar chain – gas perfluorobutane, $SF_6$ or liquid decafluoropentane



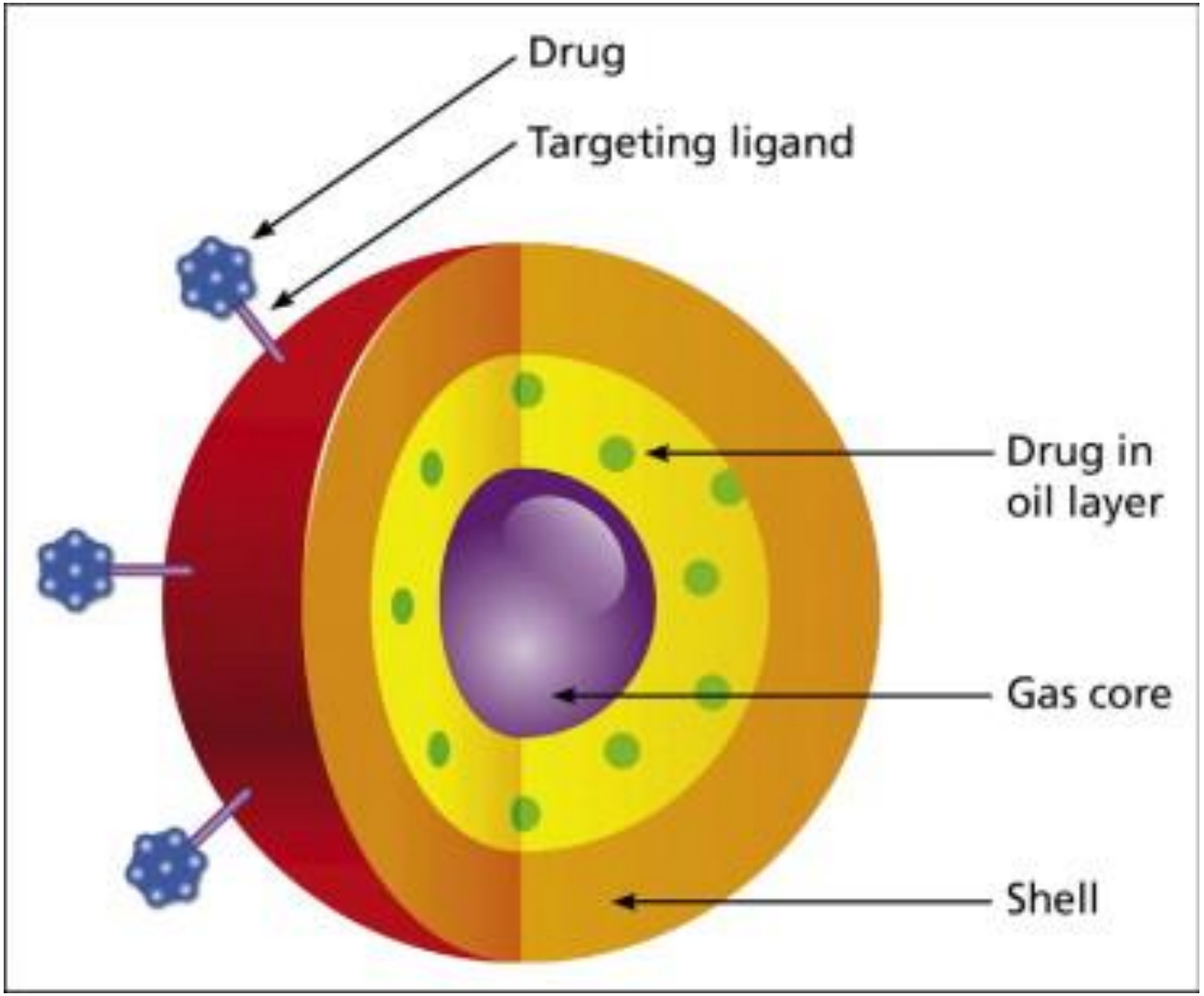

Quotation: Dijmans, et al., Eur J Echocardiogr (2004) 245-256

# Microbubbles (MBs)

- Purely intravascular contrast particles composed of a thin membrane surrounding a hollow space filled with gas



# Schematic Model of a MB

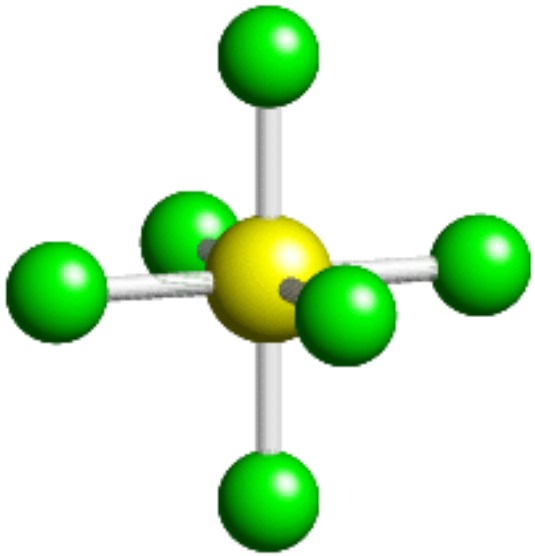


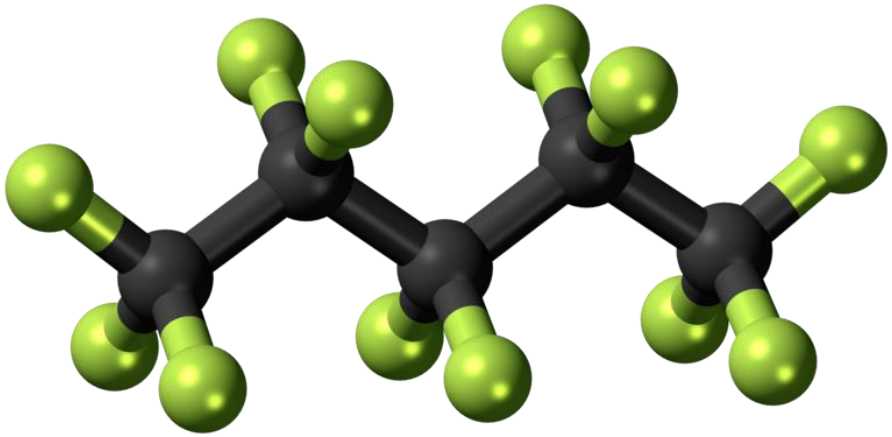
# The MB Shell

- lipid, protein, polymer
- stabilizes microbubbles to enhance the circulation time in blood after injection
- should only slightly limit the MB vibration in the US field generated by echo imaging systems

# The Filling Gas

- usually filled with a hydrophobic high-molecular gas ($SF_6$, perfluorocarbons)





# Fundamental Techniques for MB Preparation

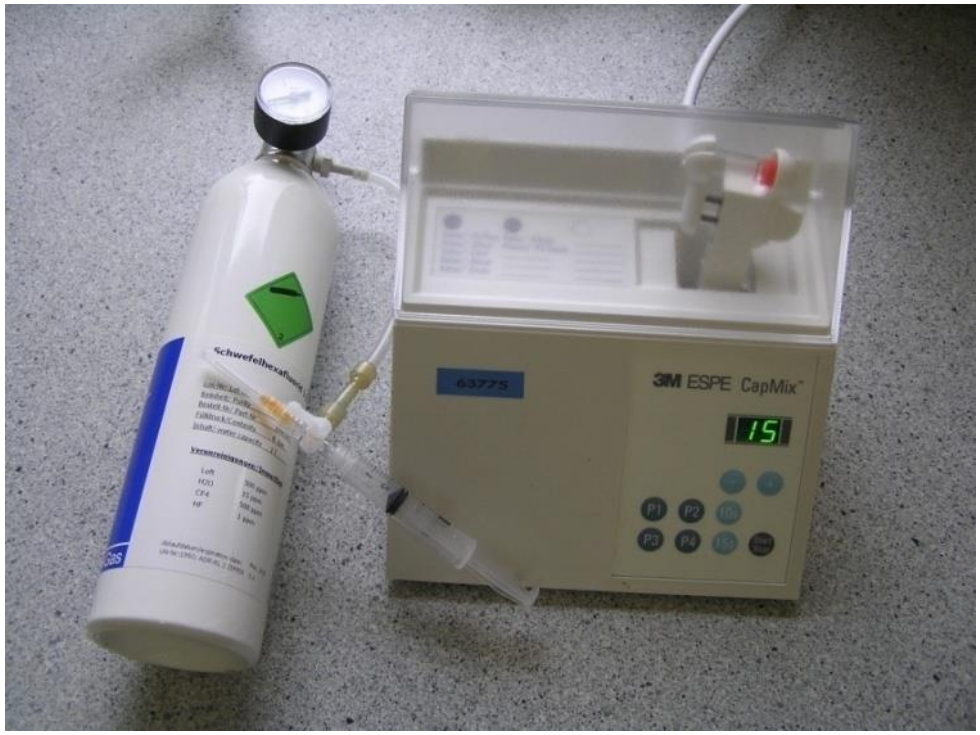
- Sonication
- Shear Mixing
- Coaxical electrohydrodynamic atomisation
- Microfluidic processing using a T-junction

# 1. Sonication

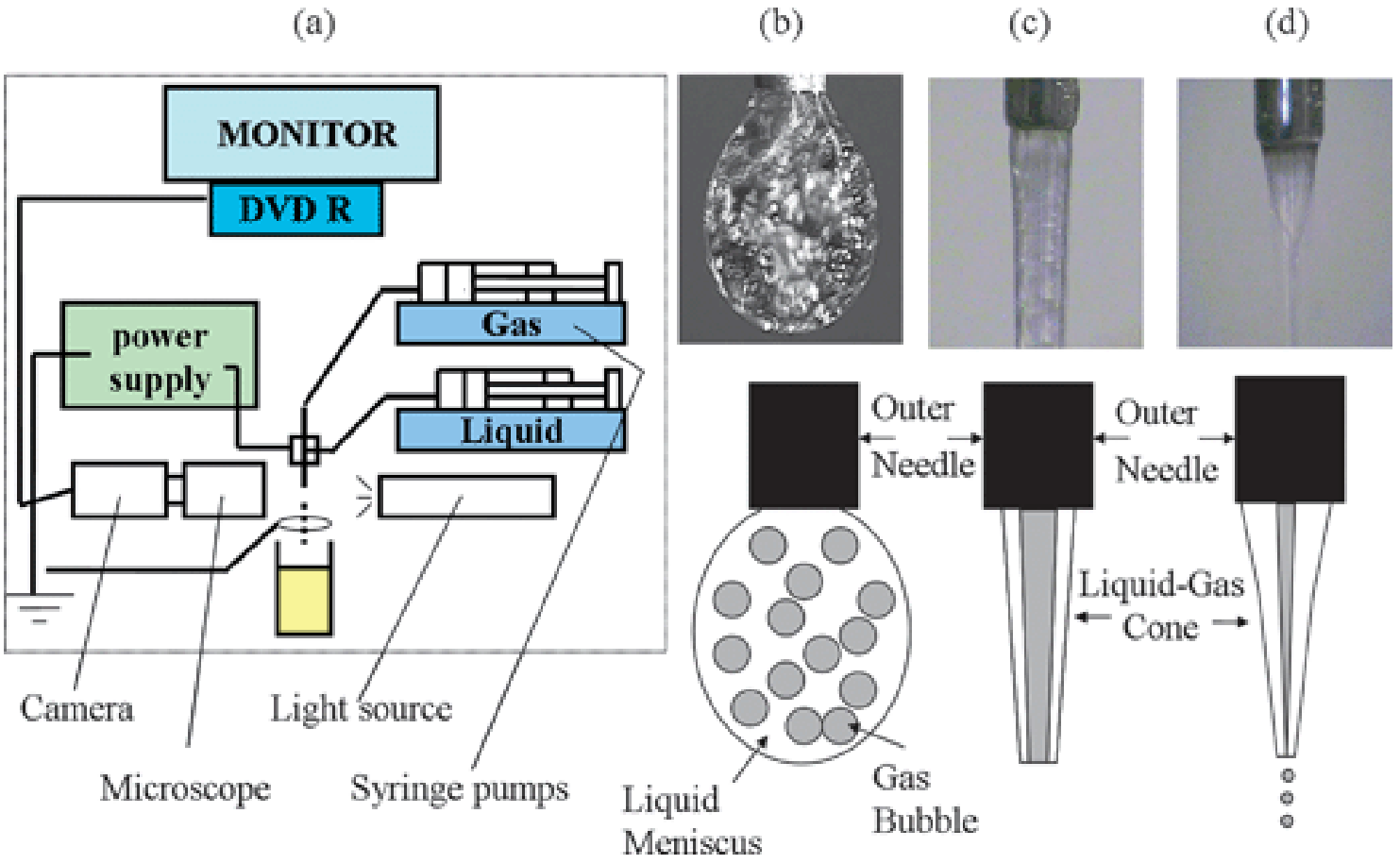
- most commonly used method for MB preparation
- use of high intensity US to produce a suspension of gas MBs in a liquid containing a suitable surfactant or polymer solution which adsorbs on the MB surface and forms a stabilizing coating
- DISADVANTAGE: produces relatively broad size MB suspensions → any large MBs must be filtered and/or removed before the injection + low productivity of MBs

## Preparation of MB by mixing

- Put liposome suspension into hermetic vial flask
- Fill the flask with gas ( sulphur hexafluoride )
- Mix intensively in 3M ESPE CapMix for 30 seconds ( f=2000 )

