Nanoparticles for intravascular applications: physicochemical characterization and cytotoxicity testing

Aim: We report the physicochemical analysis of nanosystems intended for cardiovascular applications and their toxicological characterization in static and dynamic cell culture conditions. Methods: Size, polydispersity and ζ-potential were determined in 10 nanoparticle systems including liposomes, lipid nanoparticles, polymeric and iron oxide nanoparticles. Nanoparticle effects on primary human endothelial cell viability were monitored using real-time cell analysis and live-cell microscopy in static conditions, and in a flow model of arterial bifurcations. Results & conclusions: The majority of tested nanosystems were well tolerated by endothelial cells up to the concentration of 100 μg/ml in static, and up to 400 μg/ml in dynamic conditions. Pilot experiments in a pig model showed that intravenous administration of liposomal nanoparticles did not evoke the hypersensitivity reaction. These findings are of importance for future clinical use of nanosystems intended for intravascular applications.
system cells or cancer cells to investigate the biocompatibility of nanosystems. In the recent years, increased numbers of publications appeared concerning the possible interactions between nanoparticles and endothelial cells, which are the first contact cells in the vascular wall for circulating nanoparticles. However, these reports usually focus on one type of nanosystems in the context of endothelial viability, or barrier function [6–9]. Thus, the purpose of this work was to perform comparative physicochemical and biological analyses of different types of nanoparticles intended for intravascular applications.

The candidate nanosystems included in these analyses comprise lipid nanoparticles, liposomes, polymeric nanoparticles, as well as inorganic nanoparticles, that are briefly outlined below.

Lipid nanoparticles (Lipidots™) can be considered as nano-oil droplets stabilized by a mixture of surfactants (oil-in-water emulsion). They are composed of a lipid core, herein a mixture of soybean oil and a wax, and a surfactant shell, containing a mixture of phospholipids and polyethylene glycol (PEG)-ylated surfactants [10]. Liposomes are composed of a lipid bilayer consisting of amphipathic phospholipids (primarily phosphatidylcholine) that enclose an interior aqueous space [11]. The head groups of phospholipids are often functionalized with polymerizable moieties to improve stability (e.g., PEGylated stealth liposomes [12]), or with molecular groups, which allow conjugation to antibodies or other ligands. Among the advantages of lipid nanoparticles and liposomes as drug-delivery platforms are the ease of preparation, and the reported low immunogenicity [13,14], which is expected to enable safe and repeated administration.

Polymeric nanoparticles are composed of polymers, most commonly poly(lactic-co-glycolic acid), poly(lactic acid), poly(caprolactone), poly(alkylcyanoacrylates) or chitosan. The core of the nanoparticles used in our studies (~80% of the total mass) is made of poly(isobutylcyanoacrylate) (PIBCA), which is covalently cross-linked with polysaccharides of the coating, forming a hydrophilic shell. Functionalization of these nanoparticles allows conjugation of targeting ligands, for example, fucoidan, a mimic of siayl Lewis X, the natural ligand of P-selectin [15,16], a promising molecule to target processes upregulated during destabilization of vulnerable plaques. Inorganic nanoparticle systems included in this work comprised superparamagnetic iron oxide nanoparticles. These particles consist of an iron oxide core, which is coated with organic materials such as fatty acids, polysaccharides or polymers. Iron oxide nanoparticles have good biocompatibility and contrast-enhancing properties in MRI, enabling plaque detection and characterization [17–20]. Furthermore, the magnetic properties of these particles could allow their remote targeting by means of external magnetic field [21–23].

Although the concept of nanomedicine encompasses a localized delivery of nanosystems to the diseased organs or target tissues and minimized systemic side effects, the extended circulation time, as well as multiple degradation products, may result in nanoparticle cytotoxicity [24], or immunogenicity [25]. Hence, in order to predict in vivo responses, the toxicity of any nanosystem should first be evaluated on cultured cells (e.g., endothelial cells in the case of intravenous application, and the target cells), preferably under conditions that resemble the physiological state. Although standard cytotoxicity assays have been commonly used to estimate the cellular responses to various nanosystems, many nanoparticles interfere with the available photometric assays, which may render the experimental results difficult to assess and interpret [26]. Here, we compare nanosystems’ biological effects on primary human endothelial cells, using two complementary methods for long-term in vitro monitoring in static conditions (real-time cell analysis and live-cell microscopy), as well as an in vitro model of arterial bifurcation that allows observation of endothelial cells under physiologic-like flow conditions. Furthermore, we report the initial results of the pilot studies on the complement activation-related pseudoallergy (CARPA) upon the intravenous administration of liposomal nanoparticles in a pig model.

Materials & methods

In total, ten nanoparticle systems were synthesized and investigated, including two types of liposomes (LP-NPs), three types of lipid nanoparticles (LD-NPs), two types of polymeric nanoparticles (PM-NPs) and three types of iron oxide nanoparticles (IO-NPs). The detailed description of nanoparticle characterization is provided in the Online Supplement.

Reagents

Soybean oil and Myrl™ s40 (PEGylated surfactant) were purchased from Croda, Chocques, France. Lipoid S75 and dipalmitoylphosphatidylcholine were from Lipoid GmbH, Ludwigshafen, Germany. Suppocire NB was from Gatefosse, Saint-Priest, France. 1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol, 1,2-distearyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG-2000) and 1,2-distearyl-phosphatidylethanolamine-methyl-polyethylene glycol conjugate-550 (DSPE-PEG550) were purchased from Avanti Polar Lipids, Inc. (AL, USA).
Nanoparticles for intravascular applications: physicochemical characterization & cytotoxicity testing  
Research Article

Dextran T70 was from Roth (Karlsruhe, Germany), or from Amersham Pharmacia Biotech (Freiburg, Germany), and dextran T40 from PharmaCosmos (Holbaek, Denmark). Carboxymethyl-dextran sodium salt (CM-Dextran) was purchased from Sigma Aldrich (Saint Quentin Fallavier, France) and diethylamino-ethyl-dextran 20 (DEAE-dextran) from TdB Consulting (Uppsala, Sweden). Low molecular weight Fucoidan (3–8 kDa, Fucoidan Ascophysiænt®) was from Algues et Mer (Ouessant, France). IBCA (isobutylcyanoacrylate, Glue 368) was from Orapi (Saint-Vulbas, France). Bovine serum albumin (BSA) and iron (II) chloride tetrahydrate were from Merck, Darmstadt, Germany. Lauric acid, epichlorohydrin and dextran T6 (Mw = 6 kDa) were from Sigma Aldrich, Munich, Germany. Ceric (IV) ammonium nitrate and tri-sodium citrate dihydrate were purchased from Fluka (Saint Quentin Fallavier, France). NaOH, HCl (25%), NH₃ (25%) and nitric acid (65% w/w) were from Roth. Iron (III) chloride hexahydrate was purchased from Sigma-Aldrich or from Roth. All compounds used were of pharmaceutical (Ph. Eur) or highly pure (≥99%) grade and were used without any further purification.

