



## Surfactant-free preparation of highly stable zwitterionic poly(amido amine) nanogels with minimal cytotoxicity



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### ABSTRACT

Narrowly dispersed zwitterionic poly(amido amine) (PAA) nanogels with a diameter of approximately 100 nm were prepared by a high-yielding and surfactant-free, inverse nanoprecipitation of PAA polymers. The resulting, negatively charged, nanogels (**PAA-NG1**) were functionalized with *N,N*-dimethylethylenediamine via EDC/NHS coupling chemistry. This resulted in nanogels with a positive surface charge (**PAA-NG2**). Both types of nanogels were fluorescently labelled via isothiocyanate coupling. **PAA-NG1** displays high colloidal stability both in PBS and Fetal Bovine Serum solution. Moreover, both nanogels exhibit a distinct zwitterionic swelling profile in response to pH changes. Cellular uptake of FITC-labelled nanogels with RAW 264.7, PC-3 and COS-7 cells was evaluated by fluorescence microscopy. These studies showed that nanogel surface charge greatly influences nanogel–cell interactions. The PAA polymer and **PAA-NG1** showed minimal cell toxicity as was evaluated by MTT assays. The findings reported here demonstrate that PAA nanogels possess interesting properties for future studies in both drug delivery and imaging.

#### Statement of significance

The use of polymeric nanoparticles in biomedical applications such as drug delivery and imaging, shows great potential for medical applications. However, these nanoparticles are often not stable in biological environments. Zwitterionic polymers have shown excellent biocompatibility, but these materials are not easily degradable in biological environments. With the aim of developing a nanoparticle for drug delivery and imaging we synthesized a biomimetic and readily biodegradable zwitterionic polymer, which was incorporated into nanogels. These nanogels showed excellent stability in the presence of serum and minimal cytotoxicity, which was tested in three cell lines. Because of their negative surface charge and excellent serum stability, these nanogels are therefore promising carriers for drug delivery and molecular imaging.

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### 1. Introduction

Nanoparticle size and surface chemistry are of key importance to their successful application in targeted drug delivery and imaging. In biomedical applications, nanoparticles will be surrounded by complex media, containing proteins and cells [1,2]. In this environment, the adsorption of proteins onto nanoparticles requires careful consideration, as it may lead to accelerated removal of the particle by the immune system and consequently nanoparticle accumulation in the liver and spleen [1,3,4]. To improve the half-

life and efficacy of a nanoparticle, and consequently its ability to reach the targeted site of interest, reduction of protein adsorption onto a nanoparticle's surface can be achieved by engineering the nanoparticle's surface chemistry [1,4,5]. Since polymeric nanoparticle surface chemistry, particle size, biodegradability and responsiveness are readily adapted, they have accordingly been at the centre of attention in the biomedical field [1,6–12]. Polymeric nanoparticles also show tremendous variability in particle structures and show circulation properties dependent on their physicochemical properties, such as particle size, surface charge and hydrophilicity [2,4]. Therefore, they are applicable in a wide variety of applications ranging from controlled release to targeted imaging of tissues.

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A layer of polyethylene glycol (PEG) is commonly used to introduce a hydrophilic layer and to increase steric hindrance around the nanoparticle to prevent protein adsorption and thus delaying an immunological response [1,2]. Zwitterionic polymers [13–17] have shown extremely low protein adsorption and excellent biocompatibility [18]. However, the carbon-based backbone of these zwitterionic polymers is not prone to degradation. The incorporation of degradable groups along the polymer backbone [19,20] or coupling of oligomers via degradable linkages [21,22] improves the polymer's biodegradation properties, but still prevents degradation of the polymers down to single monomeric units. Polymers, which resemble peptides, are interesting materials for biomedical applications, because of the gradually degradable amide motif in the polymer backbone. Furthermore, the chemical resemblance to proteins would grant unique properties to these polymers in terms of interaction with biological materials [23,24]. From this perspective, we [25–32] and others [33–37] have extensively studied poly(amido amine)s (PAAs) as versatile polymers for gene and drug delivery. PAAs are water-soluble peptidomimetic polymers, which are readily obtained via a Michael-type poly-addition between bisacrylamides and bifunctional amines [36]. PAAs showed minimal cytotoxicity on multiple occasions: such as in gene delivery [25,29,34,35], drug delivery [38,39], imaging [40], therapeutics [33] and tissue engineering [41]. Introduction of disulphide moieties in the PAA backbone renders these polymers bioreducible [31]. As such, bioreducible PAA polyplexes have successfully been applied as carriers for plasmid DNA, siRNA and proteins, with excellent transfection efficacies [29–32]. Regarding these properties, crosslinked PAA nanoparticles are potentially interesting materials for application in drug delivery and molecular imaging. Furthermore, incorporation of a monomer such as ethylenediamine-*N,N'*-diacetic acid introduces zwitterionic properties to the PAA [42,43].