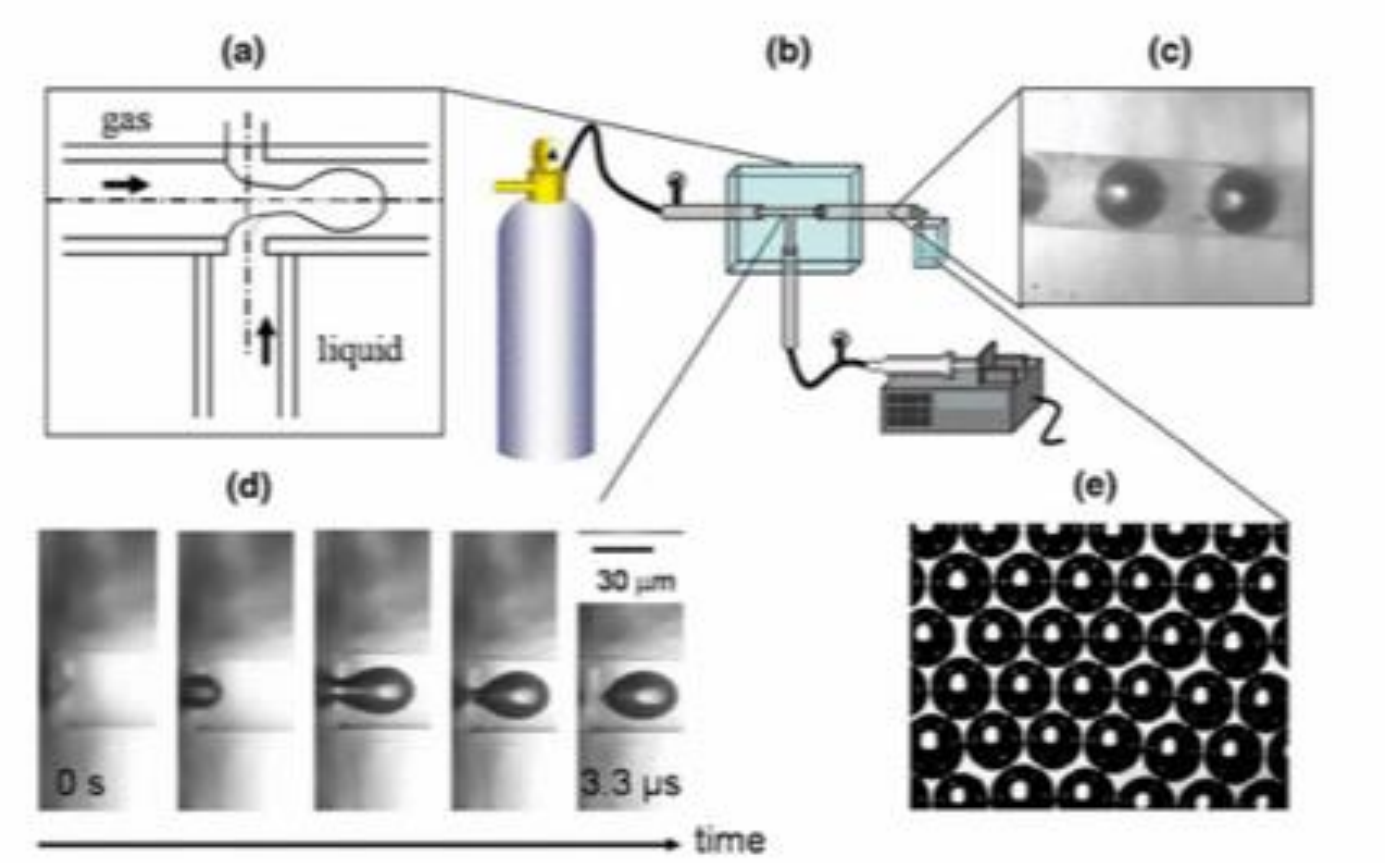



# 3. Coaxical electrohydrodynamic atomization




Schematic of the experimental set-up for coaxical electrohydrodynamic microbubbling (Farook et al. 2007).

# 4. Microfluidic Processing Using T-Junction




T-junction processing: **a** fluid flow through the junction; **b** schematic of the experimental apparatus used; **c** bubbles approaching the device exit; **d** bubble formation at the junction; **e** optical micrograph of bubbles after collection (Stride & Edirisinghe 2009).

# Methods for MB Characterization

- Microscopy: optical, confocal, TEM, and SEM
- Static Light Scattering
- Cell Counter
- Flow Cytometry

# Specific Contributions of the Research Concerned

Langmuir

ARTICLE

pubs.acs.org/Langmuir

## Preparation of Metallochelating Microbubbles and Study on Their Site-Specific Interaction with rGFP-HisTag as a Model Protein

Róbert Lukáč,[†,⊥] Zuzana Kauerová,[†,⊥] Josef Mašek,[†] Eliška Bartheldyová,[†] Pavel Kulich,[†] Štěpán Koudelka,[†] Zina Korvasová,[†] Jana Plocková,[†] František Papoušek,[‡] František Kolář,[‡] Roland Schmidt,[§] and Jaroslav Turánek*[,†]

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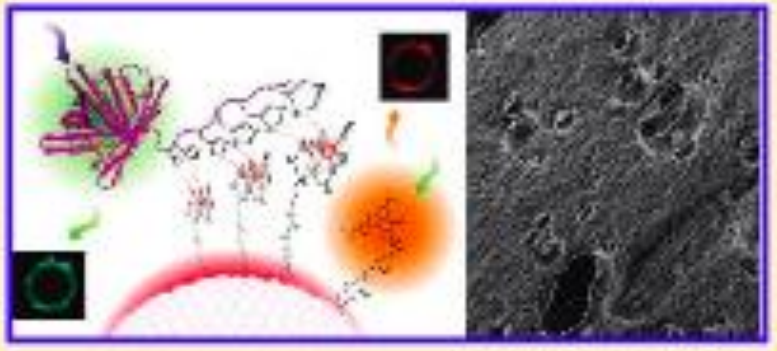
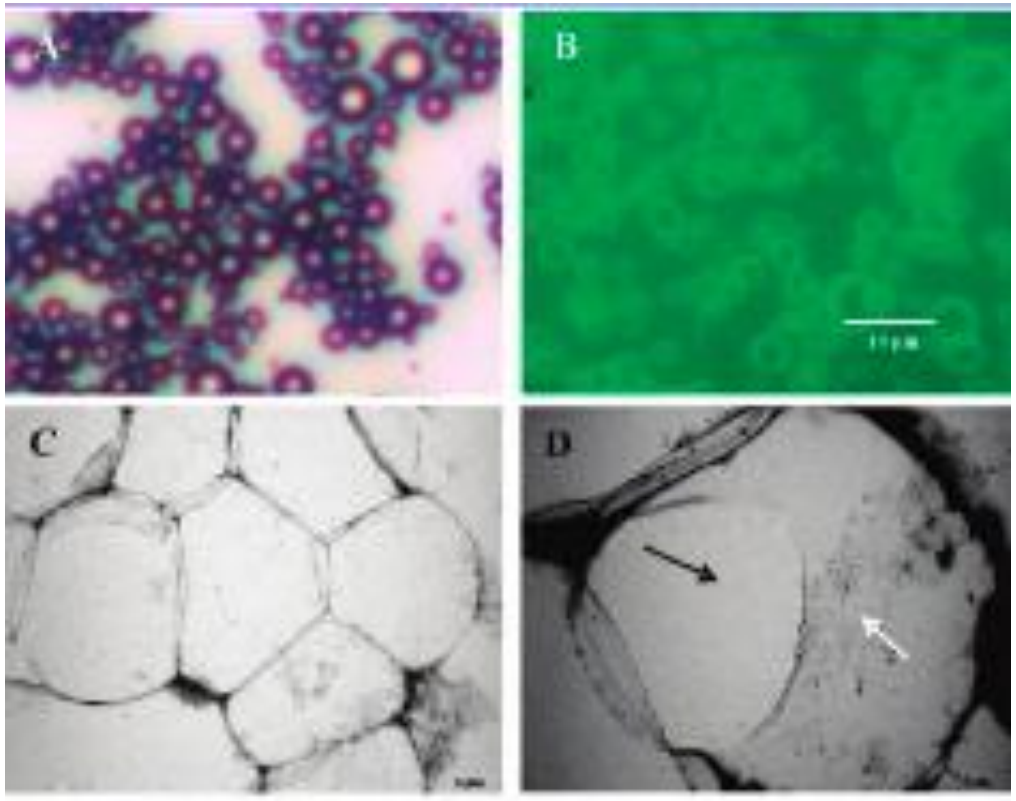
**ABSTRACT:** The histidine−metallochelating lipid complex is one of the smallest high affinity binding units used as tools for rapid noncovalent binding of histidine tagged molecules, especially recombinant proteins. The advantage of metallochelating complex over protein−ligand complexes (e.g., streptavidine−biotin, glutathiontransferase−glutathion) coesists in its very low immunogenicity, if any. This concept for the construction of surface-modified metallochelating microbubbles was proved with recombinant green fluorescent protein (rGFP) containing 6His-tag. This protein is easy to be detected by various fluorescence

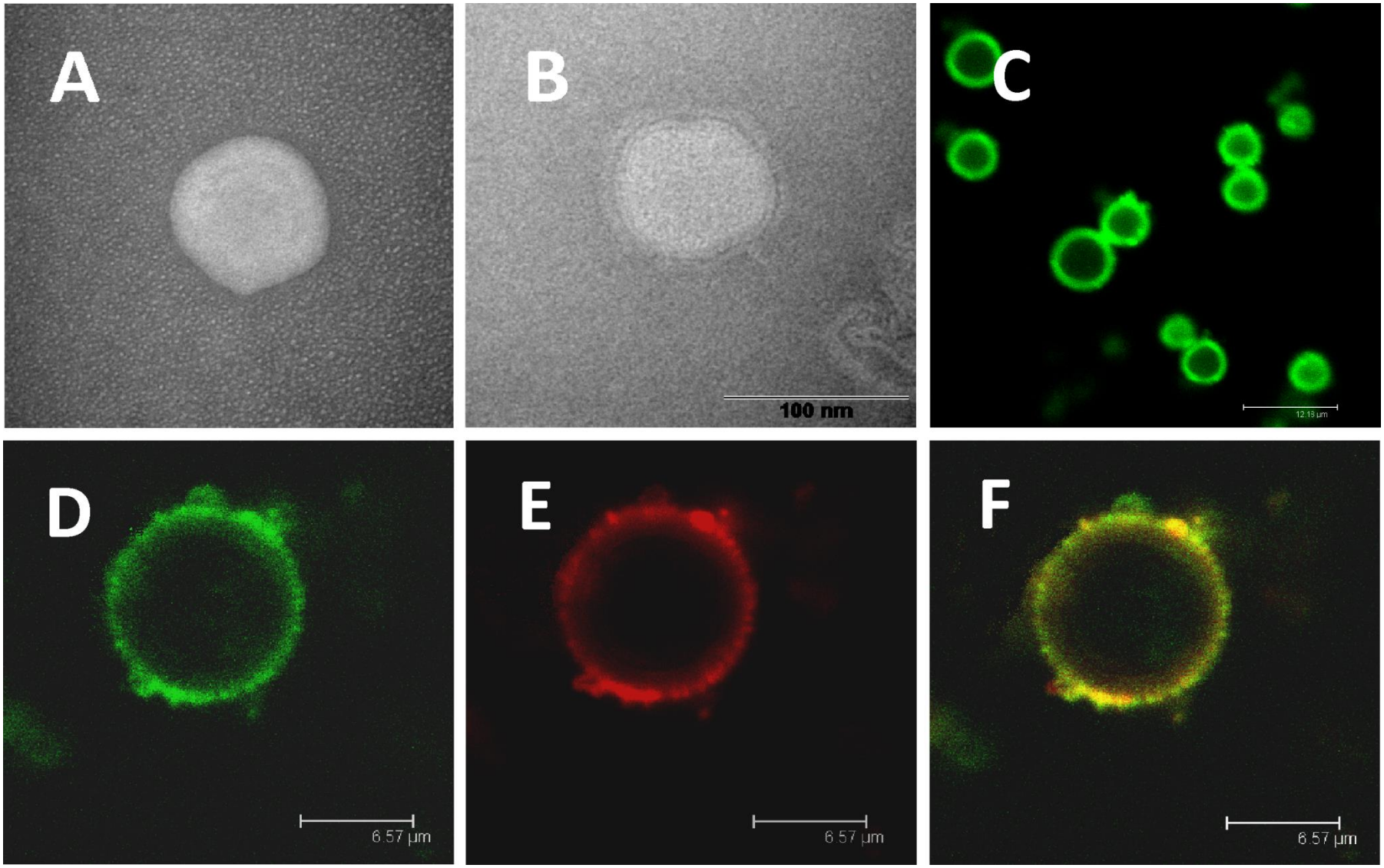


# Optical and TEM microscopy

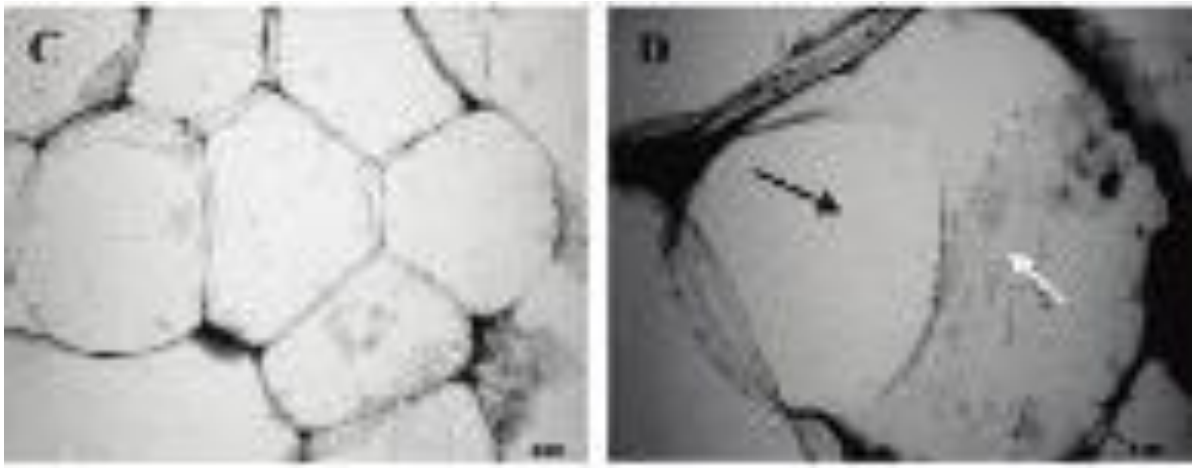


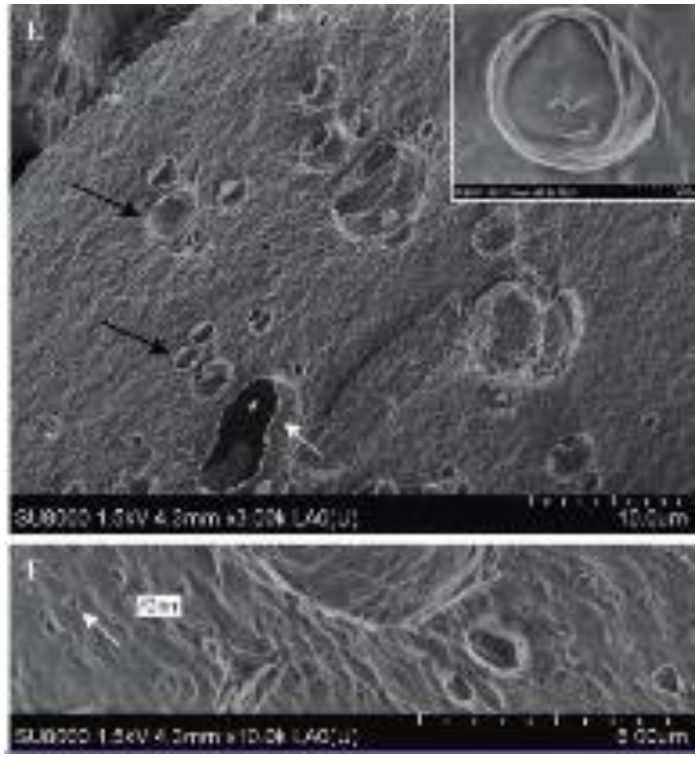

(A) Optical microscopy of DPPC/1% DOGS-NTA-Ni MB with carboxy-fluorescein-PE (1%). (B) The same MB as in (A) observed by epifluorescence microscopy. (C) TEM of MB. (D) TEM detailed picture of MB.
(Lukáč et al. 2011)

