Prothrombotic Effects of Manufactured Nanoparticles

Ph.D. Thesis

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Introduction

Nanoparticles are particles with length scales under 100 nanometres. Nanoparticles always existed in our ambient nature, but they have received a substantial boost of interest in the last 50 years with the emergence of nanotechnology. Since then, nanotechnology has grown into a prominent industry and hundreds of different variants of nanomaterials and nanotech-based products became commercially available.

The classification of nanoparticles as a new entity is justified by their physicochemical properties which are different from the bulk material and the atoms and molecules from which they are built up. Although the unique physicochemical properties of manufactured nanoparticles enable the improvement of novel applications, they might also cause unusual types of interactions with biological materials and toxic effects not yet experienced.

Nanoparticles can come in contact with the human body through inhalation, ingestion, dermal deposition, but also through injection for medical applications. Nanoparticles having entered the body can translocate into the systemic blood circulation, reach various remote organs, and may affect their function.

Haemostasis is an important physiological function of the human body maintaining the integrity of the blood vessels. Disruption of vascular integrity, resulting in the contact of human blood with any surfaces other than the inner wall of the vessels, induces thrombus formation to stop the bleeding. A well-known consequence of this mechanism is unwanted thrombus formation on the surface of implanted foreign materials in the circulation. Although nanoparticles are small, they represent a very high cumulative surface that might also influence haemostasis.

The significance of this phenomenon is underlined by previous epidemiological studies identifying ambient nano-sized particles as a major

contributor to adverse cardio-respiratory effects of air pollution, where impact on haemostasis has been found to play an important role.

The fact that nanoparticles represent a potentially thrombogenic large cumulative surface and the knowledge about the prothrombotic effects of ambient nanoparticles raise the question of whether manufactured nanoparticles also influence thrombus formation. Although circulating nanoparticles also reach the microcirculation, prothrombotic effects of manufactured nanoparticles in the microvasculature have not yet been examined.

Aims

The main objective of my research was to investigate the effects of manufactured nanoparticles on platelet activation and on thrombus formation in the macro- and microcirculation. To achieve these aims some methodological problems also had to be solved, i.e. the preparation of nanoparticle dispersions in physiological solutions and the optimisation of the platelet-granulocyte complex measurement.

1. Optimisation of the preparation method of nanoparticle dispersions

For investigations of *in vitro* and *in vivo* prothrombotic effects, nanoparticles have to be dispersed in physiological solutions. However, nanoparticles in solutions with physiological salt concentrations and pH values form coarse agglomerates. Our aim was to prepare nanoparticle dispersions in physiological solutions without coarse agglomerates by using steric stabilisation. To optimise this kind of dispersion method, we analysed the effect of the following factors:

i) ultrasound energy levels

ii) type of dispersion stabilizer: human, bovine or mouse albumin, Tween 80, or mouse serum

iii) concentration of dispersion stabilizer

iv) concentration of nanoparticles

v) sequence of preparation steps

vi) stability of the dispersion over time

vii) We also tested our method on a broad range of various types of nanoparticles.

2. Optimisation of platelet-granulocyte complex measurement

Activation of platelets does not only induce platelet-platelet adhesion but also the formation of heterotypic platelet-granulocyte aggregates. For the evaluation of platelet activation, the amount of platelet-granulocyte complexes was also measured, particularly because it is considered a parameter for biocompatibility. The measurement of platelet-granulocyte complexes with a flow cytometer is based on the simultaneous detection of fluorescent signals from both cell types. However, these double-positive signals can also originate from the coincidence of non-interacting platelets and granulocytes in the detection volume. Our aim was to develop a method that measures the real amount of platelet-granulocyte complexes without overestimating it due to coincidence.

3. Prothrombotic effects of nanoparticles

Ambient nanoparticles have been shown to exert prothrombotic effects, but manufactured nanoparticles are less well investigated in this regard. Thus the aim of this study was:

i) to characterize the effects of diesel exhaust particles, titanium dioxide rutile, and single-walled carbon nanotube nanoparticles on platelet activation and

ii) on the formation of platelet-granulocyte complexes *in vitro,*

iii) to assess their impact on thrombus formation in the macro- and

iv) microcirculation *in vivo*, and

v) to compare these effects with those induced by surface-modified polystyrene beads as benchmark particles.

Methods

1. Nanoparticles

Titanium dioxide (rutile), titanium dioxide (anatase), zinc oxide, plain polystyrene beads, carboxyl-modified polystyrene beads, amine-modified polystyrene beads, single-walled nanotubes, multi-walled nanotubes, diesel particulate matter, silicon oxide, and silver nanoparticles were used in this study.

2. Optimisation of the nanoparticle dispersion method

Titanium dioxide (rutile) nanoparticle dispersions were prepared in distilled water, phosphate-buffered saline, or RPMI 1640 cell culture medium. Different ultrasound energies ($0 - 1.65 \times 10^6$ kJ/m^3), various dispersion stabilizers (human, bovine, and mouse serum albumin, Tween 80, and mouse serum), different concentrations of nanoparticles (2 μg/ml – 2 mg/ml) and stabilizers (1.5 μg/ml – 15 mg/ml), and different sequences of preparation steps were applied. The size distribution of dispersed nanoparticles was analysed by dynamic light scattering and their zeta potentials were measured using phase analysis light scattering. Nanoparticle size was also verified by transmission electron microscopy.

