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Research paper

Polymeric nanoparticles of different sizes overcome the cell membrane barrier

Simone Lerch [a], Martin Dass [a], Anna Musyanovych [a], Katharina Landfester [a], Volker Mailänder [b,*]

[a] Max-Planck-Institute for Polymer Research, Mainz, Germany
[b] 3rd Medical Clinic (Hematology, Oncology, and Pneumology), University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

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ABSTRACT

Polymeric nanoparticles have tremendous potential either as carriers or markers in treatment for diseases or as diagnostics in biomedical applications. Finding the optimal conditions for effective intracellular delivery of the payload to the location of interest is still a big challenge. The particles have to overcome the barrier of the cell membrane. Here, we investigated the uptake in HeLa cells of fluorescent polystyrene particles with different size and surface charge. Particles stabilized with the nonionic surfactant Lutensol AT50® (132 nm, 180 nm, 242 nm, 816 nm, 846 nm diameter) were synthesized via dispersion polymerization. Cationic particles (120 nm, 208 nm, 267 nm, 603 nm diameter) were obtained by a combination of miniemulsion and seed dispersion polymerization using cationic surfactant (cetyltrimethylammonium chloride (CTMA-Cl). The particle uptake into HeLa cells was studied by confocal laser scanning microscopy and flow cytometry. Nonionic particles were – independent of their size – taken up by cells only at a barely detectable level, thus aggravating a quantitative comparison. The uptake of positively charged particles was substantially higher and therefore enabling further investigation keeping constant one of these parameters: either material amount or particles number or total interaction surface area. It was found that the uptake rather depends on the total amount of polymeric material present in the media than on the number of particles. The total particle's surface area does not correlate linearly with the uptake, thus indicating that there is no direct dependency between the total surface area and the cellular endocytotic process to overcome the biobarrier "cell membrane." A potentially novel uptake mechanism is found which can be described as an *excavator shovel* like mechanism. It is a kind of macropinocytosis dependent on actin filaments as well as dynamin, but is clathrin-independent.

1. Introduction

Tailored polymeric nanoparticles have a great potential in biomedical applications, mainly due to their manifold possibilities for functionalization and incorporation of diverse organic and inorganic materials. Different hydrophilic and hydrophobic drugs, enzymes, proteins, and imaging agents, etc. can be immobilized on the surface or encapsulated inside the particles, making them useful in specific drug targeting or diagnostics [1–6]. Design parameters that influence the particle uptake into cells are known to be the surface charge [7–13], size and shape [12,14–18], type of polymer [19–24], surface coverage [3,25], the cell line [26], and functionalization of the particles with bioactive molecules such as receptor-ligands or antibodies [27]. Particle or capsule size as given by the diameter is of importance as the amount of payload can be increased by the third power by designing these carriers with larger diameters. Therefore, larger nanoparticles are desirable in terms of maximum load that can be delivered by nanoparticles. On the other hand, polymeric nanoparticles of medium size (50–200 nm) have also been implicated with higher uptake into cells [28–30].

Further parameters determining the uptake of nanoparticles are inherent to the state of the cell and the cell type [11,31]. It is reported that non-tumorous cell lines internalize bigger particles easier than tumorous ones reflecting the different mechanisms of uptake, mostly phagocytosis versus endocytosis [32]. Macrophages have a size optimum of 1 μm and show a decreased uptake for smaller (200 nm) and bigger (10 μm) particles [13], possibly due

Abbreviations: CTMA-Cl, (1-hexadecyl) trimethylammonium chloride; cLSM, confocal laser scanning microscopy; V59, 2,2′-azobis(2-methylbutyronitrile; V50, azobisisobutylnamide dihydrochloride; Lumogen F Red®, (N,N-bis(2,6-diisopropylphenyl-)1,6,7,12-tetraphenoxyperylene-3,4,9,10-tetracarboxdiimide; DLS, dynamic light scattering; GPC, gel permeation chromatography; TEM, transmission electron microscopy; SEM, scanning electron microscopy; EIPA, 5-(N-ethyl-N-isopropyl) amiloride; 7-AAD, 7-aminoactinomycin D.

* Corresponding author. 3rd Department of Medicine (Hematology, Oncology, and Pneumology), University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstraße 1, 55131 Mainz, Germany. Tel.: +49 6131 379 248; fax: +49 6131 379 100.

E-mail addresses: simone.lerch@web.de (S. Lerch), dass_martin@gmx.de (M. Dass), anna.musyanovych@mpip-mainz.mpg.de (A. Musyanovych), katharina.landfester@mpip-mainz.mpg.de (K. Landfester), volker.mailaender@mpip-mainz.mpg.de (V. Mailänder).

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Table 1
Characteristics of fluorescent polystyrene particles obtained with nonionic surfactant Lutensol AT50® by dispersion polymerization.