# Laser Confocal Microscopy



# C. Transmission and Scanning Electron Microscopy

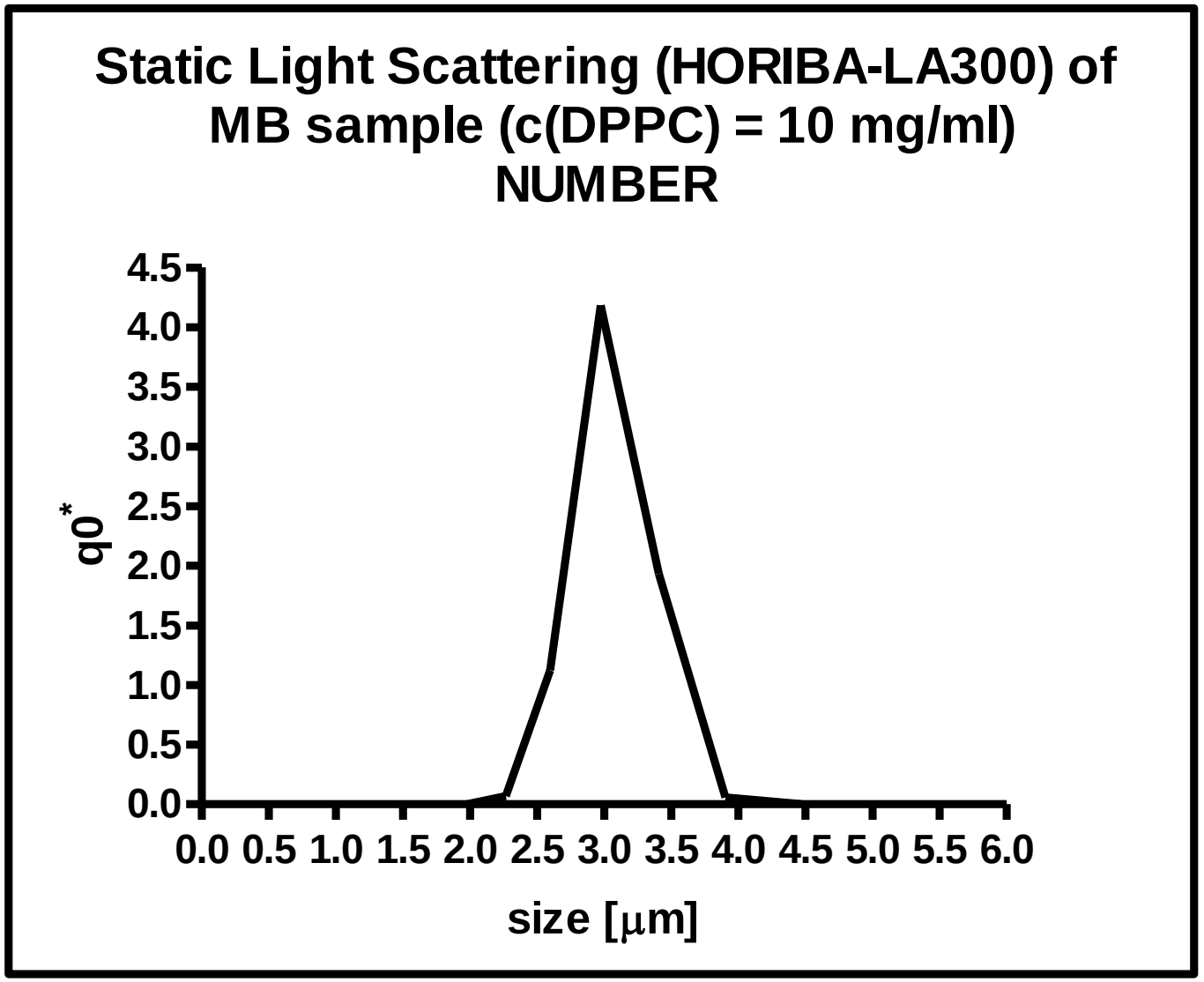




(C) **TEM** of MB. (D) **TEM** detailed picture of MB. (E) **SEM** picture of lyophilized microbubbles. (F) Residual liposome embedded in matrix (white arrow).

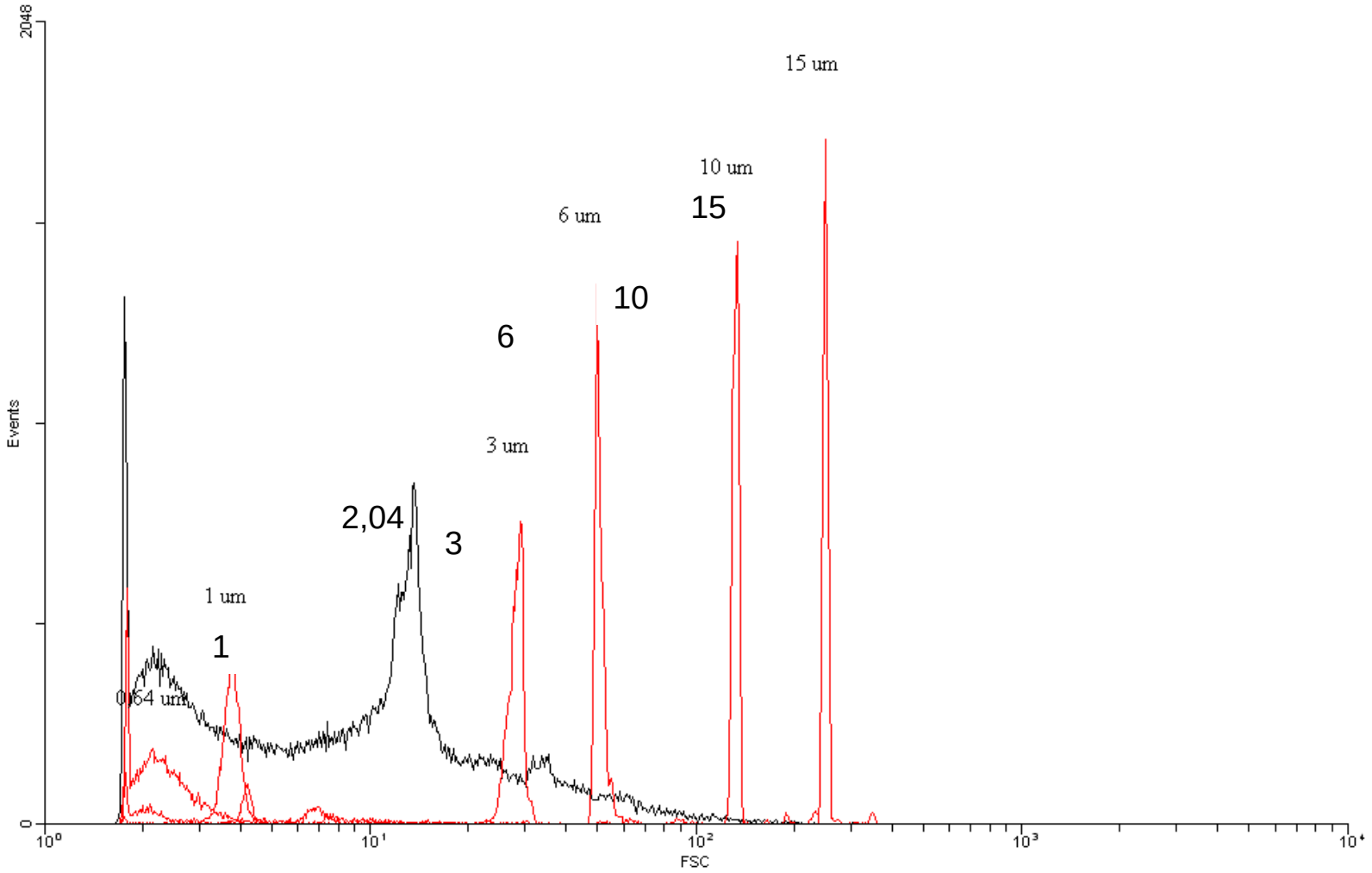

E
SU8000 1.0kV 4.3mm x3.00k LA0(U)
10.0μm

# Static light scattering

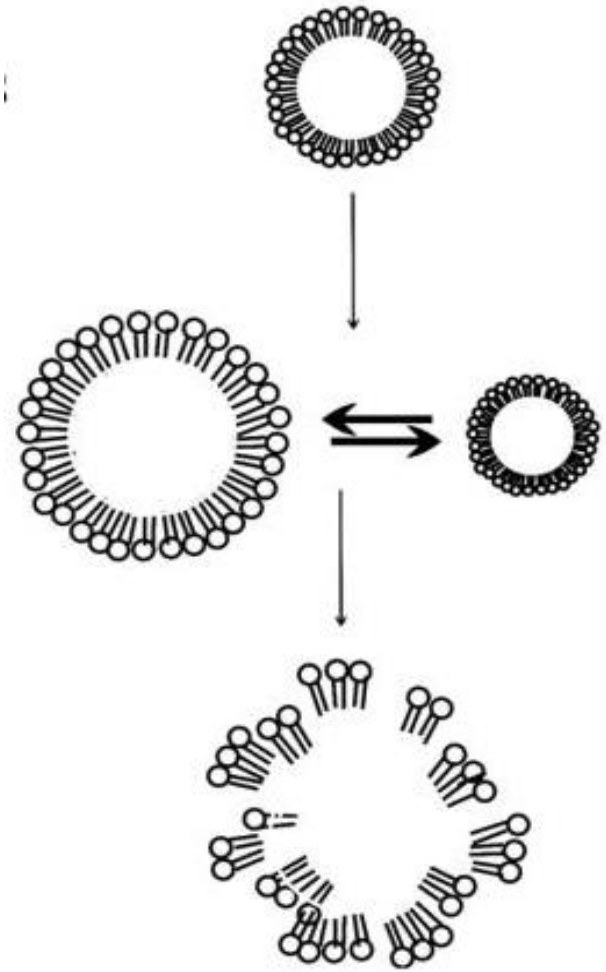


# Flow cytometry

Histogram of FSC measurement of particle size standards and the MB sample.



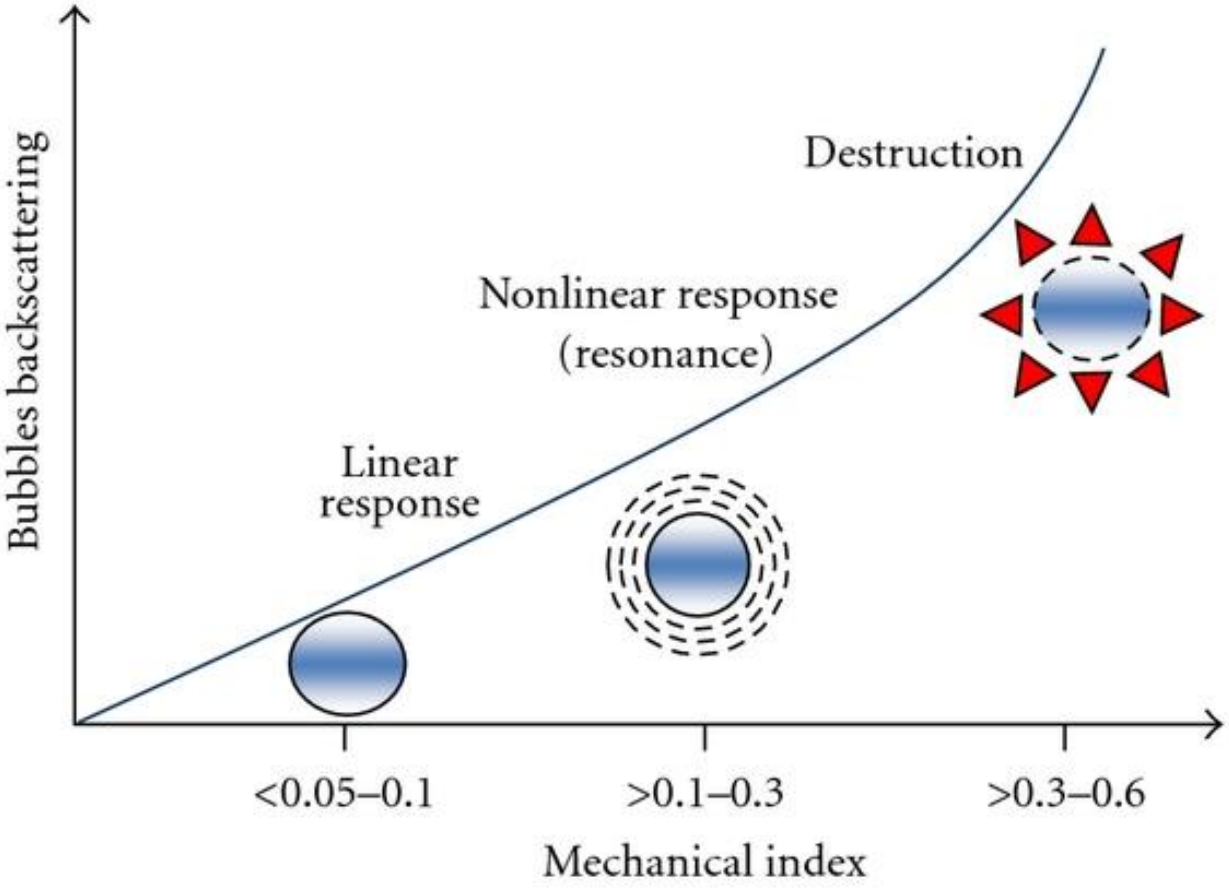
# MBs in the US Field



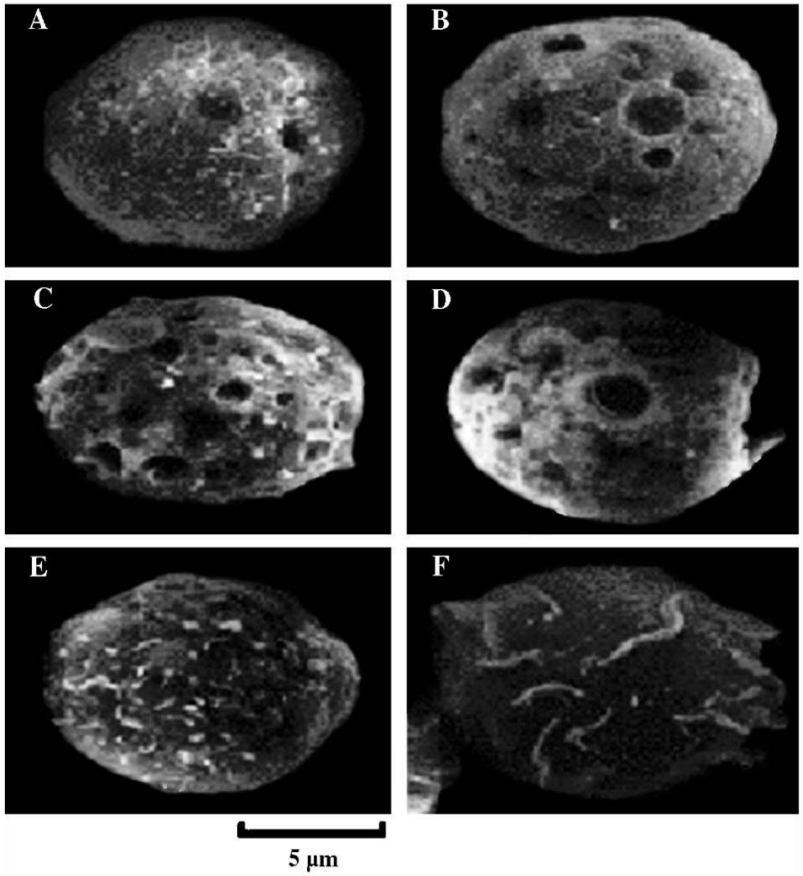
- to produce effective backscattering, MBs must be insonated by their characteristic resonant frequency

- increasing of the US frequency leads to the explosion of the MB, accompanied by a ‘shock wave’

(Quaia & Bartollota, 2005; Leighton 1997; www.escardio.org).



