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Effects of extracts prepared from modified porous poly(ether imide) microparticulate absorbers on cytotoxicity, macrophage differentiation and proinflammatory behavior of human monocytic (THP-1) cells

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Abstract

Remaining uremic toxins in the blood of chronic renal failure patients represent one central challenge in haemodialysis therapies. Highly porous poly(ether imide) (PEI) microparticles have been recently introduced as candidate absorber materials, which show a high absorption capacity for uremic toxins and allow hydrophilic surface modification suitable for minimization of serum protein absorption. In this work, the effects of extracts prepared from PEI microparticles modified by nucleophilic reaction with low molecular weight polyethylene imine (Pei) or potassium hydroxide (KOH), on human monocytic (THP-1) cells are studied. The obtained results suggested that the extracts of Pei and KOH modified PEI absorbers have no negative effect on THP-1 cell viability and do not initiate the critical differentiation towards macrophages. The extracts did not enhance transcript or protein levels of investigated proinflammatory markers in THP-1 cells, namely, TNFα, MCP1, IL6 and IL8. Based on these findings such modified PEI microparticles should be qualified for further pre-clinical evaluation i.e. in an in vivo animal experiment.

Keywords: Chronic kidney disease, haemodialysis, inflammation, porous poly(ether imide) microparticulate absorbers, THP-1 cells, uremic toxins
1. Introduction

Porous polymer-based systems are widely utilized as key components of extracorporeal medical devices. Prominent examples are polymeric membranes and particulate absorbers applied in haemodialysis or apheresis for blood detoxification therapies [1-6]. The more frequent cardiovascular events in renal failure dialysis patients might be related to an accumulation of (protein-bound) uremic toxins in the serum, which cannot be efficiently removed by existing dialysis methods, because of their hydrophobic nature and size, which is larger than the pore size of the common dialysate membrane [7,8]. Furthermore, the albumin-binding capacity was reduced in patients with chronic kidney disease (CKD) along with an accumulation of protein-bound uremic toxins [9]. Uremic toxins like indoxyl sulfate (IDS) or p-cresylsulfate (pCRS) are reported to be associated with vascular inflammation by activating monocytes, macrophages causing endothelial dysfunction [10,11].

In this context, the usage of multifunctional hydrophobic polymeric absorber materials represents an alternative approach to remove the uremic substances from the plasma of patients, whereby a hydrophilic coating of the absorbers would be beneficial to minimize protein absorption. One promising absorber candidate are highly porous microparticles prepared from poly(ether imide) (PEI) showing a high absorption capacity for uremic toxins in particular of hydroxy hippuric acid (OH-HPA), phenylacetic acid (PAA), IDS and pCRS [12]. Additionally, the surface of the porous PEI absorbers can be hydrophilized via reaction with nucleophiles such as low molecular weight polyethylene imine (Pei) or potassium hydroxide (KOH). Advancing water contact angles around 60° determined in environmental scanning electron microscopy experiments confirmed the successful hydrophilic modification with both modifiers Pei or KOH [13]. Initial hemocompatibility tests revealed a reduced hemolytic activity of KOH modified PEI microparticles when compared to the non-modified absorbers [14].
In this study, extracts prepared from differently modified PEI microparticles were examined regarding potential toxicity or inflammatory effects on human monocytic (THP-1) cells and their differentiation behavior into macrophages. The modified absorbers were prepared by post-treatment of porous PEI microparticles with Pei or KOH.

2. Materials and methods

2.1. Preparation of modified poly(ether imide) microparticles

Porous PEI microparticles were prepared by a spraying/coagulation process as described previously [13,15]. For hydrophilic modification, PEI particles were reacted at 90 °C with an aqueous solution containing 4 wt% of either low molecular weight poly(ethylene imine) (Mn = 600 g/mol) or with potassium hydroxide for 30 min. The modified products were then washed with distilled water until pH neutrality. Finally, the modified particles were purified with deionized water at 100 °C for 1 hour and vacuum dried. Prior to biological testing, the microparticles were steam sterilized at 121 °C, 2.0 bar for 20 minutes using a Systec Autoclave D-65 (Systec GmbH, Wettenberg, Germany).

