Tissue- and cell-type specific compartmentalization of surface-modified fluorescent nanoparticles: Enhanced detection with spectral imaging fluorescence microscopy

PhD thesis

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INTRODUCTION

Regarding physicochemical characteristics nanoparticles (NPs) lay between molecular structures and bulk materials. The unique characteristics of NPs arise from the small size and their consequent high surface-to-volume ratio: the proportion of surface-exposed atoms is much larger compared to bulk materials. Surface atoms have fewer neighbors, thus are prone to less cohesive energy and higher chemical reactivity. The resulting huge surface energy creates unique mechanical, electrical and thermodynamical properties, rendering NPs advantageous for various applications. The energy-states of electrons are relatively easily modified in NPs, leading to characteristic semiconductor, magnetic and optical behaviour (e.g. surface plasmon resonance, or particle size dependent color and light emission).

Nanomaterials are widely used in various consumer goods, including electronics, clothing, food and personal care products (as cosmetics and sunscreens). Nano-additives are used to improve handling, stability and efficacy of several products. In nanomedicine, NPs may find application in drug delivery, bio-imaging, diagnostics and therapeutics, since NPs can importantly modify the body distribution, metabolism and release of an associated drug. As an example, the first FDA-approved nano-drug, the nanoliposomal doxorubicin (Doxil®) can "passively target" and attack tumors due to the enhanced penetration of nanoliposomes and the retention of encapsulated doxorubicin.

For bio-imaging techniques, NP probes have been constructed for magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and near-infrared (NIR) fluorescence imaging. Given the high performance of nanostructure-based NP-probes, application in human subjects is appealing. Prior to clinical application, however, a comprehensive understanding of the biological effects of diverse types of NPs is essential including toxicity, biodistribution and pharmacokinetics.

The unique physicochemical properties that make NPs attractive for pioneering research may bring potential health hazards. Regarding the increased production, the widespread industrial, medical and domestic applications, the enhanced presence of engineered NPs in our surrounding raises serious questions concerning their interaction with biological systems. While developments in nanomedicine aim to improve specific cellular uptake

and tissue permeation of NPs, the protection of consumers and factory workers against non-intended exposure and uptake is far from solved.

For testing the safety of NPs, fast and reliable measurement systems and evaluation protocols are needed. In case of spherical nanoparticles, where the shape is not considered as an immediate hazard, the major mode of action is chemically mediated toxicity. Chemically mediated toxicity includes adverse effects originated from (1) the release of toxicants from NPs, usually ions, (2) the reactions of active surface groups and (3) the catalytic activity of NP surfaces. Due to the biologically inert core material, non-metal nanoparticles are widely used in biomedical applications. In my dissertation work, two widely produced and applied NPs with non-toxic core material were investigated in detail.

Polystyrene nanoparticles (PS-NPs) are made of a nontoxic and not carcinogenic polymer. Due to its inertness and biocompatibility, polystyrene is widely used in the everyday life and for the production of biomedical devices and laboratory equipment. Polystyrene does not degrade in the cellular environment and exhibits no short-term cytotoxicity. The biological inertness and the properties of polystyrene allowed us to use PS-NPs with different surface modifications to study the effects of surface composition on the interactions of NPs with biological material.

Amorphous silica nanoparticles (SiO_2-NPs) have been approved for oral administration for decades. It is added to food to prevent caking, poor flow, to control foaming, to clarify beverages or to carry flavors. In medical applications amorphous silica nanoparticles have attracted significant interest as promising candidates for drug delivery systems, imaging or diagnostic tools and anti-cancer therapeutics. Despite of the favorable properties and the potential medical applications, sound evidence of safety of SiO_2-NPs is still missing, and relevant risk assessment has never been satisfactorily completed.

The biological identity of NPs can be altered by interactions with the physiological environment, through the formation of a biomolecular corona. NPs readily adsorb various chemical substances from their environments due to the highly reactive surface; in contact with biological fluids NPs are rapidly covered by a selected group of biomolecules. This so-called protein corona masks the surface of the NPs. The protein corona is dynamically changing over time, representing the chemical properties and

adhesion kinetics of both the NP surface and the environment. Eventually, chemicals bound to particles surfaces will determine the body-distribution and toxicity of NPs. While NP surfaces are ultimately functionalized by the actual environment, this process can be regulated by changing the surface charge of NPs. Nanoparticles can gain, activated or passivated surfaces *via* functional groups, which attract or repel inorganic, organic and biological macromolecules. In the thesis work the cell and tissue penetration of polystyrene and silica nanospheres with equal size but with different surface compositions were investigated.