**Nanoparticle synthesis**

**Lipidots**

Lipid nanoparticles (LD-NP) were prepared by the sonication method [10]. Briefly, the lipid phase was prepared by mixing Suppocire™ NB, soybean oil and lipid S75. The aqueous phase, containing Myrj s40 (PEGylated surfactant) in phosphate buffered saline (PBS), was heated to 50°C to melt the surfactant and then mixed with the lipid phase. Following sonication for 5 min, the samples were dialyzed against PBS and sterilized by filtration through a 0.22 µm filter. The batches of particles with specified diameter were obtained by altering the lipid and surfactants ratios. Three different sizes (diameters) were formulated: 50 nm (LD-NP1), 80 nm (LD-NP2) and 120 nm (LD-NP3).

**Liposomes**

For the synthesis of sterically stabilized PEGylated liposomes (LP-NP1), POPC, cholesterol (Avanti Polar Lipids) and DSPE-PEG-2000 were used. Lipids were dissolved in chloroform-methanol 2:1 (v/v) at molar ratios of 3:2:0.15. LP-NP1 were made using a dry film rehydration technique, followed by size extrusion, as described previously [12,27]. Briefly, the organic solvent was evaporated under a stream of nitrogen to obtain a lipid film. For complete removal of solvents the film was dried in a vacuum chamber overnight. Subsequently, the dry lipid film was hydrated in PBS and size-extruded using an Avanti Polar mini-extruder (Avanti Polar Lipids, Inc.) and 100 nm polycarbonate membranes.

The LP-NP2 liposomes were prepared using the lipid injection method, by mixing the ethanolic lipid solution with the aqueous phase under magnetic stirring at 60°C. Briefly, dipalmitoylphosphatidylcholine, cholesterol (BUFA, Uitgeest, The Netherlands), and DSPE-PEG550 were dispersed in ethanol at molar ratios of 1.85:1:0.15. Subsequently, the lipid solution was transferred into PBS previously heated to 60°C. The resulting emulsion was downsized by multiple extrusion steps through polycarbonate filter membranes with decreasing pore sizes of (200–100 nm). Subsequently, ethanol and dissolved lipids were removed by dialysis against PBS.

**Polymeric nanoparticles**

Polymeric nanoparticles (PM-NP) were synthesized by a redox radical emulsion polymerization method. This method ensures the polysaccharides of the shell to assemble into a brush-like layer of coating. Here, two different coatings were used: 90% CM-Dextran/10% Fucoidan (PM-NP1); and 80% Dextran T70 (Pharmacia Biotech)/10% DEAE-dextran/10% Fucoidan (PM-NP2). Briefly, polysaccharides (0.1375 g) were dissolved in a nitric acid solution (2 × 10⁻¹ M) at 40°C and left under nitrogen bubbling for 10 min. Polymerization was initiated by adding 2 ml ceric (IV) ammonium nitrate solution (8 × 10⁻² M) in nitric acid and 0.5 ml of IBCA monomers to the polysaccharide solution under nitrogen atmosphere and vigorous stirring. The reaction was then left for 40 min at 40°C under gentle stirring, followed by cooling to room temperature. Subsequently, 1.25 ml of a trisodium citrate dihydrate solution (1.02 M) was added to the suspension and the pH was adjusted to 7.0 with NaOH. PM-NPs were purified by dialysis (molecular weight cut-off 100 kDa) against water. One final dialysis was performed against NaCl 0.9%. Nanoparticles were sterilized by filtration through a 0.45 µm filter, followed by 15 min exposure to UV radiation.

**Iron oxide nanoparticles (IO-NP)**

Lauric acid/BSA-coated magnetite nanoparticles (IONP) were synthesized by coprecipitation, subsequent in situ coating with lauric acid and formation of an artificial albumin corona as previously described [28]. Briefly, Fe (II) and Fe (III) salts at a defined molar ratio (Fe³⁺/Fe²⁺ = 2) were dissolved in 20 ml of water and stirred at 80°C under argon atmosphere, followed by addition of 20 ml of NH₃ solution (25%). The solution was heated to 90°C and 1.25 g lauric acid, dissolved in acetone, was added. The brownish suspension was left to homogenate for 30 min at 90°C. The suspen-
sion was then dialyzed multiple times against water. Subsequently, IO-NP1 were stabilized by incubation with a freshly prepared 20% BSA solution, purified by centrifugal ultrafiltration (molecular weight cutoff 100 kDa), and sterilized by filtration through a 0.22 μm filter.

For preparation of dextran-coated magnetite nanoparticles (IO-NP2), the synthesis method by Unterweger et al. was used [29]. Briefly, Fe (II) and Fe (III) salts in molar ratios (Fe$^{3+}$/Fe$^{2+}$ = 2) as well as 1.75 g of dextranT6 were dissolved in water. After cooling to 4°C under continuous stirring and argon atmosphere, 5 ml of ice-cold 25% NH$_3$ was added. After 5 min, the reaction mixture was heated and kept at 75°C for a further 40 min, followed by cooling to RT and dialysis (molecular weight cut-off 8 kDa). The mixture was then cleared from excess dextran and concentrated to a total volume of 20 ml using ultrafiltration (molecular weight cut-off 100 kDa). To stabilize the dextran coating, crosslinking was performed by adding 4 ml of epichlorohydrine dropwise to the nanoparticles suspension after alkalization with NaOH under vigorous stirring for 24 h. The solution was then dialyzed against water, concentrated by ultrafiltration and sterile filtered through 0.22 μm membrane.

IO-NP3 nanoparticles were also synthesized by the coprecipitation method. Briefly, Fe (II) and Fe (III) salts at a defined molar ratio (Fe$^{3+}$/Fe$^{2+}$ = 2) were dissolved in water under nitrogen atmosphere, followed by addition of a preheated strong alkali solution under continuous stirring. In the second step, the coating material (carboxydextran) was added to the aqueous suspension of iron oxide nanoparticles followed by heating under reflux. After cooling, the resulting material was filtered and dispersed in water, followed by dialysis against water, and sterile filtration using a 0.45 μm filter.

**Physicochemical characterization & stability on storage**

Z-averaged hydrodynamic diameter, polydispersity (PDI) and ζ-potential were determined with a Zetasizer Nano ZS (Malvern) using standard polystyrene cuvettes and disposable folded capillary cells (DTS1070), respectively. All samples were diluted prior to the measurement according to the producers’ instructions: LD-NPs, LP-NP1 suspensions were diluted 10× in deionized and 0.2 μm-membrane filtered water, LP-NP2 were diluted 10× in PBS pH 7.4, and PM-NPs and IO-NPs were diluted between 50× and 100× in ultrapure water. Samples were equilibrated to 25°C and 3 × > 10 runs of 10 s performed in 173° backscatter mode. The employed refractive indices and absorption coefficients for the different particles systems were: LD-NPs, 1.5, 0.1; LP-NP1, 1.4, 0.001; LP-NP2, PM-NPs, 1.59, 0.01; IO-NPs, 2.9, 5.18. To determine sample stability, the first time-point for physicochemical characterization was set at 1 month post synthesis date, followed by the subsequent measurements after 3 and 6 months of storage at 4°C. The detailed description of further characterization methods relevant for the respective nanoparticle types is provided in the Online Supplement.

