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Research paper

Endothelial cell response to (co)polymer nanoparticles depending on the inflammatory environment and comonomer ratio

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ABSTRACT

Endothelial cells lining the lumen of blood vessels serve as a physiological barrier controlling nanoparticle movement from the vasculature into the tissue. For exploring the effect of polymer hydrophilicity on nanoparticle interactions with human umbilical vein endothelial cells (HUVECs) *in vitro*, a series of monomodal poly[acrylonitrile-*co*-(*N*-vinylpyrrolidone)] model nanoparticles with increasing hydrophilicity as related to their increasing content (0–30 mol.%) of *N*-vinylpyrrolidone (NVP) were synthesized by miniemulsion polymerization. Nanoparticles with a low NVP content were rapidly endocytized into all cells independent from the particle dose with toxic effects only observed at high particle concentrations, while only 10–30% of the cells incorporated particles with $\geqslant$20 mol.% NVP. Since pathologies are often related to inflammation, an inflammatory HUVEC culture condition with IL-1β stimulation has been introduced and suggested to be widely applied for studying nanocarriers, since cellular uptake in this assay was clearly increased for NVP contents $\geqslant$20 mol.%. Importantly, the secretion of functional biological mediators by HUVECs was not relevantly influenced by the nanoparticles for both homeostatic and inflammatory conditions. These findings may motivate concepts for nanocarriers specifically targeted to pathologic regions. Additionally, rapidly endocytized Rhodamin B loaded particles with low NVP content may be explored for cell labeling and tracking.

1. Introduction

The fascinating concept of modulating the biodistribution and cellular uptake of particulate carriers by a matrix size reduction has put great expectations into the field of nanoparticle technology. It was indicated that certain nanocarriers may not only penetrate into the skin but may permeate through it for systemic delivery of encapsulated drugs [1]. For nanocarriers injected intravenously, a reduced vascular integrity of tumor tissue due to lesions in-between the endothelial cells led to the theory of enhanced permeation and retention (EPR), by which drug-loaded particles would extravasate and possibly distribute into the tumor tissue for a more effective treatment [2]. In addition to tumor targeting, nanoparticle delivery to specific organs has been focused on by facilitating a reported inherent capability of specific families of biomaterials to overcome intact endothelial layers such as the very effective blood–brain barrier for brain targeting [3]. Despite these remarkable findings, the research community is presently developing a more critical view on the opportunities, challenges, and limitations of nanoparticulate carriers for both topical [4] and intravenous [5] administration. One concern, which has also been recognized by the public, is a potential short-or long-term toxicity of nanoparticles [6]. Therefore, systematic studies remain of high relevance that evaluate cellular fate based on properties of nanoparticles, which may be injected as carrier systems for drug delivery, originate from environmental sources or implant abrasions, or may be employed in bioimaging to label and potentially track cells.

When focusing on an *in vivo* scenario, nanoparticles that have entered the body by one or the other pathway will, upon their distribution in the body through the vascular system, contact endothelial cells (ECs) as the top luminal cell layer in vessels. As applicable for all mammalian cells, ECs possess the capability for endocytosis, for example, for uptake of nutrients by a number of different pinocytic pathways [7], which may also be followed by nanoparticles entering the cells. Nanoparticle surface functionalization may ideally result in an accumulation in a certain pathologic tissue or in a defined uptake pathway to be followed [8]; the biological consequences of nanoparticle entry into ECs by either one or the other pinocytic pathway are currently not fully understood.

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Similarly, *in vivo* EC interaction with nanoparticles may strongly differ from standard cell culture conditions in numerous *in vitro* studies. In particular, physiological processes such as blood flow that limits the approximation of particles to the ECs [9] and reduces their uptake [10] as well as pathophysiological conditions such as inflammation are rarely considered [11]. In particular, an inflammatory environment, as can be mimicked *in vitro* by pro-inflammatory cytokines such as IL-1β [12], can be expected not only to alter paracellular transport by modulation of EC tight junctions [13], but it was hypothesized to also affect the EC endocytic activity and/or response to engulfed nanoparticles.

In addition to the assumed response of EC functionality to inflammation, different nanoparticle properties such as the hydrophobicity of the polymer matrix as related to the polymer composition are believed to be important contributors to their interaction with cells either directly or indirectly as mediated by adsorbed proteins [14]. In order to provide model materials with systematic variation of hydrophilicity, a suitable copolymer system with increasing capacities to interact with water may be a rational approach. Copolymers from monomers substituted with side chains of different hydrophobicity such as poly(alkyl-2-cyanoacrylates) [15] or poly(alkyl methacrylate) [16] are examples of polymer systems with tunable properties, but possible contributions of the dangling side chains in the interaction with cells may be difficult to evaluate. Alternatively, comonomers forming repeating units of different hydrophilicities that do not carry spacious side chains may be copolymerized. Here, based on the reported hemo- and bio-compatibility of membranes from acrylonitrile (AN)-based (co)polymers [17], poly[acrylonitrile-*co*-(*N*-vinylpyrrolidone)]s [P(AN-*co*-NVP)] were selected for this study as a family of materials, in which an increasing molar content of *N*-vinylpyrrolidone (NVP) correlates with an increasing material hydrophilicity and water uptake.