OBJECTIVES

In my dissertation work, the physicochemical characteristics and the biological interactions of fluorescent nanoparticles were investigated, including cellular nanoparticle uptake *in vitro* and tissue distribution *in vivo*. The studies were focused on the altered behavior of silica (SiO_2) and polystyrene (PS) nanoparticles coated with distinct functional groups; ranging from particles with strong negative surface charges (PS-COOH, SiO_2, SiO_2-SH) to positively charged (SiO_2-NH_2) particles or NPs with passivated surfaces (polyethylene glycol-coated PS (PS-PEG), and polyvinylpyrrolidone-coated silica (SiO_2-PVP)). Experimental work aimed to answer the following questions:

- Are polystyrene- (PS-COOH, PS-PEG) and silica nanoparticles (SiO_2, SiO_2-NH_2, SiO_2-SH and SiO_2-PVP) good models for investigating the effects of surface characteristics? Are there any biologically relevant differences between the nanoparticles except from the surface functionalization?
- How does the chemical surface composition affect the behavior of NPs in inorganic or organic solutions?
- What types of cells can interact with and internalize the distinct types of nanoparticles in serum-free *in vitro* conditions?
- How far can polystyrene nanoparticles with different surface functionalization penetrate in and clear from different organs and tissues? With special attention to physiological barriers as the blood brain barrier and the placenta.
- Does spectral imaging fluorescence microscopy provide a reliable imaging approach to distinguish particle-fluorescence from tissue autofluorescence?

METHODS

Nanoparticles

Fluorescent polystyrene nanoparticles were obtained from Spherotech Inc. (10 mg/ml NP-suspensions, Lake Forest, IL, USA) with carboxylated (PS-COOH) or PEGylated (PS-PEG, MwPEG = 300 g/mol) surfaces and nominal size of 50-70 nm. NPs were made from “Yellow” or “Nile-Red” fluorochrome-labelled polystyrene.

Fluorescent silica nanoparticles (50nm, encapsulating fluorescein-isothiocyanate (FITC)) with a core-shell structure were synthesized by Emilia Izak-Nau with modified Stöber method at Bayer Technology Services. The NPs surface was either coated with polyvinylpyrrolidone (PVP K-15, Sigma-Aldrich) or modified to generate amino and mercapto surface groups by addition of 3-aminopropyltriethoxysilane (APTES, 98 %, Alfa Aesar) and 3-mercaptopropyltrimethoxysilane (MPTMS, Sigma-Aldrich) organosilanes, respectively.

Physicochemical characterization

Size of nanoparticles was measured by dynamic light scattering (DLS) with a ZetasizerNano ZS90 (Horiba Instruments Inc., Irvine, CA) at 25°C operating a 633 nm He-Ne laser. Time dependent increase in particle diameter was monitored at 37°C in inorganic or biological environments. Shape and size of NPs were confirmed by transmission electron microscopy (TEM, JEOL JEM 1010, JEOL Ltd., Tokyo, Japan). Zeta-potential of nanoparticles was determined at 25°C by ZetasizerNano ZS90 with a folded capillary cell (DTS1070, Malvern Instruments).

Assays on protein adsorption

Proteins adsorbed by particles during 1 hour or 24-hour incubation in 10% fetal calf serum (FCS) containing minimum essential medium (MEM, Sigma-Aldrich) were analyzed by SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis). Gels were stained with silver staining 33 kit (Cosmobio Ltd., Tokyo, Japan).

Cell cultures

Primary brain cell cultures: Cell suspensions were prepared by mechanical dissociation of embryonic (E14-19) mouse forebrains, or by combined enzymatic dissociation (Neural Tissue Dissociation Kit, Miltenyi Biotec) of early postnatal (P0-P3) mouse forebrains. Single cells and were seeded onto poly-L-lysine (PLL, Sigma-Aldrich)

coated substrates. Primary cell cultures were maintained in 10% FCS-MEM, supplemented with 4mM glutamine, 2.5µg/ml Amphotericin-B (Fungizone, Sigma-Aldrich) and 40µg/ml gentamycin (Sanofi-Aventis/Chinoin) and were kept in 5% CO_2 containing humidified air atmosphere, at 37°C. Neuron-enriched cultures were prepared by treatment with anti-mitotic CAR (cytozin-arabino-furanozid, 10µM) agent, and were maintained in 10% horse serum containing MEM.