**Real-time cell analysis**

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords (kindly provided by the Department of Gynecology, University Hospital Erlangen) and cultured as described in the Online Supplement. In all experiments, HUVECs at passage 1–2 were used. The use of human material was approved by the local ethics committee at the University Hospital Erlangen (review number 237_12B from 19.09.2012). For monitoring the effects of nanoparticles on HUVEC viability, the xCELLigence system (RTCA DP Analyzer, Roche Diagnostics, Mannheim, Germany) was used [30]. Experiments were performed in 16-well E-plates (ACEA Bioscience, CA, USA), in which the impedance is measured with the help of microelectrodes localized at the bottom of the wells (for detailed description, see Online Supplement).

For the background measurement, 100 μl of cell-free endothelial cell growth medium was added to the wells. Afterwards, 50 μl of media from each well were replaced with 50 μl of cell suspension containing 1 × 10³ HUVECs. About 30 min after seeding of the cells, monitoring of impedance by the xCELLigence system was initiated. At 24 h after seeding, an additional 100 μl of media containing different concentrations of nanoparticles were added to the wells, as follows: (a) for controls, 100 μl of pure medium without nanoparticles, and (b) for the treatment samples, 100 μl of medium containing nanoparticles at concentrations 2× higher than the required final nanoparticle concentration. The final nanoparticle concentrations were as follows: 0, 12.5, 25, 50, 100, 200 and 400 μg/ml. Concentrations for iron oxide nanoparticles were calculated as total iron (Fe) concentration. The concentrations for lipid nanoparticles, liposomes and polymeric nanoparticles were calculated as total dry mass weight per volume. Cell growth was monitored every 10 min for 96 h. The experiments were performed in hexaplicate.

**Live-cell microscopy**

HUVECs were seeded in 96-well plates at 2 × 10³ cells/well in 100 μl medium. At 24 h after seeding, additional 100 μl of media containing different con-
centrations of nanoparticles were added to the wells, as described in detail above. Cell growth was monitored for 72 h using a live cell-imager (IncuCyte FLR microscope system, Essen Bioscience, MI, USA). The experiments were performed in hexaplicate.

Flow experiments
Flow experiments were performed as previously described (see [31] and Online Supplement). For the perfusion with nanoparticles, two different concentrations were used (100 μg/ml and 400 μg/ml). Subsequently, HUVECs were stained with Alexa488-phalloidin (PromoKine, Heidelberg, Germany). Cell nuclei were counterstained with DAPI (Molecular Probes, Darmstadt, Germany). Images were obtained using fluorescence microscope Zeiss Axio Observer. Z1 (Carl Zeiss AG, Oberkochen, Germany). The confluence was determined on ×10 objective magnification images using ImageJ software.

Pig model of complement activation-related pseudoallergy (CARPA)
Pilot studies in a pig model of infusion reaction to LP-NP1 were performed as described by Szebeni [32]. Briefly, domestic male Yorkshire pigs (20–25 kg) were anesthetized with isoflurane (2–3% in O₂). Intubation was performed with endotracheal tubes to maintain free airways. Oxygen saturation was monitored using a pulse-oximeter fixed on the tail, and body temperature was monitored rectally. A capnograph was connected to the tracheal tube to monitor EtCO₂ and the respiratory rate (CAP10, Medlab GmbH, Karlsruhe, Germany). To measure the pulmonary arterial blood pressure (PAP), a Swan-Ganz catheter (AI-07124, 5 Fr. 110 cm, Arrow International, Inc., Teleflex, Athlone, Ireland) was introduced into the pulmonary artery via the right external jugular vein – right atrium – right ventricle, while systemic arterial blood pressure (SAP) was measured in the femoral artery. LP-NP1 and zymosan were injected into the pulmonary artery via the left external jugular vein. The amount of test material injected is given as mg phospholipids/kg, unless otherwise indicated. Hemodynamic changes were continuously monitored at 1000 Hz sampling rate, using an AD Instruments PowerLab System with LabChart Pro v6 software. From the mean PAP, SAP and heart rate (HR) data about 20 s intervals were averaged and evaluated by AD Instruments LabChart Pro v6 software modules. The usual evaluated periods were: before the test material injection, then 20 s in every minute for 10 min, and every 5 min until the end of the reaction. Plasma levels of thromboxane B2 (TXB2, the stable metabolite of TXA2) were measured with a commercially available ELISA kit (Cayman Chemicals, MI, USA). The study was approved by the local ethics committee for animal experimentation.

Statistical analyses
The differences between the in vitro treatment groups were calculated using ANOVA on Ranks. Data were expressed as mean ± SEM, unless stated otherwise. In vivo changes in SAP and HR, as well as TXB2 were compared with time 0 (baseline) values using one-way ANOVA with Dunnett’s post hoc test. p < 0.05 was considered statistically significant.

Results

Nanoparticle characterization & stability on storage
The detailed description of physicochemical properties of respective nanoparticles is provided in the Online Supplement (Supplementary Figures 1–16, see also [10,12,27–29]). To validate the stability of physicochemical parameters upon prolonged storage, the Z-averaged hydrodynamic diameter, the polydispersity (expressed as polydispersity index, PDI) and the ζ-potential of the investigated nanoparticles were determined at different time points. The first collective analysis time-point was set at 1-month post synthesis date. The detailed characteristics of the nanoparticles are shown in Table 1. To ensure the long-term particle stability, subsequent measurements were performed on the various nanoparticles after 3 and 6 months of storage at 4°C in their respective dilution media. As shown in Table 2, no significant changes in hydrodynamic diameter, PDI and ζ-potential of the nanoparticles were found, indicating a good stability with time.