2.2. Microparticle characterization

Shape and size of the prepared microparticles were characterized by scanning electron microscopy (SEM) with a Phenom G2 pro (L.O.T. - Oriel, Darmstadt, Germany) after coating with a conductive layer. For analysis of the average particle diameters the image processing software, Image J (Version 1.48v, Wayne Rasband, USA) [16] was applied and the standard deviation (error) was determined by measuring 350 particles. In addition, the particles’ surface and cross section was visualized by scanning with a Gemini Supra 40 VP SEM (Zeiss AG, Oberkochem, Germany). The water microwettability of the prepared particles was assessed by environmental scanning electron microscopy (ESEM) utilizing a FEI Quanta FEG 250 (FEI
Deutschland GmbH, Frankfurt/Main, Germany) equipped with a gaseous secondary electron
detector operated in the wet mode according to the method described in [13]. The micro-contact
angles were estimated from the water meniscus condensed between two polymeric particles.
At least four measurements for advancing or receding angles were performed for each particle
sample and the average contact angles as well as the related standard deviation were calculated.

2.3. Preparation of PEI microparticles extracts

Dry PEI microparticles were suspended in a mixture of isopropanol and deionized water (1:1)
for 60 min and filtered with glass frit filter funnel by applying vacuum. Afterwards the particles
were suspended again in deionized water for 60 min and subsequently vacuum filtered with
extensive water rinsing. Finally, the treated particles were suspended in deionized water and
their sedimentation behavior was investigated. After rewetting microparticles were steam
sterilized at 121 °C, 2.0 bar for 20 minutes using an autoclave for biological testing. 200 mg of
rewetted steam sterilized modified PEI microparticles were suspended in 12 mL of RPMI-1640
media containing 10 % (v/v) FBS and agitated for 5 h on a rotatory shaker for proper mixing
of particles.

2.4. Cell culture

Human monocytic (THP-1) cells were procured from NCCS (Pune, India) and grown to a
confluence in RPMI-1640 medium supplemented with 10 % (v/v) fetal Bovine Serum (FBS,
Gibco, USA) and 1 % (v/v) Pen Strep (Invitrogen, USA). Cells were maintained in 5 % CO₂
incubator at 37 °C. THP-1 cells were treated with 2 mL of modified PEI particle extract for 5
h and cells were filtered with 40 μm falcon cell strainer to eliminate the interference of particles
with biological assay protocols. Cells induced with PMA (25 ng/mL) or endotoxin LPS (0.5
µg/mL) for 48 h and 3 h were used as positive controls for differentiation and inflammatory studies.

2.5. Cytotoxicity-Trypan blue assay

Cell viability of modified PEI particle extract treated THP-1 cells was assessed by trypan vital staining exclusion method [17]. THP-1 was plated at a density of 5× 10⁵/mL and pretreated with PEI particle extracts for 5h. After incubation, 20 µL of each particle-treated cell suspension was mixed with 10 µL trypan blue dye solution and observed the phenotype of cells in relevance to morphology microscopically using a hemocytometer.

2.6. Cell differentiation: Monocyte to macrophage

Effect of modified PEI microparticle extracts on monocyte to macrophage differentiation were investigated by using an inverted microscope. Monocyte to macrophage differentiation can be achieved by stimulating THP-1 cells with 25 ng/mL of phorbol-12-myristate 13-acetate (PMA) for 48 h [18,19]. Phenotype of extracts-treated cells for 5h were observed microscopically and PMA stimulated cells were used as positive control.

2.7. Transcript levels of proinflammatory markers in THP-1 cells – quantitative real-time PCR

RNA was prepared from extract-treated cells using Trizol reagent and transcribed to c-DNA by I Script c-DNA synthesis kit (Bio-Rad laboratories, Hercules, USA). The transcript levels of proinflammatory genes such as TNF-α, MCP-1, IL-6, IL-8, and IL-1 were analyzed by qRT-PCR using SYBER Green PCR reagent. The primer sequences (Eurofins genomics, Bangalore, India) of targeted proinflammatory genes are given in the Table 1 [20-22]. The 10 µl reaction volume consisted of a cDNA template and a cocktail mix (1x SYBR Green, 1x forward and
reverse primers, water). The transcript levels of all the tested genes were normalized using as internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Table 1** Primer sequences for studying gene expression of TNF-α, MCP-1, IL-6, IL-8, IL-1 and GAPDH