Sample	Lutensol AT50®, mg	D_i, nm	Lutensol AT50® molecules per nm^2 [a]	Fluorescence normalization[d]	
				a.u.[e]	Factor [f]
SL-DP5	1000	132 ± 19	0.12	564.2	2.76
SL-DP6	500	180 ± 10	0.08	230.0	6.76
SL-DP7	200	242 ± 27	0.05	495.6	3.14
SL-DP8	100	635 ± 70	n.d.[g]	n.d.[g]	
SL-DP8-420[b]		816 ± 44	0.05	379.5	4.10
SL-DP8-110[b]		846 ± 51	0.06	1237.5	1.26

[a] Determined from the GPC measurements.
[b] Particles obtained after separation through centrifugation of sample SL-DP8.
[d] Measured by fluorescence spectroscopy at three different particle concentrations.
[e] The three fluorescence intensities obtained from different particle concentrations are linearized and the slope is given as a mean to normalize the differences in fluorescence between the particles.
[f] Fluorescence intensity slope from particle SL-V502 (see Table 2) is set to 1 and all other values are adjusted accordingly.
[g] The measurements were not performed as these particles were used for further purification.

Table 2
Characteristics of fluorescent positively charged polystyrene particles obtained with cationic surfactant and water-soluble cationic initiator V50.

Sample	CTMA-Cl, mg	D_i, nm	Lumogen F Red® [c]		Fluorescence intensity slope [d]		Zeta potential
			mg per $g_{Polymer}$	Factor	a.u.	Factor	mV
SL-V502	100	120 ± 10	0.80	1.00	1555.6	1.00	+73
SL-SP	100	120 ± 12	–[b]	–[b]	–[b]	–[b]	+77
SL-S27[a]	11	208 ± 29	2.16	0.37	3164.4	0.49	+56
SL-S30[a]	5	267 ± 28	1.23	0.65	4077.2	0.38	+56
SL-S26[a]	0	603 ± 15	0.28	2.85	1122.4	1.39	+60

[a] Obtained by seed dispersion polymerization, using SL-SP as seed particles (48 ml with solid content 0.1 wt.%).
[b] The measurements were not performed, because these particles were used only as seed particles and not studied in the cell uptake experiments.
[c] Calculated from the UV–vis spectroscopy data.
[d] Measured by fluorescence spectroscopy.

to their specific role as scavenger cells in the human immune system and with phagocytosis being an important uptake mechanism. Furthermore, particles cannot only be endocytozed but also transported across the cell, that is transcytozed [33]. For the non-phagocytotic entry routes, the reasons for the variances in the uptake of particles with different size are still to be explored thoroughly. Investigations into the different mechanisms of endocytosis, for example, clathrin-dependent or caveolin-dependent endocytosis, or macropinocytosis, showed that they are strongly size-dependent [14].

Although many papers have been published with cell uptake studies, the obtained results cannot be directly compared, due to the different experimental conditions and usually not completely characterized particles. Particle properties, such as surface charge, amount of fluorescent dye, particle size and size distribution, morphology, and so on, should be well defined and characterized.

Here, we report the synthesis, characterization, and HeLa cell uptake of nonionic and cationic polystyrene particles that are identical in their chemical composition, respectively, and differ only in the mean diameter. Varying only the size of particles, it is possible to trigger the uptake kinetic into cells and to use this difference to design target-specific particles as drug delivery systems.

For the synthesis of the particles, different polymerization techniques were used. Particles stabilized with the nonionic surfactant Lutensol AT50® (132 nm, 180 nm, 242 nm, 816 nm, 846 nm) were synthesized via dispersion polymerization; Lutensol AT50® is a poly(ethylene oxide)-hexadecyl ether with an EO block length of about 50 units. Cationic particles (120 nm, 208 nm, 267 nm, 603 nm) were obtained by a combination of the miniemulsion and seed dispersion polymerization using cationic surfactant (1-hexadecyl) trimethylammonium chloride (CTMA-Cl). Previously, we have proven that fluorescent polystyrene nanoparticles obtained via the miniemulsion process are non-toxic and by being non-degradable can be easily traced in cells [34,35].

The influence of the particle size on the time-dependent uptake of HeLa cells was studied using flow cytometry and confocal laser scanning microscopy (cLSM). Further insights into the particle-cell interactions were gained by transmission electron microscopy. By blocking specific endocytotic mechanisms, such as the clathrin-dependent pathway, the formation of actin filaments, the GTPase dynamin, and macropinocytosis, the uptake mechanism was determined for the differently sized nanoparticles.

2. Experimental part

2.1. Materials

Styrene (Merck, Germany) was distilled under reduced pressure before use. All other chemicals were used without further purification: *n*-hexadecane (HD, 99%, Aldrich, Germany), 2,2′-azobis(2-methylbutyronitrile) (V59, Wako Chemicals, Germany), azobisisobutylnamide dihydrochloride (V50, Wako Chemicals), ethanol (EtOH p.a., Merck, Germany), fluorescent dye Lumogen F Red® (N,N-bis(2,6-diisopropylphenyl-)1,6,7,12-tetraphenoxyperylene-3,4,9,10-tetracarboxdiimide; BASF, Germany), surfactant Lutensol AT50® (BASF), which is a poly(ethylene oxide)-hexadecyl ether with an EO block length of about 50 units and surfactant (1-hexadecyl) trimethylammonium chloride (CTMA-Cl, 95%, Alfa Aesar, Germany). Demineralized (demin.) water was used throughout the experiments.

2.2. Synthesis

Fluorescent particles stabilized with the nonionic surfactant Lutensol AT50® and having diameters in the range between 130 and 890 nm were synthesized via dispersion polymerization. Briefly, 6 g styrene, 0.1 g oil-soluble initiator V59, 0.004 g fluorescent dye (Lumogen F