# Perforation of Cells by MB Shrapnels



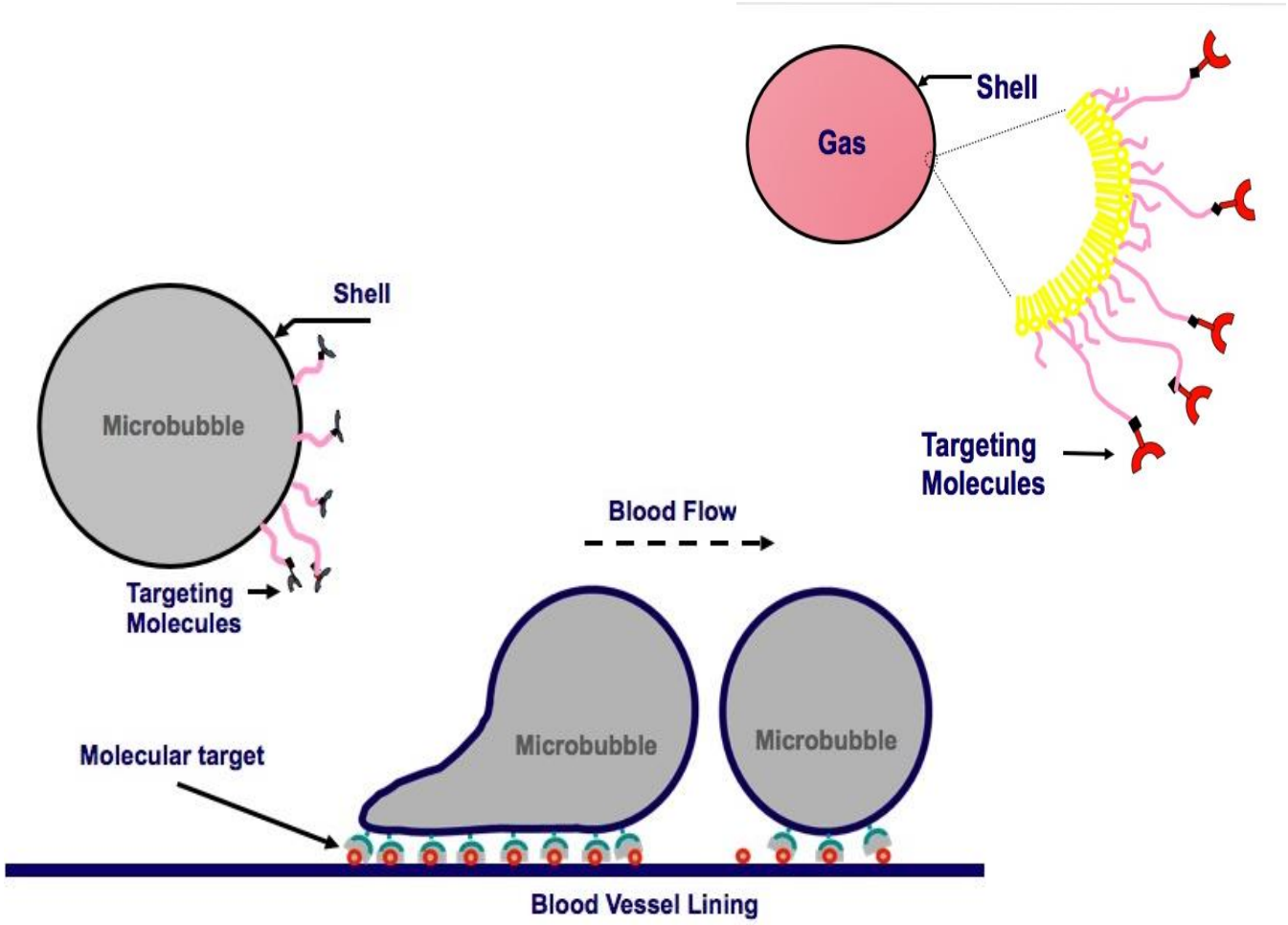
# Penetration of macromolecules into cells by sonoporation

Blue – cell nucleus, red – propidium iodide, green – fluorescein labelled dextrane )MW40 kDa)

# Functionalized MBs

- **passive targeting** - non-specific accumulation of MBs at the target site
- **active targeting** - modification of the contrast shell to allow selective binding to cellular epitopes or other receptors of interest

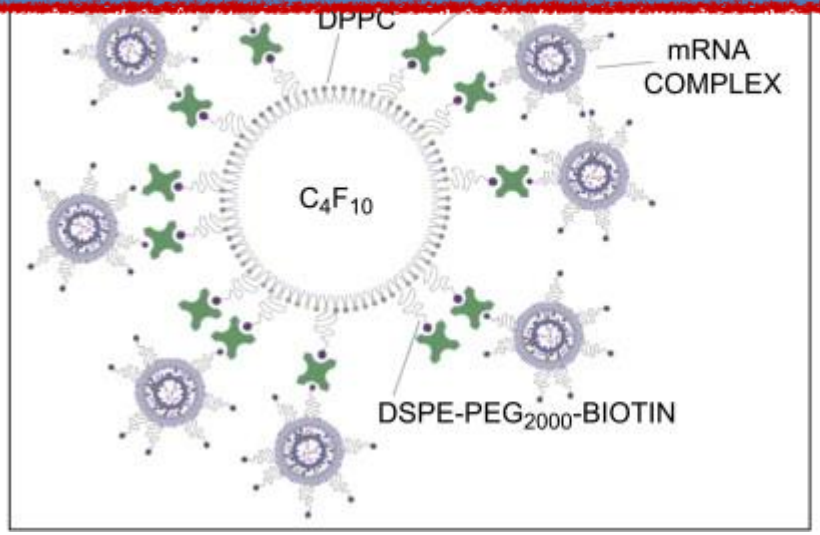
# Functionalized MBs for Targeted Delivery

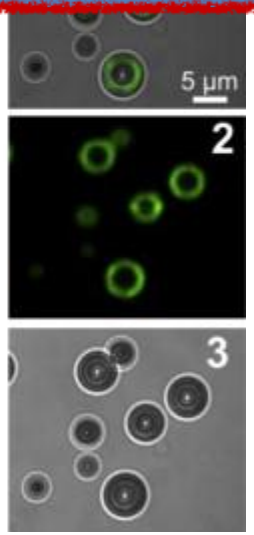


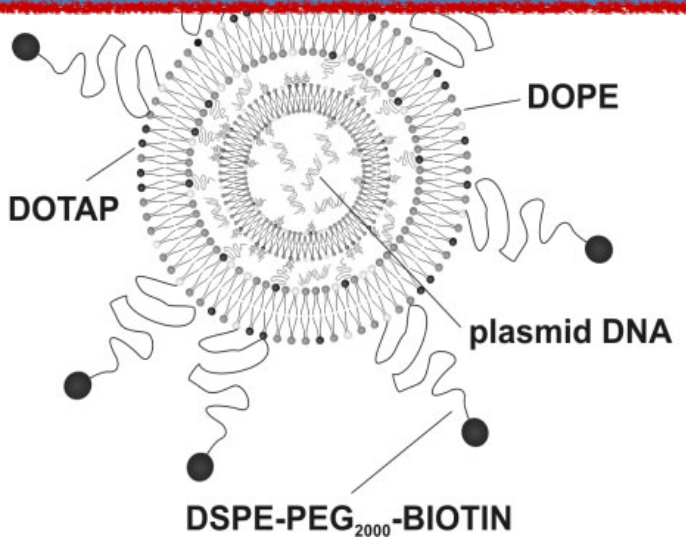
# Biotin-Avidin Coupling

- most often used non-covalent targeting technique via a hydrophobic anchor inserted into the MB monolayer shell, or via avidin-biotin sandwich (Klibanov 2005)

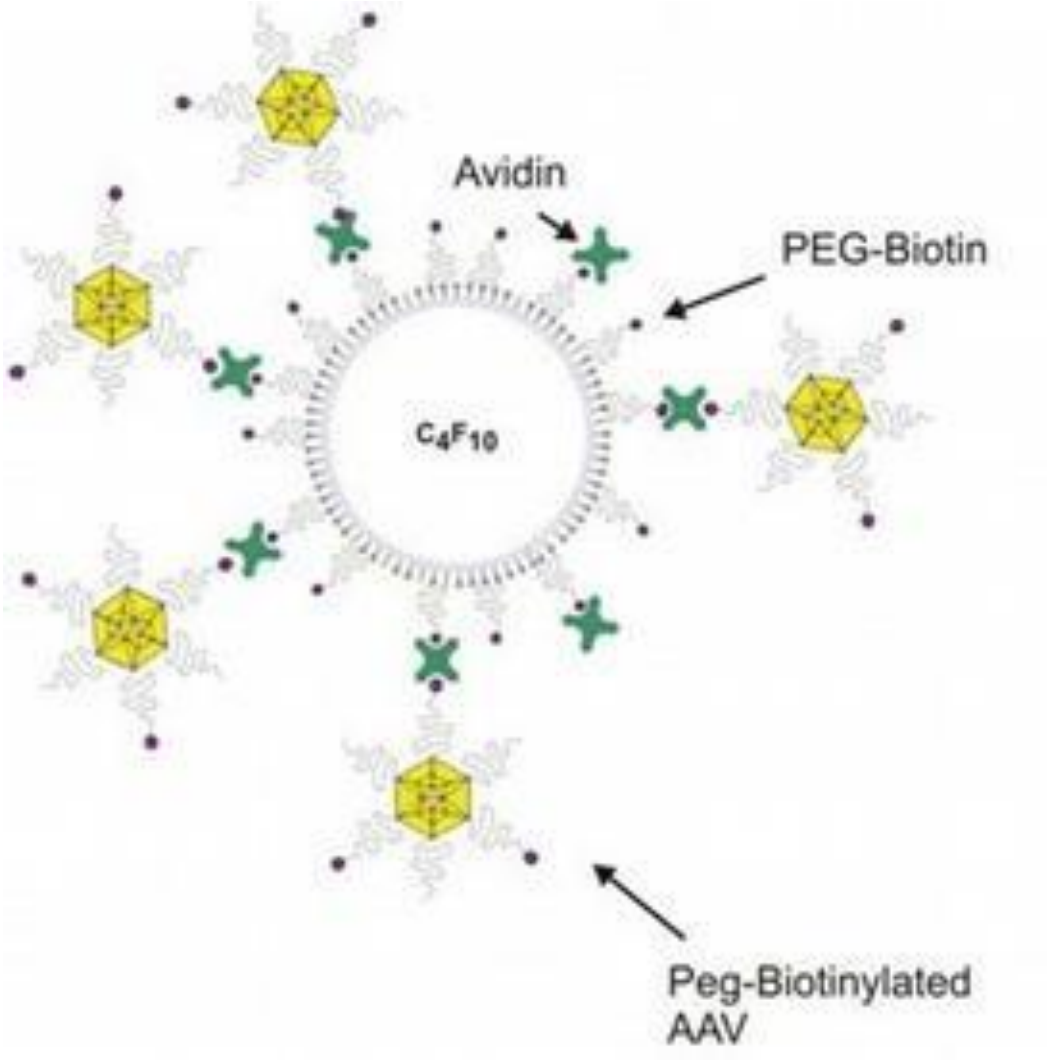
DISADVANTAGE: Avidin/streptavidin can act as foreign protein - can cause immunogenic and allergic reactions