Real-time cell analysis of nanoparticle cytotoxicity
Endothelial cells are the first-contact vascular cells for any nanosystems designed for intravascular applications. A suitable in vitro method to investigate the potential toxicity of intravenously applied nanosystems is to test their effect on HUVECs, which serve as a model system of the human endothelium. Real-time cell analysis using xCELLigence is a well-established method used, among others, for nanotoxicity studies [33]. As described in detail in Online Supplement, cell index measured with this technique reflects cell viability, number, morphology and adhesion strength. To ensure that the tested nanoparticles do not interfere with the impedance measurements, a series of control experiments was performed. Importantly, the presence of nanoparticles alone (without cells) did not affect the impedance measured by the electrodes, as shown in Supplementary Figures 17–20. In studies with HUVECs, a steady increase in cell index was observed.
over time in control (untreated) endothelial cells, but also in the cells treated with LP-NPs up to the highest tested concentration (400 μg/ml, Figure 1). There were no significant differences in the growth curves of the liposome-treated cells versus control samples, showing a similar increase of cell index until the end of the measurement at 72 h post-application. In cells treated with 100 μg/ml LD-NP1 (Figure 2A), a decrease in cell index at 48 and 72 h was observed in comparison to control, indicative of cell growth inhibition or loss of adherence. Interestingly, the decrease in cell indices became obvious at the concentration of 100 μg/ml for LD-NP1, 200 μg/ml for LD-NP2 and above 200 μg/ml for LD-NP3, suggesting that the larger lipid nanoparticles may be better tolerated by endothelial cells (Figure 2). For polymeric nanoparticles (Figure 3), the decrease in endothelial cell index relative to the preapplication values, which was indicative of cytostatic or cytotoxic effects, was induced from the concentration of 50 μg/ml for PM-NP1, and from 100 μg/ml for PM-NP2.

In cells treated with 50 μg/ml IO-NP1 (Figure 4), a significantly lower cell-index in comparison to control was observed at 48 and 72 h, indicative of cell growth inhibition. The decrease in endothelial cell index relative to preapplication values, indicative of negative effects on cell viability or adherence was induced from the concentration of 100 μg/ml IO-NP1 (Figure 4A). There were no significant differences in the growth curves of the cells treated with IO-NP2 versus control samples, showing a similar increase of cell index until the end of the measurement at 72 h post-application (Figure 4B). Since IO-NP3 were no longer stable upon dilution in the serum-containing endothelial cell

### Table 1. Physicochemical characterization of nanoparticles.

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>Z-avg d (nm)</th>
<th>PDI</th>
<th>ζ (mV)</th>
<th>SD ζ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD-NP1</td>
<td>53.3</td>
<td>0.156</td>
<td>-7.0</td>
<td>14.5</td>
</tr>
<tr>
<td>LD-NP2</td>
<td>82.8</td>
<td>0.191</td>
<td>-9.0</td>
<td>14.6</td>
</tr>
<tr>
<td>LD-NP3</td>
<td>120.1</td>
<td>0.151</td>
<td>-8.8</td>
<td>8.4</td>
</tr>
<tr>
<td>LP-NP1</td>
<td>138.6</td>
<td>0.104</td>
<td>-16.3</td>
<td>7.4</td>
</tr>
<tr>
<td>LP-NP2</td>
<td>108.8</td>
<td>0.034</td>
<td>-9.0</td>
<td>4.7</td>
</tr>
<tr>
<td>PM-NP1</td>
<td>145.1</td>
<td>0.072</td>
<td>-51.0</td>
<td>5.6</td>
</tr>
<tr>
<td>PM-NP2</td>
<td>226.9</td>
<td>0.194</td>
<td>3.3</td>
<td>5.7</td>
</tr>
<tr>
<td>IO-NP1</td>
<td>78.7</td>
<td>0.145</td>
<td>-37.3</td>
<td>12.9</td>
</tr>
<tr>
<td>IO-NP2</td>
<td>79.6</td>
<td>0.173</td>
<td>13.7</td>
<td>9.3</td>
</tr>
<tr>
<td>IO-NP3</td>
<td>57.5</td>
<td>0.217</td>
<td>-24.9</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Physicochemical characterizations were performed for various nanosystems 1 month after particle synthesis. Z-avg d: Z-averaged hydrodynamic diameter; PDI: Polydispersity (PDI = SD^2/d^2); ζ: Zeta-potential; SD ζ: Standard deviation of zeta-potential; NP: Nanoparticles; LD: Lipidots; LP: Liposomes; PM: Polymeric NPs; IO: Superparamagnetic iron oxide nanoparticles.

### Table 2. Nanoparticle stability on storage.

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z-avg d (nm)</td>
<td>PDI</td>
<td>ζ (mV)</td>
</tr>
<tr>
<td>LD-NP1</td>
<td>53.3</td>
<td>0.156</td>
<td>-7.0</td>
</tr>
<tr>
<td>LD-NP2</td>
<td>82.8</td>
<td>0.191</td>
<td>-9.0</td>
</tr>
<tr>
<td>LD-NP3</td>
<td>120.1</td>
<td>0.151</td>
<td>-8.8</td>
</tr>
<tr>
<td>LP-NP1</td>
<td>138.6</td>
<td>0.104</td>
<td>-16.3</td>
</tr>
<tr>
<td>LP-NP2</td>
<td>108.8</td>
<td>0.034</td>
<td>-9.0</td>
</tr>
<tr>
<td>PM-NP1</td>
<td>145.1</td>
<td>0.072</td>
<td>-51.0</td>
</tr>
<tr>
<td>PM-NP2</td>
<td>226.9</td>
<td>0.194</td>
<td>3.3</td>
</tr>
<tr>
<td>IO-NP1</td>
<td>78.7</td>
<td>0.145</td>
<td>-37.3</td>
</tr>
<tr>
<td>IO-NP2</td>
<td>79.6</td>
<td>0.173</td>
<td>13.7</td>
</tr>
<tr>
<td>IO-NP3</td>
<td>57.5</td>
<td>0.217</td>
<td>-24.9</td>
</tr>
</tbody>
</table>

Physicochemical parameters were obtained for the various nanosystems at 1, 3 and 6 months after particle synthesis. Z-avg d: Z-averaged hydrodynamic diameter; PDI: Polydispersity (PDI = SD^2/d^2); ζ: Zeta-potential; SD ζ: Standard deviation of zeta-potential; NP: Nanoparticles; LD: Lipidots; LP: Liposomes; PM: Polymeric NPs; IO: Superparamagnetic iron oxide nanoparticles. * No steady values could be obtained due to foaming during measurement.
medium, yielding significant nanoparticle agglomeration at concentrations above 50 μg/ml, the effect of IO-NP3 on HUVECs could not be investigated.

**Live cell imaging of nanoparticle-treated endothelial cells**

The results of real-time cell analysis were validated using live-cell microscopy. In contrast to real-time cell analysis, which estimates cell numbers, attachment and viability based on the impedance measurements, live-cell microscopy allows the observation of cell morphology, and the measurement of confluence at the same time (see also Supplementary Figures 21–24). Using this method, no differences were observed in confluence (Supplementary Figure 21) or morphology between untreated cells and the cells treated with different concentrations of LP-NPs, confirming the real-time analysis data (Figure 1A–C). Upon treatment with LD-NP1, endothelial cell numbers were only slightly affected at 100 μg/ml, but the morphology of the cells changed, starting at around 24 h of incubation, leading to an elongated phenotype at 28 h post-application. At 200 μg/ml, stronger cell elongation, and reduced number of adherent cells were observed at 24 h (Figure 2A, Supplementary Figure 22). For LD-NP2, slight morphological changes became apparent at 100 μg/ml after 30 h of incubation, which were more strongly pronounced at 200 μg/ml, in parallel with reduced cell numbers after 48 h of incubation (Figure 2B). In LD-NP3-treated cells, reduced proliferation and strong elongation were detectable at 100 μg/ml after 72 h and at 200 μg/ml after 48 h incubation (Figure 2C). Cytotoxicity was apparent for LD-NP2 at 400 μg/ml after 24 h incubation, for LD-NP3 at 400 μg/ml after 48 h of incubation and for the smallest LD-NP1 at 200 μg/ml after 24 h of incubation.