Primary microglial cultures were prepared according to Saura et al. (2003) from newborn mice. GFP-labelled microglia cells were isolated form mice expressing a green fluorescent protein (GFP) under the control of CX3CR1 gene promoter. Briefly, mixed glial cultures were prepared from the forebrains of newborn (P1-2) mouse pups by mechanical dissociation. Suspensions of single cells were seeded in 1:1 mixture of DMEM-F12 with 10% FCS (DMEM-F12-FCS) and cultured at 37°C in 5% CO_2 containing humidified air atmosphere. Astrocytes were detached from confluent mixed glial cultures by mild trypsinization and the firmly attached microglial cells were further propagated in DMEM-F12-FCS.

NE-4C neuroectodermal stem cells (ATTC CRL-2925) derived from the brain vesicles of E9 transgenic mouse embryos lacking functional p53 tumor suppressor protein. NE-4C cells were maintained in PLL-coated culture dishes, in MEM supplemented with 4 mM glutamine and 5% FCS, at 37°C and kept in 5% CO_2 containing humidified air.

Radial glia-like cells were isolated from hippocampi or subventricular zones of P21 CD1 mice *via* selective attachment to adhesive surface coated with AK-cyclo[RGDfC]. Cells were maintained in (1:1) MEM-F12 with 1% B27 and 20 ng/ml epidermal growth factor (EGF, Peprotech).

In vitro uptake of nanoparticles

For cellular uptake studies cell cultures were incubated with NP-suspensions dispersed in serum free cell culture media for 1h at 4°C or 37°C. Control cultures were incubated under the same conditions without NPs. After incubation, samples were washed with PBS and fixed with paraformaldehyde (PFA, 4% w/v, 20min) at room temperature.

In vivo NP-administration

Male mice (P25-30) and pregnant female mice on the 10th to 15th post conception days were anesthetized with a mixture of ketamine (CP-Pharma mbH) and xylazine (CEVA-PHYLAXIA). Carboxylated or PEGylated polystyrene NP were dispersed by sonication

in PBS, and 7 µl/g bodyweight aliquots were introduced into the tail vein. Animals were sacrificed by overdose of anesthetics after a 5-minute or 4-day post-injection period. Organs including brain, liver, kidney, spleen, placenta and embryos were removed and fixed with PFA (8 w/v% in PBS) for 24 hours at 4ºC. Organs and embryos were collected from animals not exposed to nanoparticles, as controls. Animal experiments were conducted with the approval of the Animal Care Committee of the Institute of Experimental Medicine of Hungarian Academy of Sciences and according to the official license (No.: 22.1/353/3/2011) issued by National Food Chain Safety Office (www.NEBIH.gov.hu).

Immunohistochemical procedures

Fixed cells or tissue sections were permeabilized with Triton X-100 (10 min, 0.1% v/v in PBS) and non-specific antibody binding was blocked by 2-hour incubation with 10% FCS containing PBS. Preparations were incubated with the primary antibodies (1:1000 in PBS-FCS) at 4ºC, overnight. For fluorescent detection, alexa-594 conjugated secondary antibodies (1:1000) were used at room temperature, for 1 h. Samples were examined using Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Jena, Germany) and Nikon A1R Confocal Laser Microscope System (Nikon, Shinjuku, Tokyo, Japan) equipped with a spectral detector unit for fluorescence spectrum analysis.

Detection of NP by spectral imaging fluorescence microscopy

Fixed cells or tissue sections were examined using Nikon A1R Confocal Laser Microscope System equipped with a spectral detector unit for spectral acquisition. For "Yellow" fluorophore labelled PS-NP 457 nm argon ion laser was used as excitation source and the emitted light was detected by the spectral detector unit from 468nm to 548nm, with a spectral resolution of 2.5nm. Regions of interest (ROIs) were analyzed in corresponding sections of nanoparticle-treated and non-treated organs. ROIs containing NPs were classified by calculating spectral ratio (SR) from the relative fluorescence intensities at reference wavelengths, corresponding to the intensity maximums. The reference wavelengths were chosen based on the positive and negative controls and contained of NP-fluorescence or the tissue autofluorescence. ROIs were considered as NP-containing if the spectral ratio was above 1 (SR > 1).