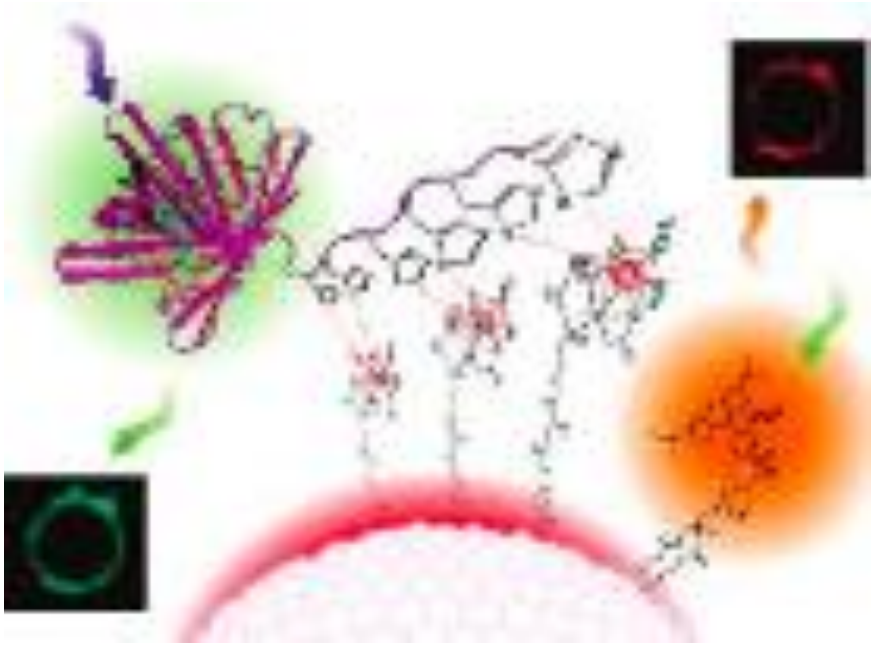



# Coupling of viruses to microbubbles



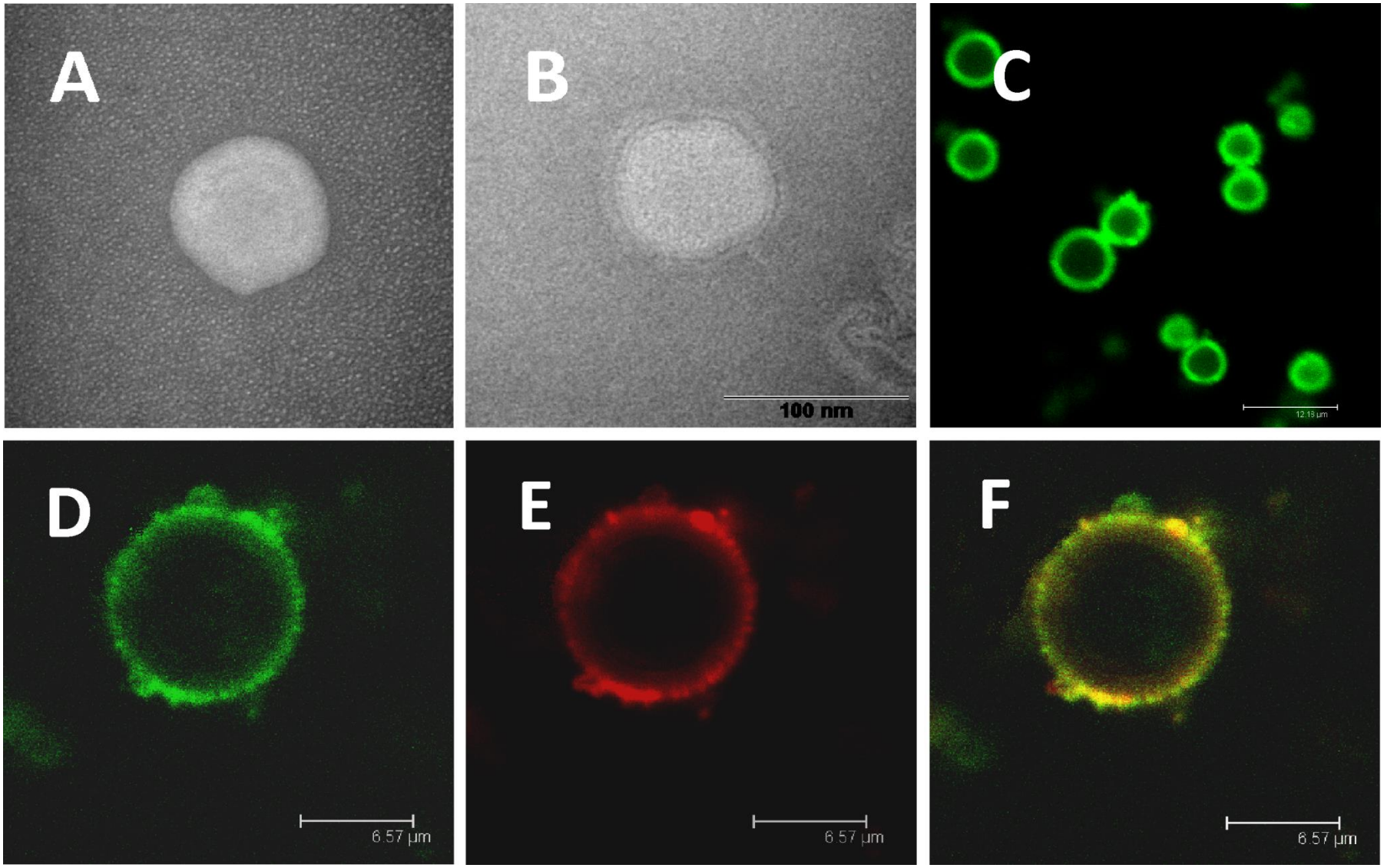
# Functionalized MBs

- **histidine-metallochelating lipid complex** - very low immunogenicity, high affinity binding units for histidine tagged molecules esp. recombinant proteins
- reversible character of the bond

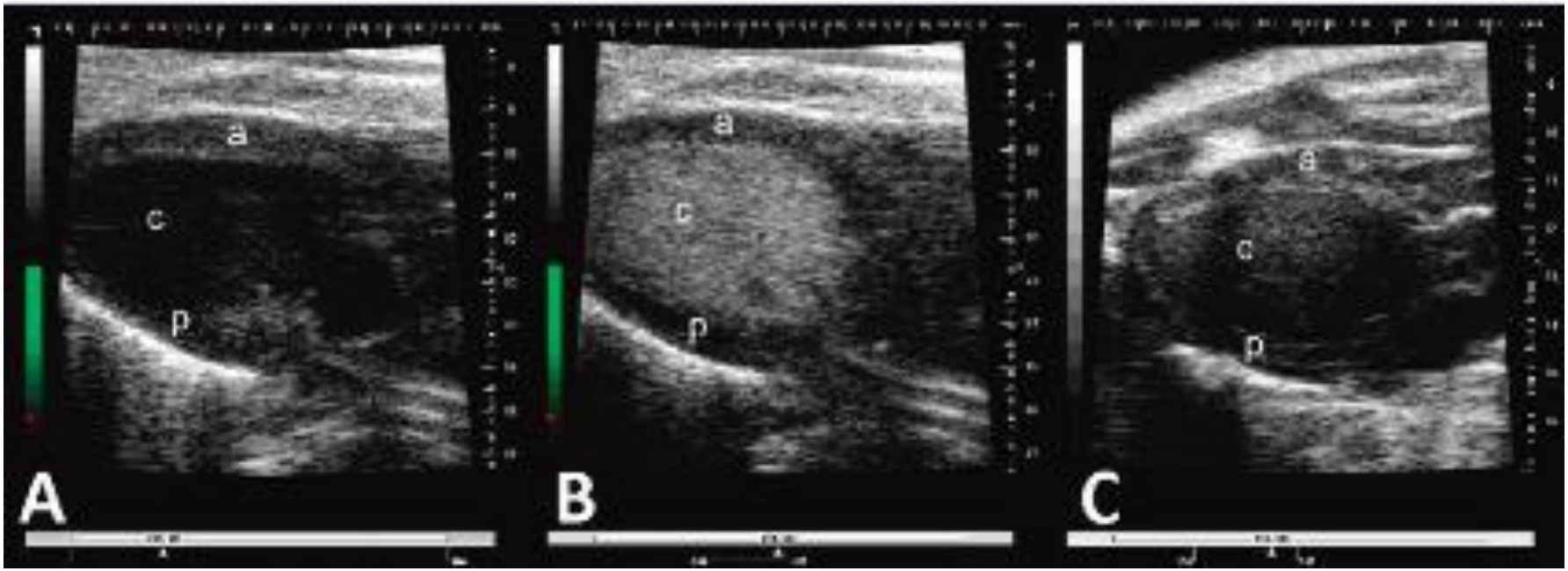


Lukáč et al. 2011

# Laser Confocal Microscopy



# CEUS




Ultrasound contrast images of the mouse heart in a long axis view. (A)Without MB. (B) After the intravenous administration of DPPC/1% DOGS-NTA-Ni MB. (C) After the intravenous administration of commercial MB by Bracco. c: left ventricle cavity; a: left ventricle anterior wall; p: left ventricle posterior wall (Lukáč et al. 2011)

# Microbubbles in the Blood Stream

- due to their size (comparable with erythrocytes), microbubbles behave as pure blood agents → capable of penetrating into small capillaries and releasing drug and genes under the action of US after reaching the area of interest

SOURCE: news.sciencemag.org

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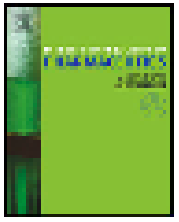

Pharmaceutical Nanotechnology

# A prototype 'Infucon' device for continuous infusion of microbubbles in vivo

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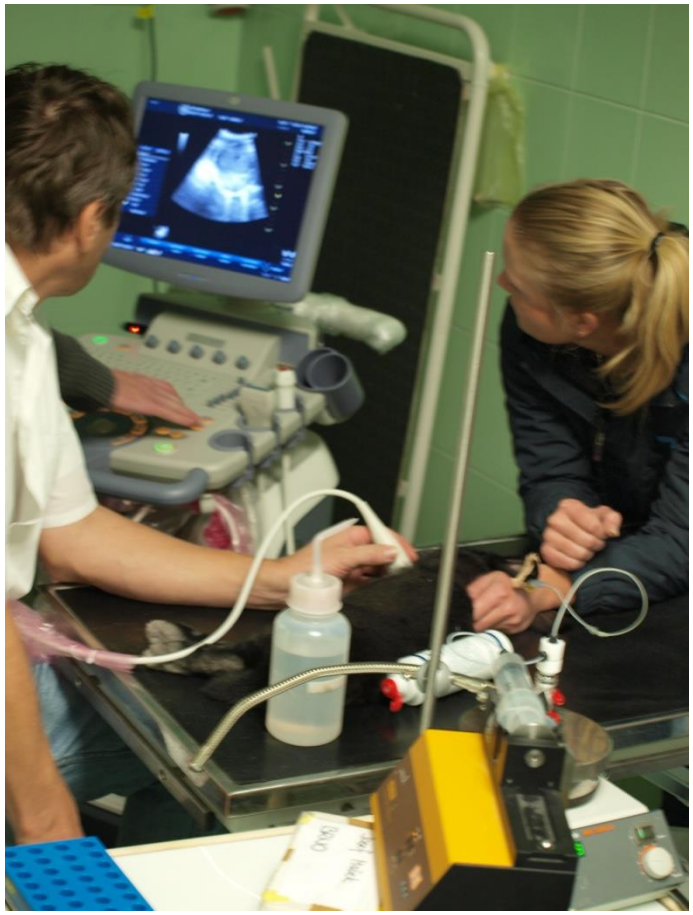
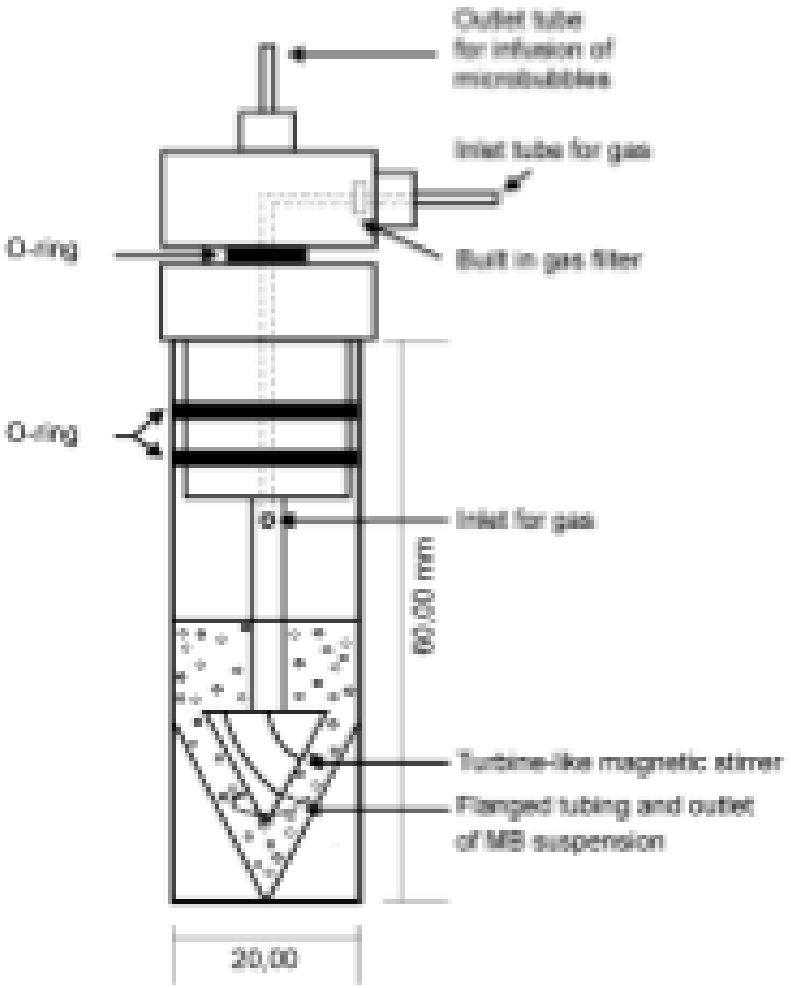
ABSTRACT

A device for continuous infusion of microbubbles (MBs) 'Infucon' has been designed, constructed and tested on rabbits. The device prevents MBs from flotation and accumulation in the layer directly below the surface in the syringe injection during i.v. application. Homogenous i.v. application of MBs was tested on 16 male New Zealand White rabbits (average weight about 3.5 kg). Two sorts of MBs were used – a set of commercial SonoVue diagnostic microbubbles (Bracco) and pegylated DPPC microbubbles (PegMBs), which had been prepared in our laboratory. Sulphur hexafluoride was used as a filling gas. The application of MBs by continuous infusion via Infucon prolonged the ultrasound signal period in the heart of the rabbit to 12 min in comparison to about 1 min observed in bolus application. No adverse effects were observed on the tested rabbits after the MB application via Infucon. The principle employed in the prototype device Infucon could be used for development of the device intended for clinical applications.

# Infucon



# To Prevent the MB Flotation




- = a special aim when constructing the device ‘Infucon’
- prevents the tendency of MBs to float and accumulate directly under the surface
- Kauerová et al. 2012

A PROTOTYPE 'INFUCON' DEVICE FOR CONTINUOUS INFUSION OF MICROBUBBLES *IN VIVO*

Zuzana Kauerová[a], Róbert Lukáč[a], Pavel Kohout[b,c], Josef Mašek[a], Štěpán Koudelka[a], Jana Plocková[a], Marta Vašíčková[c], Michal Vlašín[c] and Jaroslav Turánek[a]