Upon treatment with PM-NP1, strong reduction in cell number (reflected by decreased confluence, see Supplementary Figure 23) was visible at 50 μg/ml, whereas the presence of dead cells was observed at 100 μg/ml (Figure 3A). Treatment with 50 μg/ml PM-NP2 did not significantly affect cell numbers or morphology. Cytotoxic effects were observable from 100 μg/ml (Figure 3B). In the case of iron oxide nanoparticles, decreased cell numbers were observed upon treatment with 100 μg/ml IO-NP1, and a strong growth inhibition accompanied by cell shape change was induced at 200 μg/ml (Figure 4A, Supplementary Figure 24). In contrast, up to 400 μg/ml of IO-NP2 were well tolerated by endothelial cells and did not affect the morphology or confluence of cells as compared with untreated controls (Figure 4B).

Taken together, the results of real-time cell analysis and live-cell microscopy indicated that for the majority of the tested nanosystems, there were no significant toxic effects on HUVECs up to the concentration of 100 μg/ml. Because of the biological/cytotoxic effects observed at and above 100 μg/ml for LD-NPs, PM-NPs and IO-NPs, subsequent studies under flow conditions were performed to investigate the effects of circulating nanosystems in physiological-like settings.

**Nanoparticle effects on ECs in dynamic cell culture conditions**

In physiological conditions, endothelial cells are constantly exposed to shear stress induced by the flow of blood and its viscosity, and their responses to stimuli are determined by the patterns of shear stress. Whereas laminar flow protects endothelial cells from harmful stimuli, nonuniform shear stress induces endothelial activation [31]. Furthermore, recent studies showed that the endothelial uptake on untargeted nanoparticles greatly depends on the presence and magnitude of shear stress [34–36]. Therefore, experiments under flow conditions are necessary to estimate the cell responses in physiological-like settings. *In vitro*, the toxic effects of circulating substances manifest themselves as endothelial cell shrinking and detachment. Consequently, the viability and confluence of the cells upon treatment with nanoparticles, as well as their morphology and cell–cell contacts can be assessed by immunofluorescent staining. We therefore perfused a HUVEC monolayer with medium containing 100 or 400 μg/ml of nanoparticles for 18 h and subsequently compared the nanoparticle effects on cells exposed to different types of shear stress (laminar vs non-uniform shear stress).

In contrast to static conditions, all LD-NPs (Figure 5) and IO-NP1 (Figure 6) were well tolerated by the cells up to 400 μg/ml and did not affect endothelial cell viability and morphology, nor induced cell detachment due to shear stress exposure. In the case of PM-NP1, the circulating nanoparticles induced endothelial cell rounding at 100 μg/ml, and resulted in massive cell detachment both in the laminar and nonuniform shear stress region at 400 μg/ml (Figure 6A). The negative effects of circulating PM-NP2 on HUVECs remained observable at 100 μg/ml (Figure 6B), similar as seen in static conditions.

**Reaction to liposomal nanoparticles in a pig model of CARPA**

Evaluation of cardiovascular changes upon intravenous bolus injection of LP-NP1 at two different doses (0.1 and 0.5 mg phospholipid/kg) was done in domestic pigs. After the negative control injection (5 ml saline), the first test dose (0.1 mg phospholipid/kg) was diluted in 5 ml of sterile PBS and injected as a
bolus in the external jugular vein of anesthetized animal (see online supplement for the outline of the pig model). The saline injection caused no cardiovascular changes. Following the injection of LP-NP1 at 0.1 mg/kg dose, mild PAP increase was observed (from 16.6 to 20.9 mmHg), without any other changes (Figure 7A).

The steady increase of HR was not nanoparticle-related. To test for the presence of tachyphylaxis (desensitization), the same dose was repeatedly injected 30 min later. This repeated 0.1 mg/kg bolus injection caused no reaction, just like the subsequent injection of 5× higher dose (0.5 mg/kg LP-NP1), indicating that there

**Figure 1. Biological effects of liposomes on endothelial cells grown in static conditions.** HUVECs were treated with (A) LP-NP1 and (B) LP-NP2 for up to 72 h. Left panel: Real-time cell analysis. Cell index is displayed as x-fold of untreated controls. Right panel: Live-cell microscopy images at ×10 objective magnification. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs control (one-way ANOVA); n = 4.

**Figure 2. See facing page.** Biological effects of lipid nanoparticles on endothelial cells grown in static conditions. HUVECs were treated with (A) LD-NP1, (B) LD-NP2 and (C) LD-NP3 for up to 72 h. Left panel: Real-time cell analysis. Cell index is displayed as x-fold of untreated controls. Right panel: Live-cell microscopy images at ×10 objective magnification. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs control (one-way ANOVA); n = 3.
was full tachyphylaxis. The positive control, zymosan at 0.1 mg/kg evoked severe PAP increase and a short lasting SAP decrease. The most characteristic PAP changes during the three LP-NP1 and zymosan injections, expressed as the % of the preinjection values, are shown in Figure 7B.

Pulmonary hypertension is closely associated with elevations of plasma thromboxane in response to zymosan-induced complement activation [37,38]. TXB2 measurement in blood samples collected before injections and during the reactions showed an approximate 40% TXB2 elevation in the later phase (10 min) following the first 0.1 mg/kg LP-NP1 injection (Figure 7C). Upon repeated injection, the same dose caused neither PAP nor TXB2 elevation (not shown), which confirmed the presence of tachyphylaxis.