<table>
<thead>
<tr>
<th>S.no</th>
<th>Genes</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>TNF-α</td>
<td>5´-CCCAGGGAACCTCTCTCTCTTAATC-3´</td>
<td>5´-ATGGGCTACAGGGCTTGTCACT-3´</td>
</tr>
<tr>
<td>2.</td>
<td>MCP-1</td>
<td>5´-GCCAAGGAGATCTGCTGCTGAC-3´</td>
<td>5´-CATGGAATCTGTAACCCACTTC-3´</td>
</tr>
<tr>
<td>3.</td>
<td>IL-6</td>
<td>5´-TGGATTCAATGAGGAGACTTC-3´</td>
<td>5´-CAGGAAGTGCAGCAGCTTC-3´</td>
</tr>
<tr>
<td>4.</td>
<td>IL-8</td>
<td>5´-GTGTAAACATGACTTCCAAGCTGG-3´</td>
<td>5´-GCACCTTCACACAGAGCTGC-3´</td>
</tr>
<tr>
<td>5.</td>
<td>IL-1</td>
<td>5´-GGATAACGAGCAGCTTATGTCACG-3´</td>
<td>5´-GGACATGGAGAACACACTTGG-3´</td>
</tr>
<tr>
<td>6.</td>
<td>GAPDH</td>
<td>5´-CACCAACTGCTTAGCACCACCC-3´</td>
<td>5´-TGGTCATGAGTCCTTTCACG-3´</td>
</tr>
</tbody>
</table>

2.8. *Protein levels of inflammatory markers in THP-1 cells - ELISA*

Cell culture supernatants of THP-1 cells were collected after 5h treatment of PEI microparticles by centrifugation at 3000 rpm for 3 min and quantified the release of proinflammatory cytokines and chemokines such as tumor necrosis factor (TNF-α), monocyte chemotactic protein (MCP-1) and interleukins (IL-6, IL-8) using ELISA kits (BD-Bioscience, San Diego, USA) as per manufacturer’s protocol. LPS (0.5 ng/mL) induced THP-1 cells were used as positive control for comparison [22].

2.9. *Statistics*

All data were expressed as mean values of minimum three independent experiments ± standard deviation.
3. Results and Discussion

In the present study we investigated human monocytic (THP-1) cells, which were exposed to extracts prepared from of steam sterilized Pei and KOH modified PEI microparticles.

3.1. Microparticle characteristics

All modified microparticles exhibited a nice spherical shape. Pei-modified particles had an average diameter of 110±25 µm, while for KOH-modified a diameter of 140±30 µm was found. The obtained particle diameters after modification are identical with that of the respective batches of the non-modified PEI particles used for post-modification.

![Fig. 1. Scanning electron microscopy images of PEI microparticles, modified with polyethylene imine (PEI-Pei) and KOH (PEI-KOH) visualizing a single particle, the particles’ surface, and ESEM images visualizing the advancing water contact angles obtained in microwetting. The white arrows indicate the water meniscus in between the particles.](image)

The first two columns of Fig. 1 display SEM images visualizing individual particles as well as the nanoporous structure at the particles’ surface for 30 min post-modified particles PEI-Pei
and PEI-KOH. An almost similar average pore size of 90 ± 30 nm was analyzed from the SEM images for PEI-Pei, while a higher pore size of 230 ± 70 nm was obtained for PEI-KOH, suggesting a more efficient imide bond cleavage reaction of the molecular modifier KOH when compared to the macromolecular modifiers Pei. In the right column of Fig. 1 ESEM images of the micro-wetting experiments are shown. In such experiments, almost identical advancing contact angles were found for both modified particles with 64 ± 5° (PEI-Pei) and 60 ± 2° for PEI-KOH. The respective receding contact angles were 23 ± 3° and 22 ± 6°. The obtained water contact angles, clearly demonstrated the hydrophilic nature of the modified particles when compared to the advancing contact angle of 112 ± 10° reported for non-modified PEI microparticles [14]. The obtained characteristics of the Pei and KOH modified particles is in good agreement with that reported in our previous work [13,14].

3.2. Effect of microparticle extracts on cell viability

The trypan blue exclusion method was utilized to assess potential cytotoxic effects of PEI-Pei and PEI-KOH extracts on THP-1 cells. Trypan blue is a vital stain, which traverses the membrane of dead cells and stains the cytosol blue in colour whereas intact membranes of viable cells exclude the passage of the dye as a result the cytosol remains un-stained. The resulting microscopic data shown in Fig. 2 reveal a high THP-1 cell viability of around 99% in all tests with both extracts. Based on these findings it can be concluded that the tested extracts do not exhibited any toxic effects to THP-1 cells, similarly as found for extracts of non-modified PEI microparticles [20], confirming that both chemical post-treatments with Pei and KOH do not alter the cell compatibility of the absorbers.
Fig. 2. Cell viability of THP-1 cells pre-treated with modified PEI microparticle extracts for 5 h was determined by Trypan blue exclusion method. Control (A-C) represents viable cells; D-F depicts dead cells and G-L represents live cell assemblies after treatment with the differently modified PEI microparticles for 5 h.