3. Optimisation of platelet-granulocyte complex measurement

Mixtures of non-interacting fluorescent beads as well as EDTA anticoagulated or citrated blood samples were analyzed in the flow cytometer in the presence and absence of fluorescent beads at various dilutions. Experimental data were evaluated by mathematical means using Poisson distribution.

4. Effect of nanoparticles on platelet activation *in vitro*

Platelet P-selectin expression and platelet–granulocyte complexes were measured by flow cytometry after incubation of whole blood with amine- or carboxyl-modified polystyrene beads, diesel or titanium dioxide nanoparticles (0.1 mg/ml) or single-walled nanotubes (0.001-0.1 mg/ml). Platelet aggregometry was performed with platelet-rich plasma in the presence of the above nanoparticles.

5. Effect of nanoparticles on thrombus formation *in vivo*

Upon systemic administration of polystyrene beads (0.5 mg/kg) or nanoparticles (1 mg/kg) to anesthetized mice, ferric chloride-induced thrombus formation was measured in small mesenteric arteries using intravital microscopy. In separate experiments, polystyrene beads (0.5 mg/kg), diesel (1 mg/kg), titanium dioxide rutile (1 mg/kg), or single-walled nanotubes (0.01–1 mg/kg) were injected into anaesthetised mice and light/dye-induced thrombus formation was investigated in the cremasteric microcirculation.

6. Statistics

For data analysis ANOVA with adequate post hoc test, t-test, the least squares method and regression analysis were used. P values < 0.05 were considered significant.

Results

1. Optimisation of the nanoparticle dispersion method

To avoid creating coarse agglomerates when preparing dispersion of nanoparticles in physiological solutions, the following aspects were found to be important:

i) Usage of a sonication energy high enough to deagglomerate the particles (>4.2×10^5 kJ/m^3);

ii) Addition of albumin or serum as stabilizers at a concentration sufficient to cover the nanoparticles (1.5 mg/ml albumin or an amount of serum resulting in a similar albumin concentration for dispersions with less than 0.2 mg/ml nanoparticle concentration);

iii) The optimal sequence is first to sonicate the nanoparticles in distilled water, then to add the stabilizer, and finally to add buffered salt solution to the dispersion.

2. Optimisation of platelet-granulocyte complex measurement

Based on our findings, we suggest three methods to avoid overestimation of platelet-granulocyte complexes caused by coincidence in the flow cytometer:

i) Calculate coincidence from the detection volume and platelet concentration and correct the measured platelet-granulocyte double positivity with these data.

ii) Measure coincidence by adding fluorescent beads to the blood sample and correct the measured platelet-granulocyte double positivity with these data.

iii) Measure platelet-granulocyte complexes at dilutions high enough to render coincidence negligible.

3. Prothrombotic effects of nanoparticles

Based on the investigation of ambient and manufactured nanoparticles on platelet activation *in vitro* and on thrombus formation in small arteries and in the microcirculation *in vivo*, we concluded that:

i) Amine-, but not carboxyl-modified polystyrene beads increase P-selectin expression of platelets and the amount of platelet-granulocyte complexes. Neither of the modified polystyrene beads induces platelet aggregation.

ii) Amine-modified polystyrene beads decrease and carboxyl-modified polystyrene beads increase the time of thrombus formation in mesenteric arteries, but neither of them has an impact on thrombus formation in the microcirculation.

iii) Diesel exhaust particles and TiO_2 (rutile) nanoparticles do not influence platelet activation

iv) Diesel exhaust particles and TiO_2 (rutile) nanoparticles injected into healthy mice have no effect on thrombus formation.

v) Single-walled carbon nanotubes increase P-selectin expression of platelets and the amount of platelet-granulocyte complexes and induce platelet aggregation.

vi) Single-walled carbon nanotubes exert prothrombotic effects in mesenteric arteries as well as in the microcirculation.

Conclusion

Nanoparticle preparation method was optimised to produce dispersions in physiologic solutions without coarse agglomerates. Furthermore, we developed a method that avoids overestimation of platelet-granulocyte complexes caused by coincidence in the flow cytometer.

The main investigation of my dissertation was aimed at the prothrombotic potential of manufactured nanoparticles. Our results demonstrate that amine modification of polystyrene beads increases platelet activation and mesenteric thrombus formation, while carboxyl modification of the same nanobeads does not change platelet activation and inhibits thrombus formation in the mesenteric arteries. Diesel or titanium dioxide (rutile) nanoparticles do not exert prothrombotic effects. In contrast, single-walled carbon nanotubes induce platelet activation and aggregation *in vitro* and augment thrombus formation in the mesenteric arteries as well as in the microcirculation *in vivo*.

The increasing utilisation of nanomaterials in technological and medical applications warrants an assessment of the risk of these manufactured materials on human health. Our most important result is that our studies strongly highlight the fact that some nanoparticles are thrombogenic in the macro- as well as in the microcirculation. Since prothrombotic effects cannot be estimated from the physicochemical parameters of the nanoparticles, screening methods would be necessary.

List of Publications

Publications used in the dissertation

1. **Bihari P**, Holzer M, Praetner M, Fent J, Lerchenberger M, Reichel CA, Rehberg M, Lakatos S, Krombach F. (2010) Single-walled carbon nanotubes activate platelets and accelerate thrombus formation in the microcirculation. Toxicology, 269(2-3):148-54. (IF: 3.641)
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