Figure 3. Biological effects of polymeric nanoparticles on endothelial cells grown in static conditions. HUVECs were treated with (A) PM-NP1 and (B) PM-NP2 for up to 72 h. Left panel: Real-time cell analysis. Cell index is displayed as x-fold of untreated controls. Right panel: Live-cell microscopy images at ×10 objective magnification. Data are expressed as mean ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001 vs control (one-way ANOVA); n = 5 for PM-NP1; n = 3 for PM-NP2.
Discussion

Detailed in vitro characterization facilitates the prediction of nanoparticle behavior in more complex physiological conditions, and is a prerequisite for human use [39]. We therefore investigated 10 nanoparticle systems, including lipid nanoparticles (Lipidots), liposomes, polymeric nanoparticles and iron oxide nanoparticles with regard to their physicochemical features, stability and biological effects. Important parameters affecting nanoparticle properties include size, charge and PDI [40]. Size is one of the critical factors that affect the circulation time and bioavailability of nanoparticles. Surface charge, indicated by the ζ-potential, has a strong influence on nanoparticle sta-

Figure 4. Biological effects of iron oxide nanoparticles on endothelial cells grown in static conditions. HUVECs were treated with (A) IO-NP1 and (B) IO-NP2 for up to 72 h. Left panel: Real-time cell analysis. Cell index is displayed as x-fold of untreated controls. Right panel: Live-cell microscopy images at ×10 objective magnification. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs control (one-way ANOVA); n = 6 for IO-NP1; n = 3 for IO-NP2.
A) LD-NP1

B) LD-NP2

C) LD-NP3

**Note:** The images do not display accurate text content due to image quality. The legend and labels are not visible.
Nanoparticles for intravascular applications: physicochemical characterization & cytotoxicity testing

Research Article

Figure 5. See facing page. Biological effects of circulating lipid nanoparticles on endothelial cells grown under flow conditions. HUVECs were grown in bifurcating slides until confluence and perfused for 18 h with medium containing LD-NP1 (A), LD-NP2 (B), or LD-NP3 (C) at 100 and 400 μg/ml. Left panel: Fluorescent images of representative laminar and nonuniform regions at 20× objective magnification are shown. F-actin was visualized with Alexa 488-conjugated 488 (green) and nucleus with Hoechst 33342 (blue). Right panel: The graphs show a semiquantitative analysis of the confluence in laminar (green bars) and nonuniform region (red bars), determined on x10 objective magnification images using ImageJ software. Nanoparticle-untreated controls (white columns) were set to 100%. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs corresponding control.

bility in biological fluids. Moreover, positively charged nanoparticles enhance cellular uptake and can induce cytotoxicity [40,41]. Nanoparticles with a ζ-potential above (+/-) 30 mV are usually considered as colloidally stable, since the surface charge prevents their aggregation, but ζ-potential cannot be regarded as an absolute predictor of nanoparticle stability, especially in biological fluids as the ζ-potential is dependent on solvent composition. Steric repulsion, such as the hindrance provided by a PEGylated coating of the nanoparticle surface, can also provide high colloidal stability despite a nearly neutral ζ-potential [42].

Nanoparticle agglomeration is thus influenced by their physicochemical properties, but also by extrinsic factors, for example, temperature, as well as pH, osmotic strength and the presence of serum in the dispersion media. For clinical applications, nanoparticle agglomeration may be a key factor limiting their use in patients, as it affects bioavailability, and thus efficacy. Aggregated nanoparticles are no longer nano-sized, and undergo a rapid recognition by the reticuloendothelial system followed by the clearance via liver or spleen. Moreover, the presence of agglomerates in circulation may cause serious undesirable side effects, such as clogging blood or lymphatic vessels [41]. In our study, although the results of physicochemical characterization of all investigated nanosystems in their respective dilution media indicated a good colloidal stability also upon prolonged storage, one type of nanoparticles (IO-NP3) was prone to agglomeration in serum-containing media, and was therefore excluded from biocompatibility testing. This underlines that only a careful nanoparticle analysis enables the design of a stable, clinically safe nanosystem.

The potential toxicity of nanoparticles is a major concern that must be excluded prior to their application in humans [39]. As nanoparticles may interfere with the available photometric methods for testing cell viability and/or metabolic activity [26,43–44], it is important to use in parallel at least two different methods for toxicity testing, to enable the verification of the results. In our studies, the biological effects of nanoparticles on endothelial cells were analysed in vitro using two real-time cell-monitoring methods. The obtained data underscore the importance of applying different methods to assess the toxicity of nanoparticles, as one single method may increase the risk of bias. For example, the impedance measurements indicated a negative effect of LD-NP1 at 100 μg/ml on HUVECs (cell-index significantly lower in comparison to control), hinting to a reduced endothelial cell numbers, whereas the results of live-cell microscopy indicated a change in cell morphology, possibly resulting in weaker adherence of the cells, that is responsible for the measured impedance differences. Smaller lipid nanoparticles (LD-NP1) had more pronounced effect on cell elongation and adherence of the cells than larger lipid nanoparticles. This could be due both to the effect of higher surfactant concentration and the small size facilitating the uptake. Concerning the mechanisms of nanoparticle-induced toxicity, we did not observe acute cell death accompanied by the rupture of plasma membrane, which would be indicative of necrosis. Based on the morphological features, including blebbing and cell shrinkage occurring over longer incubation periods, most probably the apoptotic processes were responsible for cell death induced at the concentrations of, and above, 100 μg/ml of the tested nanoparticles. To gain a more detailed insight into the mechanisms of toxicity, future studies including annexin V and caspases staining would be necessary.

Compared with iron oxide nanoparticles, which have been extensively investigated for their effects on endothelial cells, very scarce information are available regarding the endothelial toxicity of solid lipid nanoparticles or polymeric nanoparticles composed of PIBCA. Lipid particles composed of cetyl palmitate and polysorbate 80 have been well tolerated by human cerebral microvascular endothelial cell line up to 1500 μg/ml [6], although the selected exposure time was very short (4 h). Polymeric nanoparticles coated with fucoidan and dextran have previously been tested by Lira et al. [45] on macrophage and fibroblast cell lines, showing the IC_{50} of 9.6 μg/ml after 48 h incubation, but no data are available in the literature concerning their endothelial effects. Stealth liposomes of various compositions are generally well tolerated by endothelial cells [46,47], which is in agreements with our present observations. The largest pool of data related to endothelial toxicity is thus far available for iron oxide nanoparticles (reviewed in [48,49]). Based on the existing literature, the presence and the type of coating is a
<table>
<thead>
<tr>
<th></th>
<th>PM-NP1</th>
<th></th>
<th>PM-NP2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminar</td>
<td><img src="image1" alt="Laminar images" /></td>
<td>Nonuniform</td>
<td><img src="image2" alt="Nonuniform images" /></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td><img src="image3" alt="Control images" /></td>
<td>100 µg/ml</td>
<td><img src="image4" alt="Control images" /></td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td><img src="image5" alt="Images" /></td>
<td>400 µg/ml</td>
<td><img src="image6" alt="Images" /></td>
<td>400 µg/ml</td>
</tr>
<tr>
<td>400 µg/ml</td>
<td><img src="image7" alt="Images" /></td>
<td></td>
<td><img src="image8" alt="Images" /></td>
<td></td>
</tr>
</tbody>
</table>

### Confluence (%)

<table>
<thead>
<tr>
<th></th>
<th>PM-NP1</th>
<th></th>
<th>PM-NP2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image9" alt="Confluence graphs" /></td>
<td></td>
<td><img src="image10" alt="Confluence graphs" /></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>IO-NP1</th>
<th></th>
<th>IO-NP2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminar</td>
<td><img src="image11" alt="Laminar images" /></td>
<td>Nonuniform</td>
<td><img src="image12" alt="Nonuniform images" /></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td><img src="image13" alt="Control images" /></td>
<td>100 µg/ml</td>
<td><img src="image14" alt="Control images" /></td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td><img src="image15" alt="Images" /></td>
<td>400 µg/ml</td>
<td><img src="image16" alt="Images" /></td>
<td>400 µg/ml</td>
</tr>
<tr>
<td>400 µg/ml</td>
<td><img src="image17" alt="Images" /></td>
<td></td>
<td><img src="image18" alt="Images" /></td>
<td></td>
</tr>
</tbody>
</table>