In order to systematically study the interaction of P(AN-*co*-NVP)-based nanoparticles with ECs, monomodal, narrow particle size distributions were envisioned, which ideally should be formed by combining copolymer synthesis and particle formation in an integrated process. The synthesis of AN-based nanoparticles has been systematically studied, mostly based on (macro)emulsion/dispersion polymerization [18–20], which in some cases involved the use of solvents or metal based catalysts with assumed toxicity and/or may result in particles with a larger size distribution. Several of these challenges can be overcome by acrylonitrile copolymerization in miniemulsions [21], in which less surfactant is required when compared to the high detergent levels used for polyacrylonitrile nanoparticle synthesis in microemulsions [22] and a hydrophobic costabilizer impedes Ostwald ripening of nascent droplets/particles, thereby enabling narrow size distributions.

In the present study, a series of copolymer nanoparticles from P(AN-*co*-NVP) with low cytotoxicity for a murine fibroblast cell line [23] have been synthesized in a miniemulsion process and doped with Rhodamin B dye to enable visualization and quantification of their interaction with primary human umbilical vein endothelial cells (HUVECs). Importantly, besides a detailed characterization of nanoparticle composition and morphology, the response of HUVECs to nanoparticle exposure was analyzed not only under standard cell culture conditions, but additionally also in an inflammatory environment, thus mimicking conditions possibly present under pathologic conditions.

2. Materials and methods

2.1. Materials

The comonomers acrylonitrile (AN; ⩾99.0%, distilled at 77 °C prior to use) and 1-vinyl-2-pyrrolidone (NVP; ⩾99%, purified on syringe column packed with inhibitor removers) as well as the initiator 2,2′-azobis-(2-methyl-butyronitrile) [AMBN; ⩾98.0%], the stabilizer sodium dodecyl sulfate (SDS; ⩾99.0%), the dye Rhodamin B, and the solvent hexadecane (⩾99.8%; Fluka brand) were purchased from Sigma–Aldrich (Steinheim, Germany). All other chemicals were of analytical grade.

2.2. Synthesis of nanoparticles

In order to achieve highest purity of nanoparticles, the synthesis based on an oil-in-water (o/w) emulsion and the subsequent purification was performed with depyrogenized glassware in a biological safety cabinet under laminar air flow. The continuous w-phase consisted of 38 ml of water substituted with 80 mg of SDS. The o-phase was prepared by first dispersing 0.333 mg Rhodamin B in 200 mg hexadecane and adding a total of 2 g of AN/NVP monomers with different molar ratios. After pre-emulsification by magnetic stirring at 650 rpm in a pressure tube (100 ml, 17.8 cm length × 38.1 mm O.D.) for 1 h, 40 mg of AMBN was added and the mixture was sonicated for 4 min in an ice bath for nanodispersion of the o-phase droplets (Sonopuls HD 2017 with a 70 G probe, amplitude 90%; Bandelin, Berlin, Germany). The reaction was continued at 77 °C in closed vessels under magnetic steering at 600 rpm for 7 h. Purification by dialysis was performed with diluted aliquots (25 ml + 65 ml sterilized water) in ethylene oxide-sterilized dialysis tubing (Visking type 20/32, MWCO 14,000 Da cutoff; Carl Roth, Karlsruhe, Germany) for 6 d at room temperatures with frequently replaced 2.5 ml sterilized water as external medium. Rhodamin B did leak neither during dialysis nor during release experiments at 37 °C into the medium as analyzed by HPLC. Depending on the percent relative molar feeding ratio *R* of NVP with $R = n_{NVP} \cdot (n_{NVP} + n_{AN})^{-1}$, the copolymer nanoparticles were denoted as P(AN-*co*-NVP) *R*, for example, P(AN-*co*-NVP) 10 as prepared by using 1.654 g AN and 0.346 g NVP monomers during synthesis.

2.3. Chemical composition of nanoparticles

The composition of nanoparticles as obtained after synthesis was characterized by ^{1}H NMR on a 500 MHz Bruker Avance spectrometer (Karlsruhe, Germany) in dimethyl sulfoxide-d6 (Sigma Aldrich, Steinheim, Germany). ^{1}H NMR (500 MHz, DMSO-d6) δ (ppm) = 4.38–4.18 (1H, d), 3.38–3.29 (2H, g), 3.25–2.98 (1H, b), 2.92–2.60 (2H, e), and 2.35–1.70 (6H, f + a + c). Additionally, elemental analysis (EA) was performed with a vario EL III (Elementar, Hanau, Germany) with a thermal conductivity detector in a helium atmosphere.

2.4. Particle size, charge, and morphology

Particle sizes were determined by dynamic light scattering on a Delsa™Nano C (Beckman Coulter, Krefeld, Germany) with dilution of 50–100 µl of the nanoparticle samples in 1 ml water. Measurements were performed at 20 °C in quartz glass cuvettes, and scattering pattern analyzed at an angle of 165° with the Contin model.

Particle size and morphology were additionally evaluated by scanning electron microscopy (SEM) on a Gemini SupraTM 40 VP (Carl Zeiss NTS, Oberkochen, Germany) at 10 kV with a secondary electron detector. Sample preparation involved their spreading on a silicon waver using a spin coater at 5000 rpm (Laurell Spin Coater Modell 650) and their sputtering with iridium.

Analysis of particle powders by Wide-Angle X-ray Scattering (WAXS) was performed at room temperature on a D8 Discovery (Bruker, Karlsruhe, Germany).