RESULTS

Physicochemical characterization of nanoparticles

Thorough characterization of nanoparticles was crucial to understand the differences in interactions with the living material of NPs with distinct functional groups. Therefore, the physical and chemical parameters of particles were determined, including their hydrodynamic and dry size, aggregation properties, and protein adsorption. We have also monitored how these parameters change in distinct inorganic or biological environments, including solutions used during particle handling and solutions that mimic the characteristics of body fluids.

Dynamic light scattering measurements revealed that both polystyrene and silica nanoparticles gave stable, monodisperse suspensions in storage conditions and that the functionalization did not affect the dispersion stability in inorganic solutions. DLS measurements showed that differently functionalized NPs did not differ significantly in size; however the surface functionalization of NPs resulted in distinct surface charges (zeta potentials). Transmission electron microscopic images confirmed the similar size and the spherical shape of NPs.

In contrast to storage conditions and inorganic solutions, a time-dependent, heavy aggregation of NPs was found in serum-free DMEM, which contains notable amount of amino acids and sugars. The kinetics of particle enlargement was consistent with an immediate deposition of material on particle surfaces and a large-scale aggregation thereafter. The phenomenon could be reduced by coating the particle surfaces with chemically inert polymers, like PEG or PVP. SDS-PAGE electrophoresis data showed the rapid adsorption of proteins to NP-surfaces and verified reduced protein adsorption of PEG- or PVP-coated nanoparticles.

The incubation of nanoparticles with 10% fetal bovine serum containing DMEM evoked an immediate size increase, but prevented the large-scale aggregation of nanoparticles thereafter. The observation indicated that serum components were immediately adsorbed by particle surfaces, but instead of cross-linking particles, the protein corona could stabilize the suspension of dispersed particles.

***In vitro* cellular uptake of nanoparticles**

The *in vitro* cellular uptake experiments demonstrated that neurons, astrocytes and progenitor cells did not take up polystyrene or silica nanoparticles, although surface attachment of silica particles was often visible.

In striking contrast, phagocytic cells like microglia accumulated significant amounts of NPs regardless of the particle's core material. The chemical composition of particle surface, however, importantly modified the biological interactions of NPs. PEGylation or PVP-coating significantly reduced the accumulation of particles. Furthermore, nanoparticles were not accumulated inside the cells at 4°C, indicating that particles were internalized *via* activity dependent cellular processes.

While fluorescence microscopic studies and confocal Z-stack analyses led to important conclusions, the high cellular autofluorescence, together with the relatively weak particle fluorescence called for some more sensitive and more reliable detection method.

Optimization of NP-detection by spectral imaging fluorescence microscopy

Visualization of fluorescent NPs even with high-resolution confocal microscopy has been notoriously difficult; because of the particle size (typically being between 1 and 100 nm) is below the Abbe's diffraction limit of ~ 250 nm. Moreover, the detection of fluorescent NPs is further hindered by the high autofluorescence intensity of biological samples, which does not allow visualization of individual particles or even small aggregates due to the low signal-to-noise ratio.

The challenges in the visualization of nanoparticles prompted us to adapt spectral imaging fluorescence microscopy. The basis of this technique is that the emitted light is detected separately at consecutive wavelengths throughout a defined spectrum range by using a multidetector array confocal microscopic arrangement. Experimental data showed that the spectrum of light emitted by particles was stable in various conditions, including serum-free and serum containing solutions, as well as in contact with histological mounting material and tissue slices. The fluorescence spectrum was not modified by the surface functionalization of NPs.

Targets for spectrum analysis ("regions of interest, ROIs) were identified and analyzed in corresponding sections of nanoparticle-treated and non-treated organs. To identify NP-containing ROIs a spectral ratio was calculated by relating the intensity of NP-

fluorescence to the tissue autofluorescence at wavelengths representing the maximums of corresponding light emissions.

Spectral fluorescence microscopy combined with spectral analysis and confocal z-stack imaging could identify particle derived fluorescence in various cells. The technique demonstrated that microglia cells internalized the plain SiO_2, SiO_2-NH_2 and the SiO_2-SH NPs, while SiO_2-PVP particles were rarely found inside cells; and neural stem cells, astrocytes, or neurons did not internalize NPs. The observations support, that spectral imaging fluorescence microscopy is a valuable tool to monitor fluorescent nanoparticles in samples where high autofluorescence is an issue.

***In vivo* distribution of nanoparticles**

The anatomical distribution of fluorescently-labeled polystyrene nanoparticles was assessed after a single intravenous injection in male and pregnant female mice. Carboxylated or PEGylated particles were administered to mice through a tail vein injection.