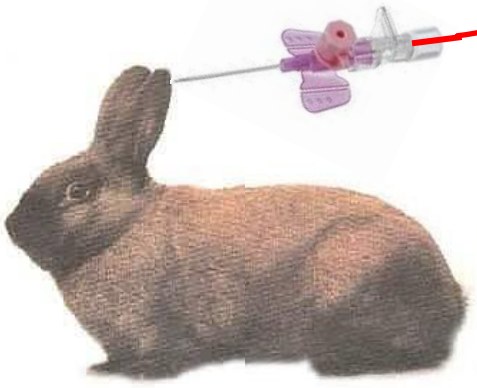
Int. J. Pharmaceutics IF 3.5

# Infucon cell

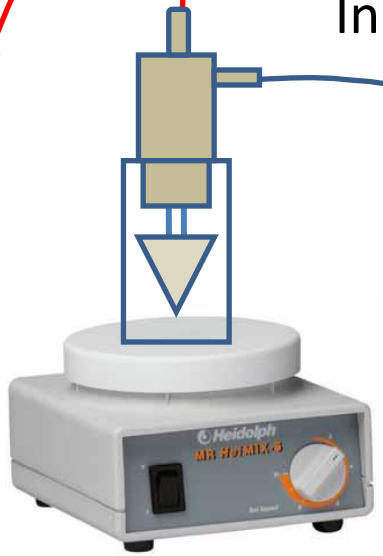
Outlet capillary

Inlet capillary

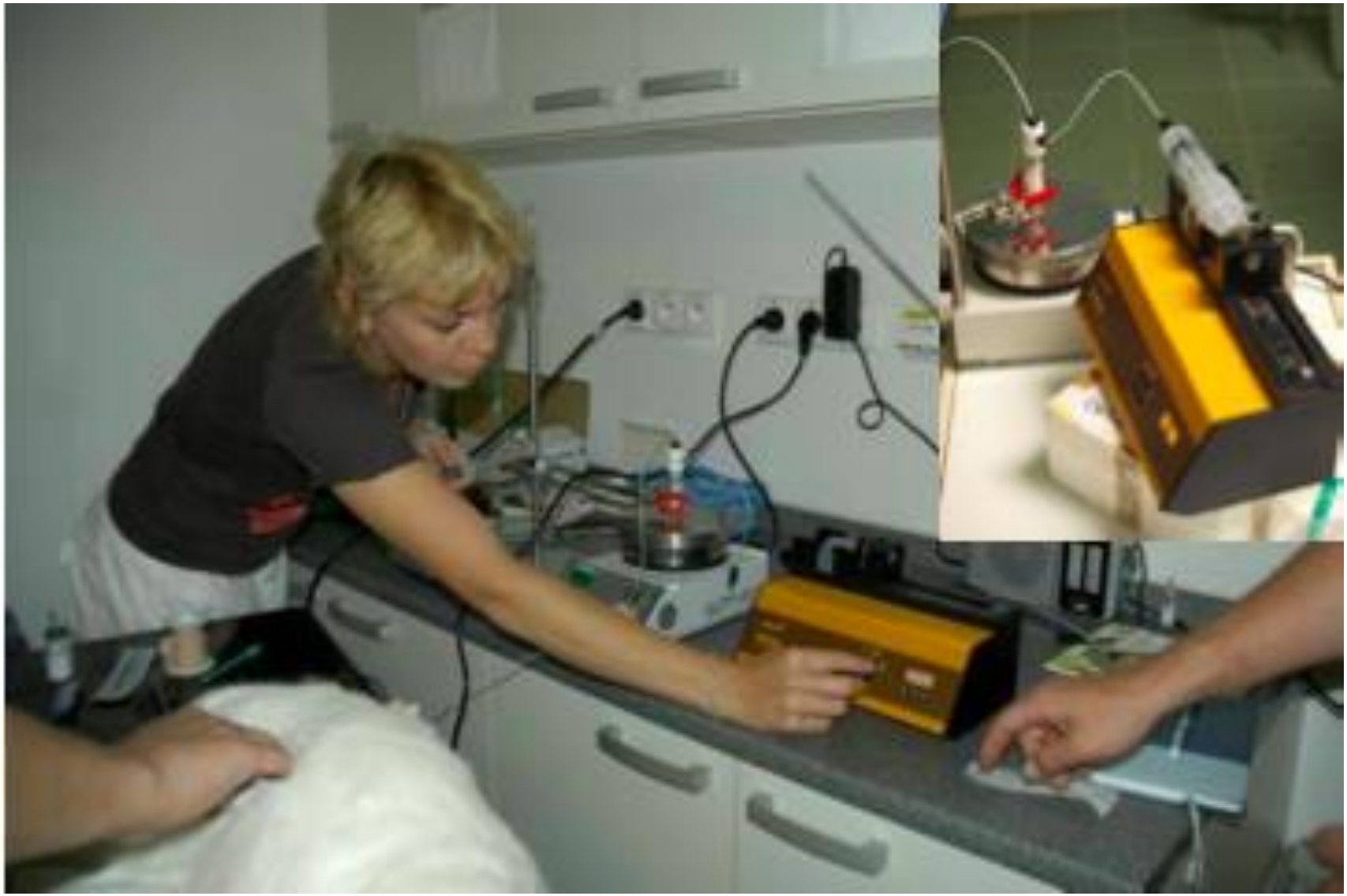
Cannula

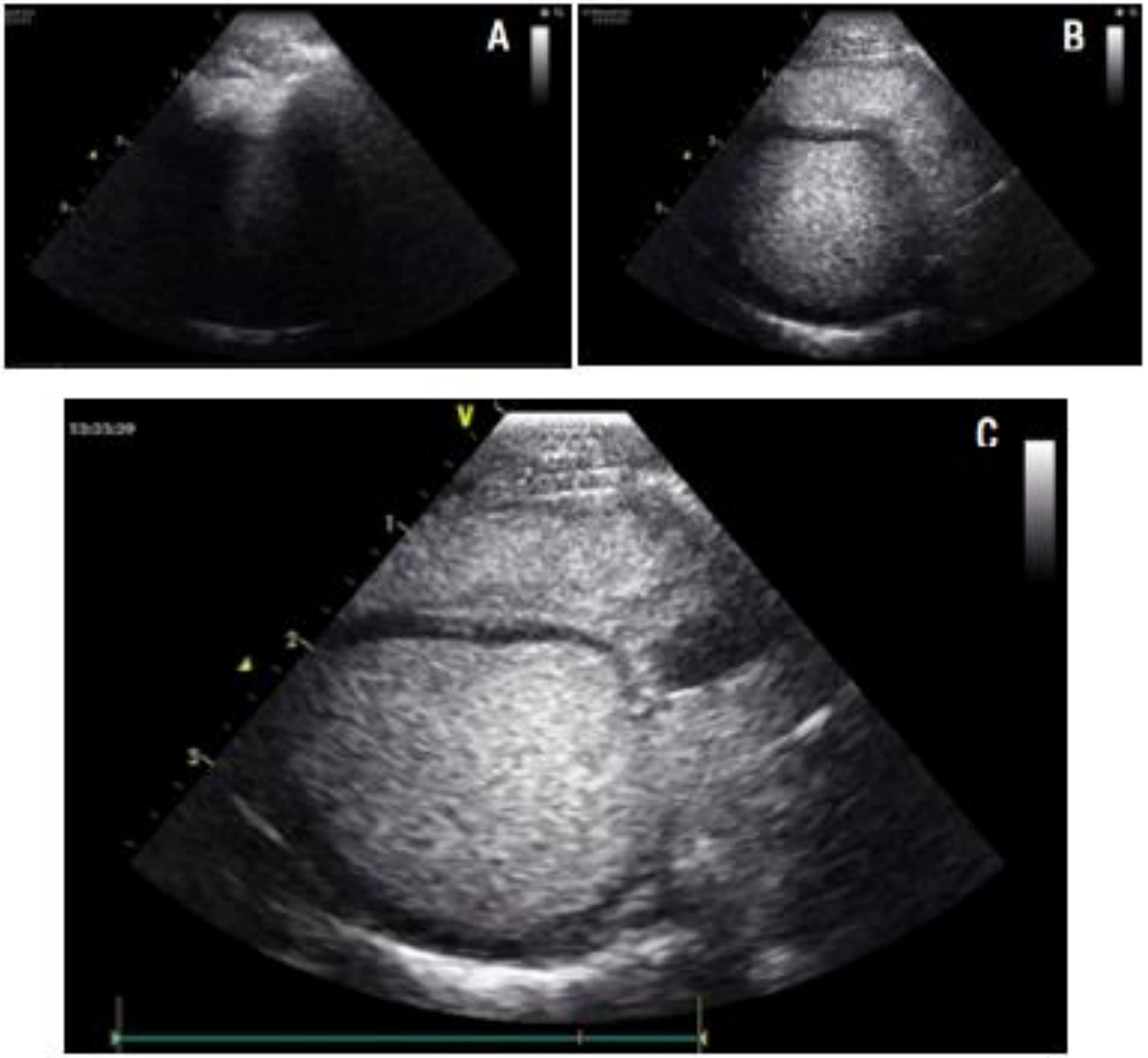


Infusion pump with gas




Magnetic stirrer






Ultrasonographic imaging of the rabbit heart: A) control B) and C) after the application of MB-based contrast agents (Kauerová et al. 2012)

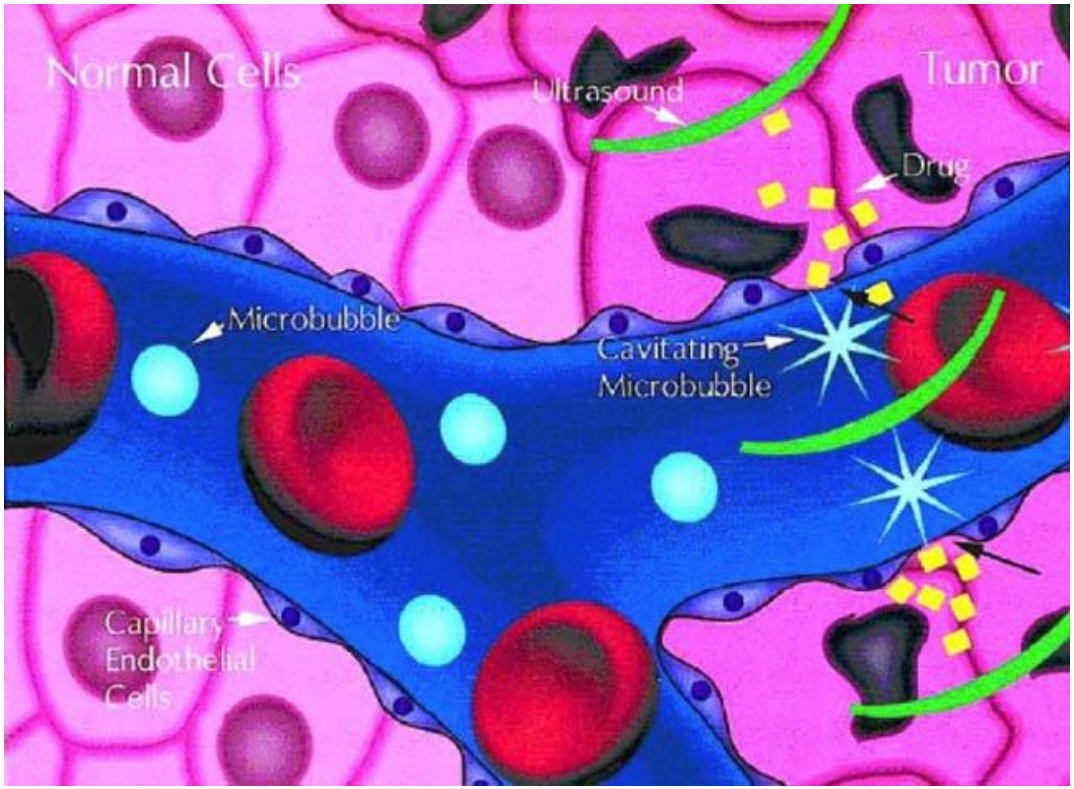
# ‘Infucon’ Results

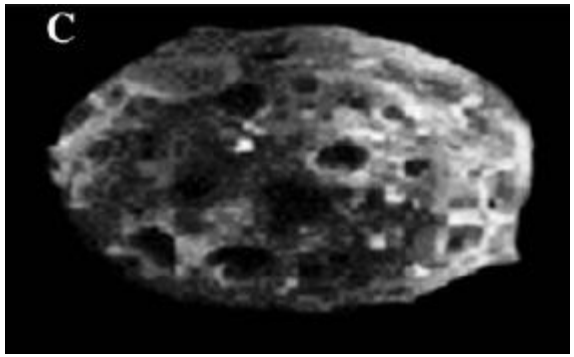
- A prototypic device for continuous infusion of MBs for clinical use was introduced, reducing the negative impact of intravenous application of MBs on their physical-chemical features
- Commercialisation of the prototype is udner development

# Clinical Use of MB Targeting

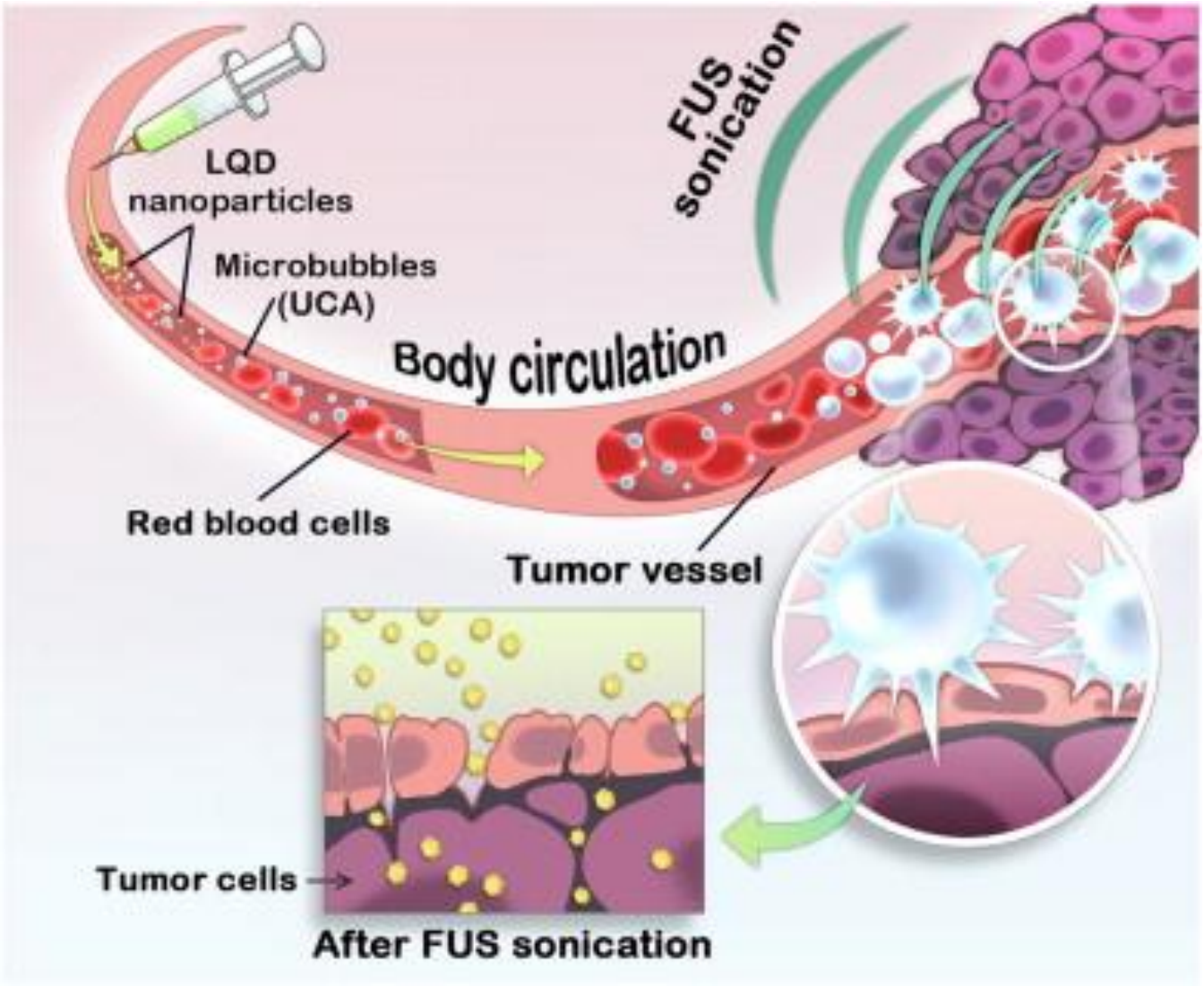
- visualization of inflammation or transplant rejection
- imaging of the angiogenesis and atherosclerosis
- Doppler studies (improvement of signal-to-noise ration in microcirculation)
- **drug and gene delivery**

# How it works




Quotation: Skyba et al., Circulation *(1998)* 290-293 and Unger et al., Echocardiography (2001) 355-361




www.sciencedirect.com (Linh et al. 2010)