### Confluence (%)

<table>
<thead>
<tr>
<th></th>
<th>IO-NP1</th>
<th></th>
<th>IO-NP2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image19" alt="Confluence graphs" /></td>
<td></td>
<td><img src="image20" alt="Confluence graphs" /></td>
<td></td>
</tr>
</tbody>
</table>

The images illustrate the effects of PM-NP1 and PM-NP2 at different concentrations (100, 400 µg/ml) and control conditions on the confluence of cells in laminar and nonuniform flow environments. The graphs show a significant decrease in confluence with increasing NP concentrations, indicating potential cytotoxic effects.
decisive factor for the biocompatibility of these particles, with good endothelial compatibility of dextran- and PEG-coated iron oxide nanoparticles up to 500 μg/ml over 24 h, as reported by Yu et al. [50]. The net effect of iron oxide nanoparticles was also related to the amount of cellular uptake, which differs strongly among different formulations [51,52]. These data are in accordance with our observations, indicating very good biocompatibility of dextran-coated IO-NP2 at all tested concentrations, and little endothelial toxicity of lauric acid/albumin-coated IO-NP1 below 100 μg/ml.

It must be noted that the nanoparticle toxicity was tested up to a very high concentration (400 μg/ml). Such high doses (above 100 μg/ml) are not expected to occur in the systemic circulation in vivo, but may be encountered locally at the region of administration and should therefore be considered in analyses. Caution is also necessary when interpreting the results obtained with iron oxide nanoparticles versus other nanoparticle types, as the concentrations of the former are normalized to the total iron content, which corresponds to a much higher total dry mass weight. We have applied long-term monitoring techniques instead of single-point measurements, to ensure the toxicity readouts over extended time. Although some nanoparticles, particularly those intended for imaging applications, will be expected to circulate for relatively short time, thus far no detailed pharmacokinetics and clearance data are available. Hence, the long-term effects must be investigated both for intended imaging and therapeutic nanosystems to ensure their safety also over extended periods of time.

In endothelial cells, constantly exposed to the blood flow, shear stress-activated mechanisms are one of the major modulators of the physiologic functions, but little is known about the influence of hemodynamic factors on the endothelial responses to circulating nanoparticles. In vitro assays in dynamic conditions corresponding to the physiological environment of endothelial cells are thus of critical importance, as the susceptibility to atherosclerosis is governed by the specific patterns of shear stress. In general, our data indicate that in case of nanoparticles, the cell culture assays under static conditions may overestimate the potential toxicity. This results from the inherent property of nanoparticles, namely their sedimentation, which occurs over time and leads to increased effective concentrations of nanoparticles in the nearest vicinity of cell monolayer. As shown in our studies, this effect is responsible for the majority of the cytostatic and cytotoxic effects observed below the concentration of 200 μg/ml. Only for one nanoparticle type (PM-NP2), the concentrations affecting cell growth and viability in static conditions (100 μg/ml) also induced cell detachment under flow conditions, other nanosystems being well-tolerated under flow up to 400 μg/ml. This may be related to the fact that except IO-NP2, PM-NP2 are the only positively charged nanoparticles, characterized furthermore by a relatively large Z-average size and a tendency to aggregate. Collectively, these features may negatively affect endothelial cell growth and viability at concentrations of 100 μg/ml and higher. Moreover, the recently reported data as well as our present studies indicate that physiologic flow is one of the important factors that must be considered when designing drug delivery nanosystems, as the internalization of untargeted nanoparticles by endothelial cells differs greatly between static and dynamic conditions [34–36].

Due to their size, nanoparticles may remain in the circulation for several hours or more, and their in vivo behavior and interactions with cellular and extracellular substrates may induce undesired effects, including hemolytic reactions, and/or complement activation. Apart from clinical efficacy and safety, diagnostic and therapeutic nanosystems should therefore offer the possibility of repeated intravenous/intra-arterial administration without inducing anaphylactoid (hypersensitivity) reactions. In this context, the clinical significance of CARPA-genic reaction upon the intravenous administration of nanosystems lies not only in the severe, occasionally lethal cardiopulmonary distress but also in a heightened risk that the nanomedicines become immunogenic, preventing their multiple applications [32]. In order to minimize such risks, the nanoparticles included in our analyses are currently entering the CARPA tests in the pig model [53]. The results of a pilot study involving the intravenous bolus administration of LP-NP1 confirmed the in vitro data, indicating a favorable safety profile and low immunogenicity of these nanoparticles. In the next stage of the
Cardiovascular changes after iv. bolus injections of LP-NP1 and zymosan

**A**

- **SAP (mmHg), HR (beats/min)**
- **SAP**
- **PAP (mmHg)**
- **HR**

<table>
<thead>
<tr>
<th>Saline</th>
<th>LP-NP1 0.1 mg/kg</th>
<th>LP-NP1 0.1 mg/kg repeated</th>
<th>LP-NP1 0.5 mg/kg</th>
<th>Zymosan 0.1 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>100</td>
<td>125</td>
<td>150</td>
<td>175</td>
</tr>
</tbody>
</table>

PAP % changes after LP-NP1 and zymosan injection

**B**

- **LP-NP1 0.1 mg/kg**
- **LP-NP1 0.1 mg/kg repeated**
- **LP-NP1 0.5 mg/kg**
- **Zymosan 0.1 mg/kg**

TXB2 and PAP changes after LP-NP1 injection

**C**

- **TXB2 (pg/ml)**
- **PAP (mmHg)**

<table>
<thead>
<tr>
<th>TXB2 (pg/ml)</th>
<th>PAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-5</td>
</tr>
</tbody>
</table>
The potential clinical impact of nanotechnology in imaging and drug targeting applications. The majority of nanosystems were well tolerated by endothelial cells, and did not induce major toxic effects in vitro up to the concentration of 100 μg/ml in static, and up to 400 μg/ml in dynamic cell culture conditions. These findings indicate an overall favorable biocompatibility profile of the tested nanosystems and their potential for cardiovascular imaging and drug targeting applications.