Five minutes after nanoparticle injection, high abundance of both PS-COOH and PS-PEG NPs were found in the kidney, liver and the spleen, e.g. in organs, known to be responsible for elimination of toxic agents from the body. In the brain and the placenta (protected by physiological barriers) only PS-COOH NPs were deposited, and PS-PEG NPs were rarely found.

In the brain, aggregated PS-COOH particles were concentrated in large vessels and capillaries, whereas the parenchyma was devoid of NPs. PEGylation reduced the attachment, indicating weaker interactions of PEGylated NPs with the vessel walls. The attachment of PS-COOH was restricted to short-term exposure; PS-COOH NPs were completely cleared from the brain four days after nanoparticle administration.

In the placenta, PS-COOH NPs, but not PS-PEG NPs were seen in the lacunas, but importantly, neither type of nanoparticles was found in embryonic tissues, indicating a proper placental barrier function. Both types of particles cleared out completely from the placenta within 4 days.

In striking contrast to the brain and the placenta, both PS-COOH and PS-PEG NPs were present in the kidney 5 minutes after NP-administration. Particles were found in the glomeruli and also in the interstitium around the tubuli. Four days later, PS-COOH NPs

were cleared from the kidney, while a few PS-PEG NPs were still visible within the glomeruli.

In the liver, high densities of both PS-NPs were found regardless of the functionalization, and PS-COOH and PS-PEG NPs were detected even after 4-day survival.

In the spleen, PS nanoparticles with both functional groups were detected in high densities. After 5-minute exposure, NPs were mainly restricted to the marginal zone, which is a region enriched in monocytes/macrophages. Immunocytochemical analyses directly demonstrated the presence of both NPs within the intracellular vesicles of Iba-1-positive phagocytic cells. After 4 days a characteristic redistribution of particles was detected regardless of functionalization: both, COOH-PS and PEG-PS NPs translocated to the white pulp, in the meantime, the total abundance of particles did not decrease during a 4-day period.

CONCLUSIONS

- The cellular uptake and biodistribution of nanoparticles is essentially modified by the chemical composition of the NP's surface.
- Passivating NP-surfaces with PEGylation or PVP-coating reduces the amount of adhered proteins, alters the protein corona composition, scales down the aggregation in organic solutions and reduces the interactions with biomolecules and cells.
- Cellular interactions of NPs are highly dependent on the types of cells and the surface characteristics of NPs:
    - Neurons and neural stem cells did not take up 50-70 nm silica or PS-NPs in serum free *in vitro* conditions.
    - In astrocyte enriched primary cultures, GFAP positive astrocytes did not internalize silica or PS-NPs, even if strong attachment of charged silica NPs to cell surfaces was observed. The phenomenon was reduced by PEGylation or PVP-coating of the particles.
    - Microglia cells internalize significant amounts of silica and PS-NP by energy-dependent endocytic mechanisms, while PEGylation and PVP-coating markedly reduced the process.

- Chemical properties of the nanoparticle surfaces interfere with the *in vivo* fate of PS-NP including short-term invasion and longer-term accumulation:
    - Initial attachment to blood vessel walls is reduced by PEGylation.
    - Barrier functions of the blood-brain-barrier and the placenta completely prevented the penetration of particles into the brain parenchyma and embryonic tissues, respectively.
    - Particles were retained in the reticuloendothelial organs regardless of surface functionalization. Strong accumulation of PS-COOH and PS-PEG was detected in the liver, in the macrophages of the spleen marginal zones, and some particles were found also in the intraglomerular mesangium of the kidney. The accumulation and long-term storage of nanoparticles in the reticuloendothelial systems rise important questions on the long-term health-risk even of otherwise non-toxic particles.
- Spectral imaging fluorescence microscopy allowed detecting the tissue- and cell-type-specific accumulation of fluorescent polymer nanoparticles in a time-frame which cannot be achieved with electron microscopy. The method was instrumental in the analysis of in vivo distribution of PS-NP, where the high autofluorescence of native tissues hinders the identification of particle fluorescence.

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PUBLICATIONS

Publications related to the PhD dissertation

- Kenesei K, Murali K, Czéh Á, Piella J, Puntes VF, Madarász E. Enhanced detection with spectral imaging fluorescence microscopy reveals tissue- and cell-type-specific compartmentalization of surface-modified polystyrene nanoparticles. Journal of Nanobiotechnology, 2016 Jul 7;14(1):55. Impact factor: 4.239

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