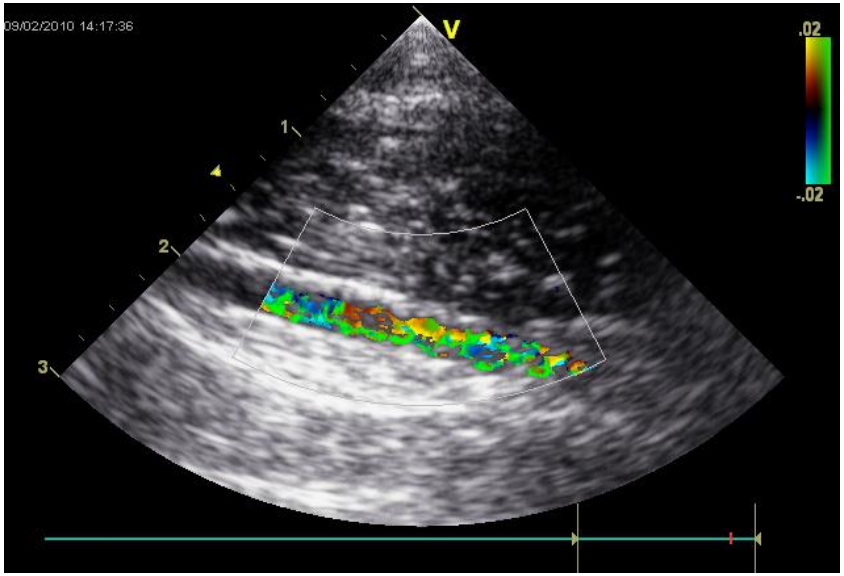
Canadian Journal of Veterinary Research


# The use of specific contrast ultrasound using sterically stabilized microbubbles for early diagnosis of thromboembolic disease in a rabbit model

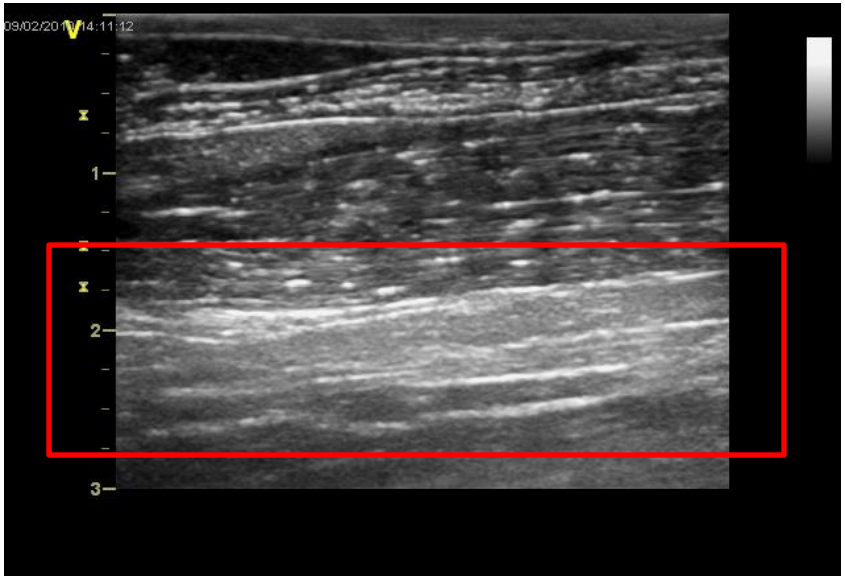
| | |
|---|---|
| Journal: | *Canadian Journal of Veterinary Research* |
| Manuscript ID: | 2012-0044.R2 |
| Manuscript Type: | Scientific Articles |
| Date Submitted by the Author: | 05-Mar-2013 |
| Complete List of Authors: | Vlasin, Michal; University of Veterinary and Pharmaceutical Sciences, Section of Small Animals Diseases; International Clinical Research Center (ICRC), St. Anne's University Hospital Brno,<br>Lukac, Robert; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy<br>Kauerova, Zuzana; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy<br>Kohout, Pavel; University of Veterinary and Pharmaceutical Sciences, Section of Small Animals Diseases; International Clinical Research Center (ICRC), St. Anne's University Hospital Brno,<br>Masek, Josef; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy<br>Bartheldyova, Eliska; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy<br>Koudelka, Stepan; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy<br>Korvasova, Zina; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy<br>Plockova, Jana; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy<br>Hronova, Nikola; University of Veterinary and Pharmaceutical Sciences, Section of Small Animals Diseases<br>Turánek, Jaroslav; Veterinary Research Institute, Toxicology, Pharmacology and Immunotherapy |

# Animal testing

Image of *aorta abdominalis in vivo*

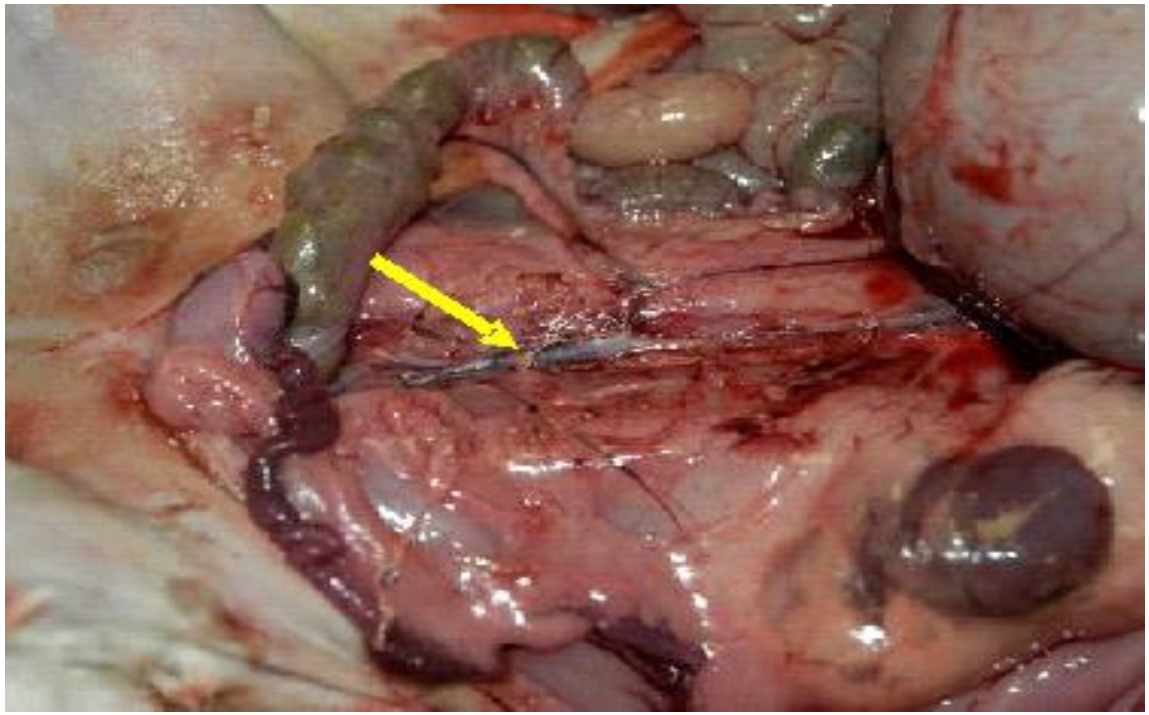


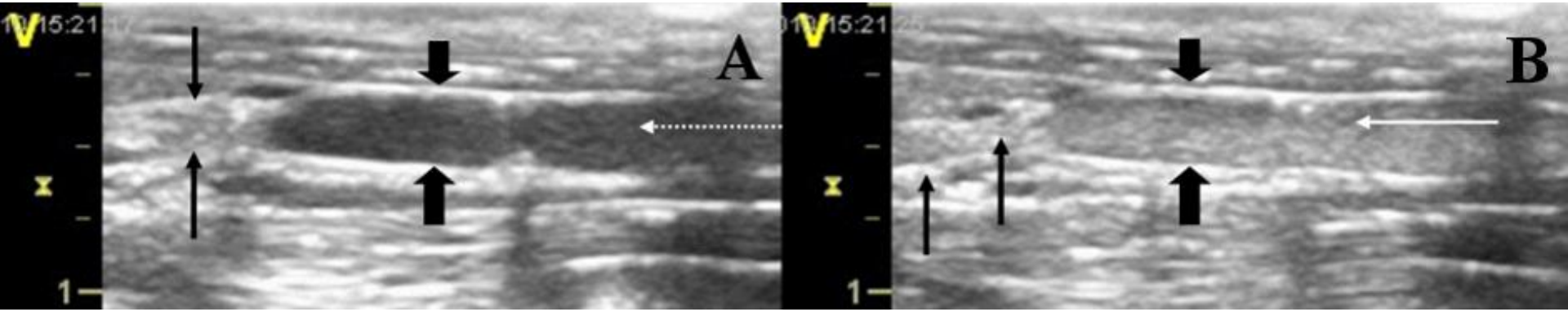

before the injection of microbubbles.




after the injection fo microbubbles.

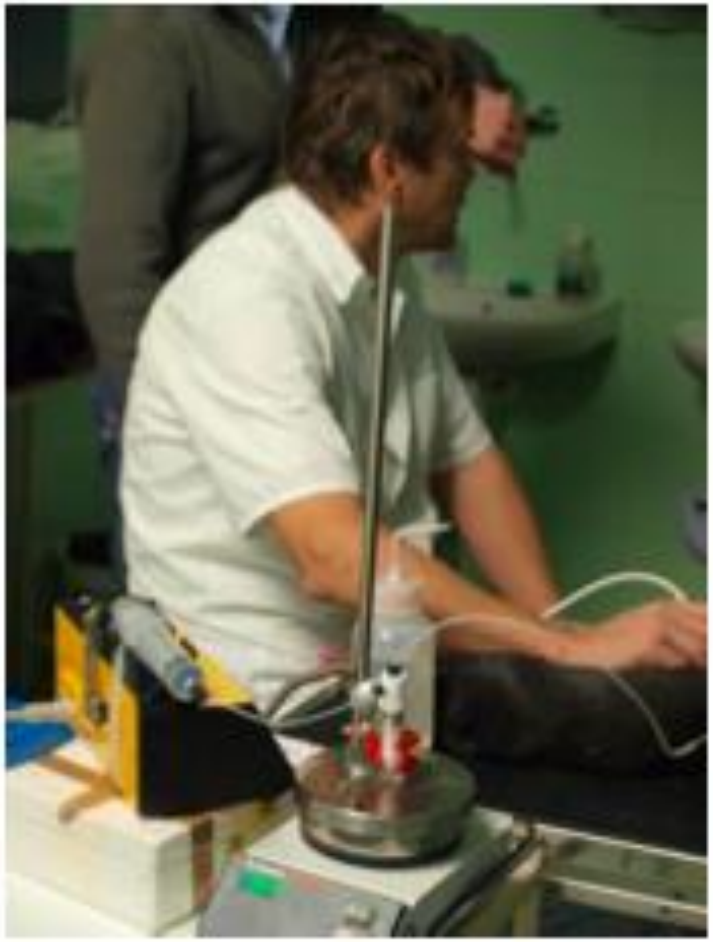
# Ultrasound diagnosis of experimental embolisation in rabbits

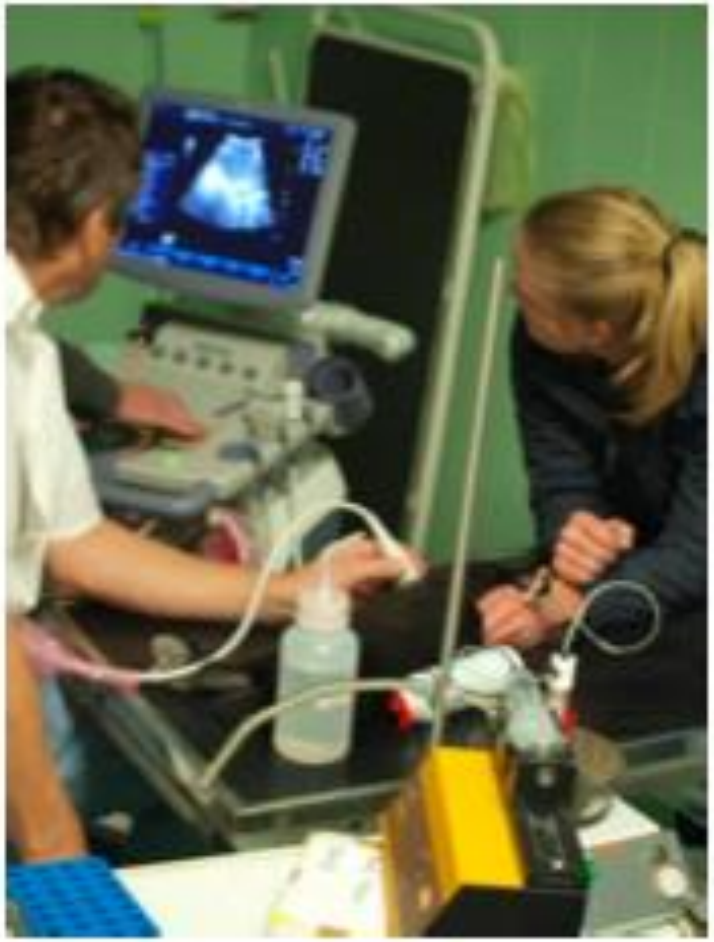





**Vlasin et al. 2013, Canadian Journal of Veterinary Medicine**

# To the team of Dr. Vlašín





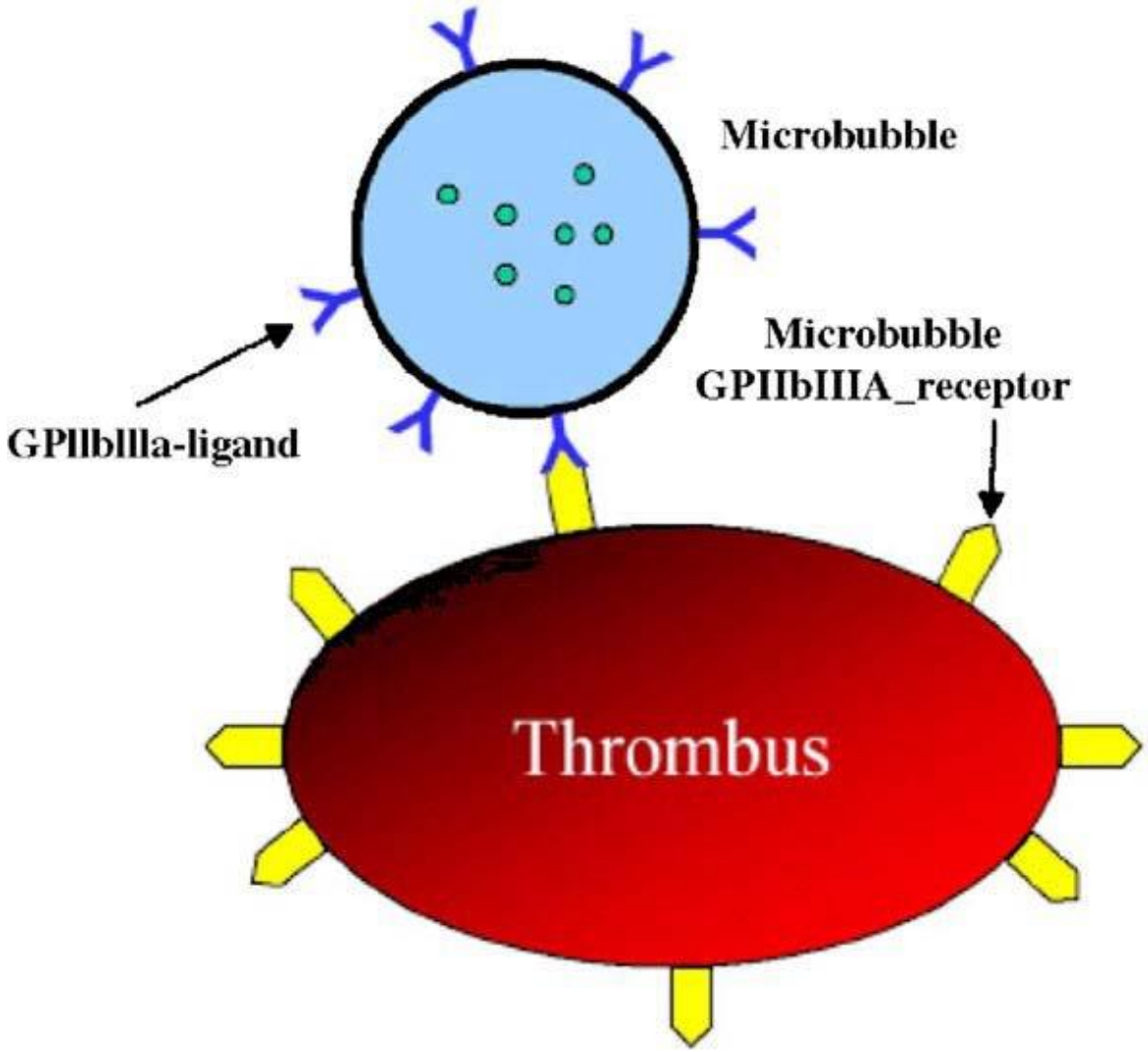
# Advantages

- Compared to a control group, the application of MBs as US contrast medium **decreased the time to clot identification ten times**
- The **transparency of the image is increased** when MBs are applied (2/3 of images - the highest degree of transparency)