Future perspective
The potential clinical impact of nanotechnology in terms of detection and management of cardiovascular diseases is enormous. But in spite of the promising results obtained in the vast number of bench investigations that have been published in the recent years [4], no specific nanoparticle-based system has been approved for diagnosis or therapy of atherosclerosis in humans. The reasons for that are mainly the safety requirements related to nanoparticulate medicines, the lacking regulatory guidelines and insufficient standardization in the matters of particle characterization and nanotoxicity testing. To ensure clinical safety, the intravascular diagnostic and drug-delivery systems must first be subject to a close toxicologic scrutiny in vitro. Our studies represent an attempt to advance this field by utilizing a systematic approach to the comparative analysis of nanoparticle effects on primary human endothelial cells. Importantly, no interference resulting from the presence of nanoparticles was observed in real-time cell analysis method, indicating the suitability of this technique for the future nanotoxicology studies. Furthermore, the comparison of nanoparticle effects on cell viability in static culture conditions and the effects of circulating nanoparticles on endothelial monolayer under physiologic-like shear stress allowed the conclusion that the majority of tested nanosystems have very good biocompatibility profiles. To understand how the physicochemical features of nanoparticles can affect the specific cellular responses, we are currently investigating the functional effects of the described nanosystems in endothelial and monocytic cells. These studies are expected to provide further important information concerning the mechanisms of nanoparticle-elicited cellular effects. Our pilot studies in the pig model confirmed the safety of liposomal formulation (LP-NP1) in vivo, and constitute an important step toward further development and functionalization of these particles for the purpose of intravascular imaging and targeted drug delivery. In the future, substantial amount of in vivo studies will be necessary before the nanosystems with proven in vitro safety and efficacy can be translated into clinical trials. But despite multiple safety and regulatory constraints, the future progress in diagnosis and treatment of cardiovascular disorders is expected to benefit strongly from the development of novel nanotechnology-based strategies.

It must also be noted that cardiovascular disease, including various clinical manifestations of atherosclerosis and thrombosis, is but an example of the disease the therapy of which may profit from intravascular application of nanoparticulate drug carriers. In fact, the majority of the clinically-relevant nanocarriers, such as anticancer and anti-inflammatory nano-drugs, are expected to require intravascular administration. Although the main focus of our work is the diagnosis and therapy of atherosclerosis, the nanosystems investigated in this study constitute a versatile platform, adjustable also for the intravascular drug-delivery in other disease conditions.

Financial & competing interests disclosure
This work was supported by the EU (‘NanoAthero’ project FP7-NMP-2012-LARGE-6–309820), the DFG (CI 162/2–1), and received the financial support from ANR-13-LAB1–0005–01 ‘Fuc-oChem.’ LETI/DTB5 is part of the Arcane Labex program, funded by the French National Research Agency (ARCANE project no ANR-12-LABX-003). The authors thank Prof. M. Beckmann (Department of Gynaecology, University Hospital Erlangen, Germany) for providing umbilical cords, N Jaziri for help with HUVEC isolation and P Dörfler for help with flow experiments. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.
Executive summary

Background
- Detailed physicochemical and biological characterization of nanosystems in vitro is necessary to ensure their safety in more complex physiological conditions.

Methods
- We investigated 10 diverse nanosystems, comprising liposomes, lipid nanoparticles, polymeric and iron oxide nanoparticles to assess their long-term stability and biological effects on endothelial cells (ECs).
- EC viability in static conditions was monitored using real-time cell analysis and live-cell microscopy.

The majority of tested nanosystems were well tolerated by ECs up to the concentration of 100 µg/ml in static, and up to 400 µg/ml in dynamic conditions.
- In static conditions, nanoparticle sedimentation was responsible for the majority of the cytostatic and cytotoxic effects observed below nanoparticle concentration of 200 µg/ml.
- The results of a pilot study in a pig model showed that intravenous administration of liposomal nanoparticles did not evoke the hypersensitivity reaction, indicating a low immunogenicity of these nanoparticles.

Conclusion
- The majority of tested nanosystems had an overall favorable biocompatibility profile, constituting good candidates for cardiovascular imaging and drug targeting applications.

References

Papers of special note have been highlighted as: • of interest;
** of considerable interest
** The in-depth review of the pathophysiology of atherosclerosis as an inflammatory disease.
• This review highlights the recent advances in the preclinical and clinical applications of nanoparticulate agents for cardiovascular diagnostics and therapy.
**Nanoparticles for intravascular applications: physicochemical characterization & cytotoxicity testing**

Research Article


• This article presents the results of the world’s largest animal study to date, investigating the efficacy of magnetic drug targeting using mitoxantrone-loaded SPIONS in tumor-bearing rabbits.


**Important contribution to the issue of nanoparticle interference with toxicity assays. This article also provides guidance on controlling for such interference to improve the accuracy of nanotoxicity assessments.**


**This review provides basic information on complement activation-related pseudoallergy (CARPA), including a short history, incidence, classification of CARPA-genic drugs and symptoms, and the mechanisms of C activation via different pathways.**


43 Keene AM, Alloway RJ, Sadrich N, Tyner KM. Gold nanoparticle trafficking of typically excluded compounds...
across the cell membrane in JB6 Cl 41–5a cells causes assay

44 Monteiro-Riviere NA, Inman AO, Zhang LW. Limitations
and relative utility of screening assays to assess engineered
nanoparticle toxicity in a human cell line. *Toxicol. Appl.

45 Lira MCB, Santos-Magalhaes NS, Nicolas V et al.
Cytotoxicity and cellular uptake of newly synthesized

46 Coimbra M, Banciu M, Fens MH et al. Liposomal
pravastatin inhibits tumor growth by targeting cancer-related

47 Orlando A, Re F, Sesana S et al. Effect of nanoparticles
binding beta-amyloid peptide on nitric oxide production
by cultured endothelial cells and macrophages. *Int. J.

48 Soenen SJ, De Cuyper M. Assessing iron oxide nanoparticle
toxicity *in vitro*: current status and future prospects.
*Nanomedicine (Lond.)* 5(8), 1261–1275 (2010).

• This work presents an overview of different types of iron
oxide nanoparticles developed for biomedical research with
particular focus on their internalization and cytotoxicity.

49 Mahmoudi M, Hofmann H, Rothen-Rutishauser B,
Petri-Fink A. Assessing the *in vitro* and *in vivo* toxicity
of superparamagnetic iron oxide nanoparticles. *Chemical

50 Yu M, Huang S, Yu KJ, Clyne AM. Dextran and
polymer polyethylene glycol (PEG) coating reduce both
5 and 30 nm iron oxide nanoparticle cytotoxicity in 2D
and 3D cell culture. *Int. J. Mol. Sci.* 13(5), 5554–5570
(2012).

51 Buyukhatipoglu K, Clyne AM. Superparamagnetic
iron oxide nanoparticles change endothelial cell
morphology and mechanics via reactive oxygen species
(2012).

52 Li M, Kim HS, Tian L, Yu MK, Jon S, Moon WK.
Comparison of two ultrasmall superparamagnetic iron
oxides on cytotoxicity and MR imaging of tumors.
*Theranostics* 2(1), 76–85 (2012).

53 Székenyi J, Bedocs P, Csukas D, Rosivall L, Burger R,
Urbanics R. A porcine model of complement-mediated
infusion reactions to drug carrier nanosystems and
(2012).