The effects of modified particle extracts on cell differentiation was studied by differentiation of monocytes (THP-1 cells) to macrophages via stimulation with PMA. Adherent irregular shaped macrophage-like structures are obtained 48h after induction with PMA (Fig. 3 D-F). However, such kind of differentiation could not be observed when the cells were cultured after exposure with PEI-Pei and PEI-KOH microparticle extracts for 5h (Fig. 3 G-L), similarly to the behavior of the cells in control experiments. Therefore, it can be concluded that extracts of the modified PEI microparticles do not affect the differentiation behavior of THP-1 cells to macrophages, like previously reported for extracts of non-modified PEI microparticles [20]. Monocyte to macrophage differentiation is the crucial step which leads to progression of
various chronic diseases [23] as macrophages are key orchestrators of inflammation via releasing cytotoxic mediators like ROS/RNS [24] and cytokines upon activation [25].

Fig. 3. Representative microscopic images visualizing the macrophage differentiation of THP-1 cells. Control A-C represents monocytes; D-F depicts differentiated macrophages upon exposure to PMA for 48h and G-L represents undifferentiated monocytes after treatment with the differently modified PEI microparticles extracts for 5 h.

3.3. Effect of microparticle extracts treated cell on inflammatory markers

Gene expression levels of TNF-α, MCP-1, IL-6, IL-8 and IL-1 transcripts were quantified (fold change) using real time PCR (qRT-PCR). Bacterial endotoxin, LPS upregulated the expression of proinflammatory genes whereas both modified PEI particle extract treated THP-1 cells did not show any upregulation (Fig. 4). The secretion of the protein proinflammatory markers TNF-α, MCP-1, IL-6 and IL-8 was detected at basal levels in extract treated THP-1 cells similar to control or untreated cells when quantified by ELISA whereas bacterial endotoxin LPS enhanced the release of the above protein proinflammatory markers by several folds as shown
in Fig. 5. Inflammatory response is characterized by the secretion of various proinflammatory cytokines and chemokines [26]. TLRs act as signaling sensors for various pathogen associated molecular patterns (PAMPs) and activates several downstream signaling molecules leading to activation of transcription factors like NF-κB, AP-1 and IRF3 [27]. These inflammation associated transcription factors translocate into the nucleus and activate the expression of various cytokines or chemokines (TNF-α, MCP-1, IL-6, IL-8 and IL-1) by binding to their promoters in the nucleus and leads to their secretion. These secreted proinflammatory agents were abundantly localized in atherogenic lesions which contribute to the initiation and progression of chronic inflammatory disease atherosclerosis aiding in the recruitment, rolling and adhesion of monocytes via upregulating adhesion or sticky molecules like vascular cell adhesion molecules (VCAM-1), intracellular adhesion molecules (ICAM-1) and endothelial selectin (E-selectin) present on the surface of endothelium [28-30]. It is indispensable to ensure that chemically modified PEI micro particles are free of pathogenic contaminants and also do not release any toxins, which otherwise elicit host response following device implantation including both acute and chronic inflammation and lead to failure of tissue engineered medical device implantation in diseased human subjects. Therefore, we analyzed the inflammatory response of extracts of modified PEI particles in THP-1 cells and we observed that particles did not activate any of the inflammatory markers at both protein and transcript levels. Summarized, the obtained results showed that the investigated extracts prepared from Pei or KOH modified PEI microparticles did not influence the viability, the differentiation, and the proinflammatory behavior of human monocytic cells.
Transcript levels of proinflammatory markers relative to GAPDH in THP-1 cells

- TNF-α
- MCP-1
- IL-8
- IL-6
- IL-1
Fig. 4. Effect of extracts of modified poly(ether imide) (PEI) microparticles on transcripts of inflammatory markers. THP-1 pre-treated with PEI microparticle extracts for 5h. At the end of the treatment, RNA was isolated using Trizol reagent. Gene expressions of TNF-α, MCP-1, IL-8, IL-6 and IL-1. Data represent mean ± SD of 3 independent experiments and *p<0.001 compared between treated and untreated.
Fig. 5. Effect of modified PEI microparticle extracts on inflammatory markers of THP-1 cells. Protein levels of TNF-α, MCP-1, IL-6 and IL-8 were quantified by ELISA. Data represent mean ± SD of 3 independent experiments and *p < 0.001 compared between treated and untreated cells.

4. Conclusion

The obtained results showed that hydrophilic modified PEI microparticles, prepared by post-modification with poly(ethylene imine) or KOH did not exhibit negative effects on the viability, the differentiation, and the proinflammatory behaviour of human monocytic cells. Therefore, such modified PEI microparticles will be further explored regarding their hemocompatibility and applicability in in vivo experiments.

5. Acknowledgements

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This paper is dedicated to the 70th birthday of Prof. Friedrich Jung.
References