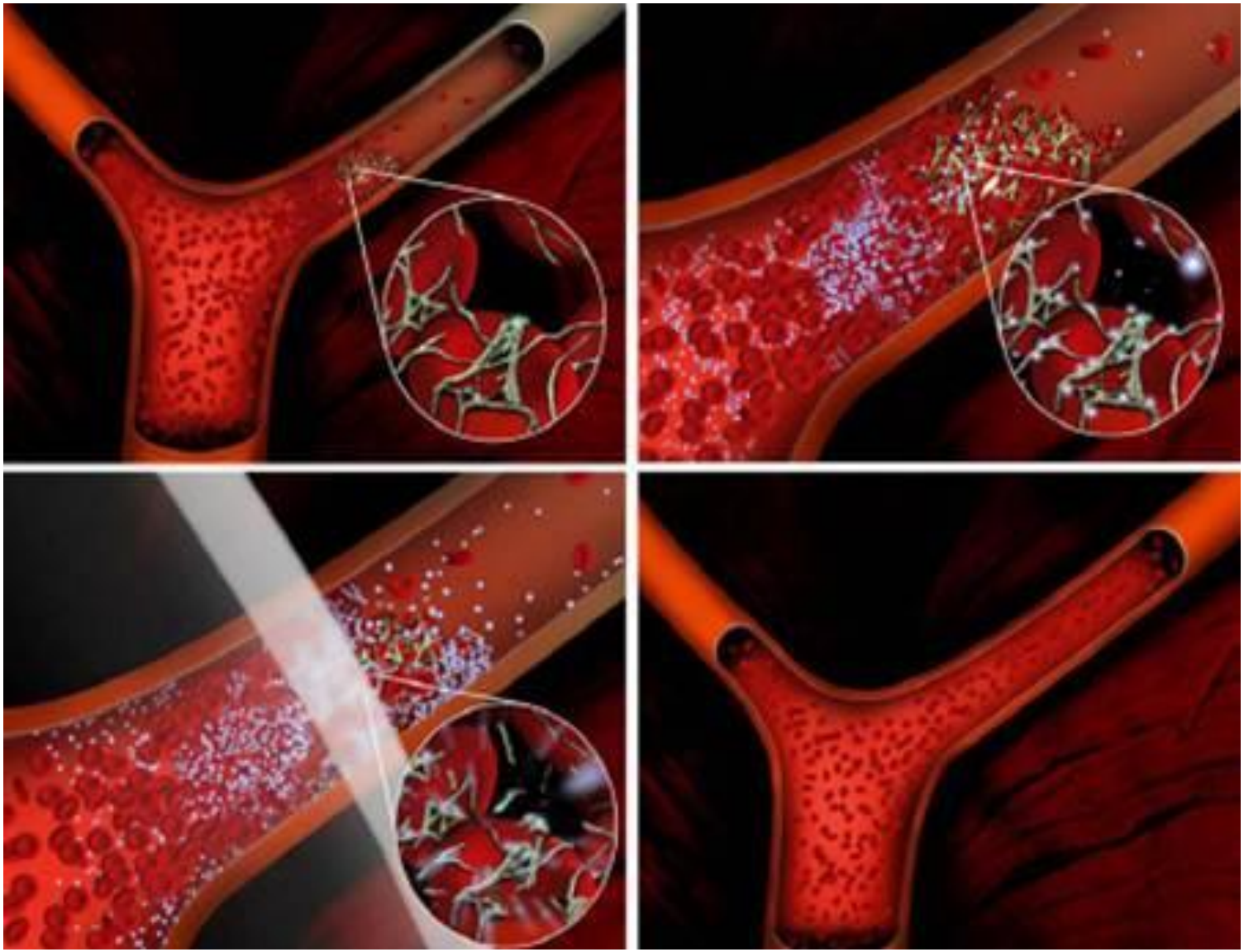
# *Vet. Can. J.* Results

- Standard DPPC MBs were produced and characterized before the application into the rabbit model
- The contrast enhanced sonography using was performed, revealing clear and easily recognizable images of the clots in thromboembolism
- The time to clot identification decreased ten times

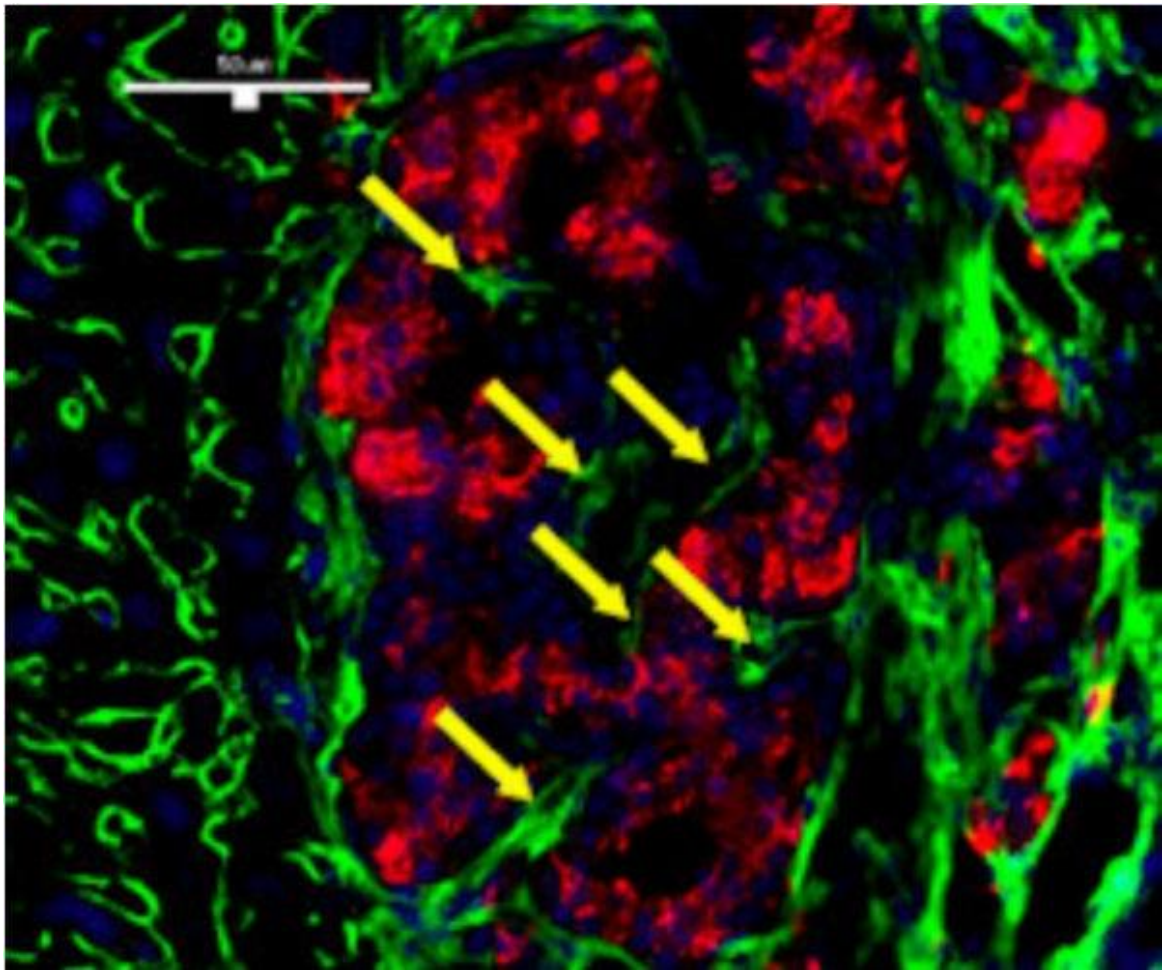
# Sonothrombolysis



# Sonothrombolysis



# Revascularization of graft islets



(day 30). Red: human insulin; green: CD31 - **Platelet endothelial cell adhesion molecule** ; blue: DAPI; arrow: vessel in islets

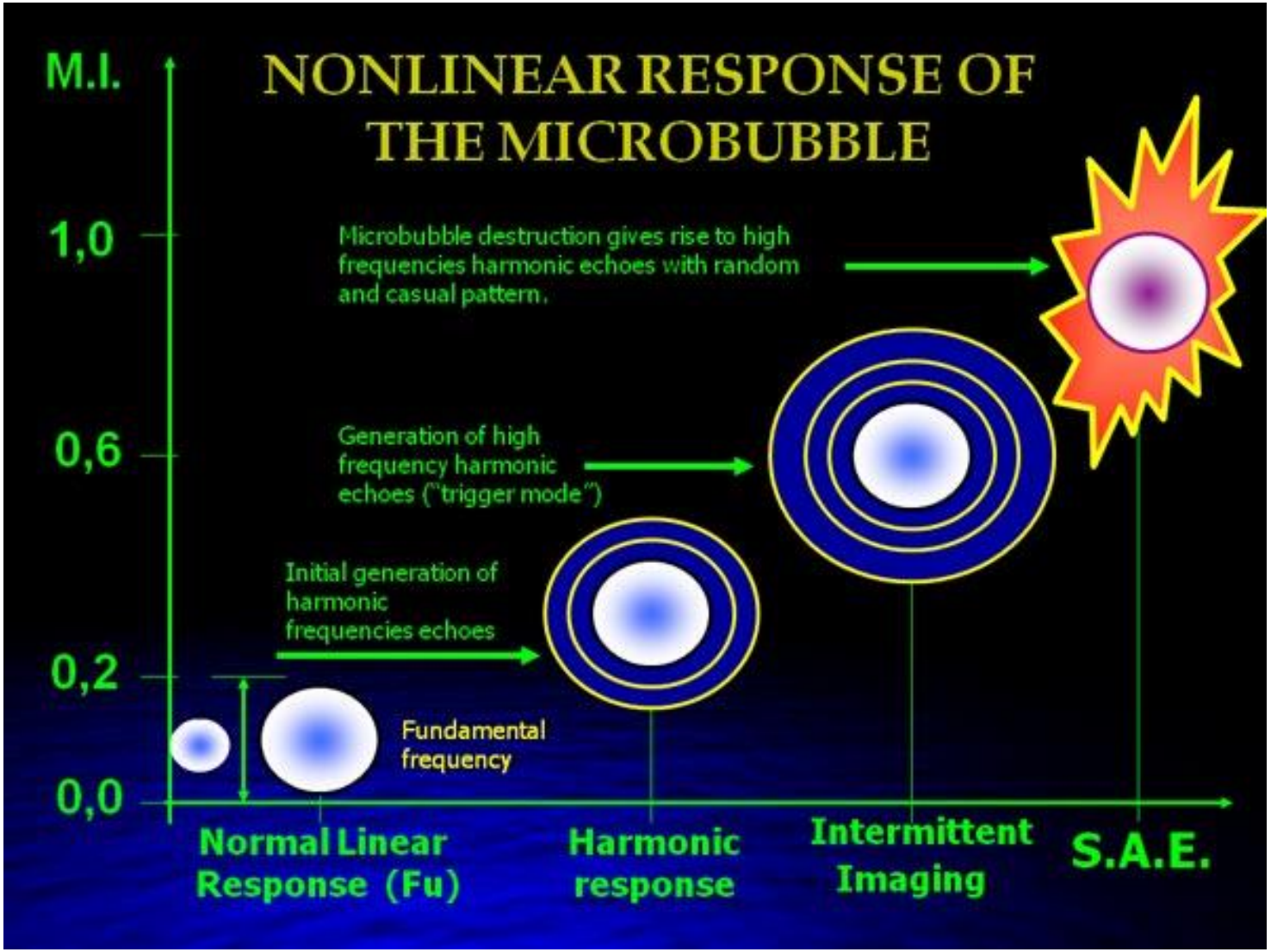
# Conclusion

- Delivery of therapeutic agents (DNA/RNA constructs, proteins, chemotherapeutic agents)
- Stable in blood circulation; US imaging.
- Visualization, size and count characterization by diferent techniques
- Size 2 – 8 μm, count $10^8$ MBs/ml
- Developed DPPC microbubbles can be modified by addition of: DOGS-Ni lipid for His-Tag binding (metalochellating binding)

# Contribution of the Grant Projects

- KAN200520703 *The Use of Ultrasound in Nanomedicine* (GAAV, finished successfully in 2011)
- KAN200100801 *Bioactive Biocompatible Surfaces and New Nanostructure Composites for Medicine and Pharmacology* (GAAV, finished successfully in 2012)

- GAP304/10/1951 *Nanoliposomes for Development of Recombinant Vaccines and Targeted Immunotherapeutics* (GAČ, till 2013)
- GAP503/12/G147 *Centre of Excellence for Nanotoxicology* (GAČR, till 2018)
- FNUSA-ICRC no. CZ.1.05/1.1.00.02.0123 from the European Regional Development Fund to M.V.


M.I.
NONLINEAR RESPONSE OF THE MICROBUBBLE
1,0
0,6
0,2
0,0
Microbubble destruction gives rise to high frequencies harmonic echoes with random and casual pattern.
Generation of high frequency harmonic echoes ("trigger mode")
Initial generation of harmonic frequencies echoes
Fundamental frequency
Normal Linear Response (Fu)
Harmonic response
Intermittent Imaging
S.A.E.