Dispersed nano-sized hydrophilic polymer networks, i.e. nanogels, are nanostructures consisting of a water-soluble matrix and are promising vehicles for the delivery of a variety of different therapeutic agents [44–48]. Swelling and shrinkage of nanogels can be induced by changes in pH or temperature [3,49–51], which has been demonstrated to be a useful tool for triggered release [8,45,52,53] and stimuli-sensitive imaging [54–57]. Nanogels are commonly prepared by polymer crosslinking in an emulsified system [3]. However, the use of surfactants and emulsifiers complicates the purification of the prepared particles. Furthermore, to prepare a stable emulsion with the desired droplet size typically requires high amounts of energy, which may damage a possible payload, for example through denaturation of proteins [46,58]. A frequently used method to prepare solid polymeric nanoparticles is nanoprecipitation, also known as solvent displacement, where at a specific solvent/non-solvent ratio the polymer will aggregate into nanoparticles [59–61]. Nanoprecipitation may also occur by adding an aqueous polymer solution to a water-miscible organic non-solvent. Subsequent crosslinking of the nanoprecipitated polymer leads to the formation of stable, narrowly dispersed nanogels [9,46]. Moreover, Haag and coworkers have reported the formation of polyglycerol nanogels for protein encapsulation by inverse nanoprecipitation [46,62]. This inverse nanoprecipitation method [10,46,62] was chosen as a facile high-yielding method to form PAA nanoparticles without the use of surfactants.

Here we report the formation of stable nanogels composed of crosslinked zwitterionic PAAs by inverse nanoprecipitation. These zwitterionic nanogels are designed to display less non-specific interactions with proteins and cells, which is beneficial for particle's application in targeted imaging and drug delivery [2]. After crosslinking, the nanogel surface charge was increased by conjugation with cationic moieties. The PAA nanogels were characterised with <sup>1</sup>H NMR spectroscopy, dynamic light scattering, and scanning

electron microscopy. Their pH-responsiveness was studied, as well as their effects on cell viability of RAW 264.7, PC-3 and COS-7 cells. Finally, cell uptake properties were studied by fluorescence microscopy.

## 2. Materials and methods

### 2.1. Materials

*N,N'*-methylenebisacrylamide (99%), triethylamine ( $\geq 99\%$ ), sodium acetate ( $\geq 99\%$ ), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride ( $\geq 98\%$ ), *N*-hydroxysuccinimide, ethylenediamine ( $\geq 97\%$ ), hydrochloric acid – 37%, *N,N'*-dimethylethylenediamine ( $\geq 99\%$ ), ethylenediamine dihydrochloride (98%), sodium hydroxide ( $\geq 98\%$ ), 0.1 M PBS – monosodium phosphate monohydrate (3.9 mM,  $\geq 99\%$ ) disodium phosphate dihydrate (6.1 mM, 99.5%), sodium chloride (137 mM,  $\geq 99.5\%$ ), potassium chloride (2.7 mM,  $\geq 99\%$ ) – Rifampicin ( $\geq 97\%$ ) and FITC ( $\geq 90\%$ ) were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Ethylenediamine-*N,N'*-diacetic acid ( $\geq 98\%$ ), methanol (reagent grade), acetone (reagent grade) and DMSO (reagent grade) were purchased from VWR (Amsterdam, the Netherlands). Phosphotungstic acid hydrate (Acros), was purchased from Fisher (Landsmeer, the Netherlands). Spectra/Por<sup>®</sup> dialysis tubing with a MWCO of 1 kDa was purchased from Spectrum Labs (Breda, the Netherlands) and SnakeSkin<sup>™</sup> dialysis tubing with a MWCO of 10 kDa was purchased from Fisher (Landsmeer, the Netherlands). MTT, RPMI medium with phenol-red, RPMI medium without phenol-red, DMEM/F-12 medium without phenol red, RPMI-1640 medium (Gibco), DMEM (Gibco) and Ham's F12K medium (Gibco), PBS, Saponin-O and Greiner 48-wells cell culture plates were purchased from Invitrogen (Bleiswijk, the Netherlands). RAW 264.7 (RAW) cells were obtained from the American Type Culture Collection (ATCC catalogue No. TIB-71), COS-7 cells were obtained from the European Catalog of Animal Cultures (ECACC Catalogue No. 87021302) and PC-3 cells were obtained from Sigma Aldrich. Milli-Q water was obtained from a Millipore Advantage A10 Ultrapure Water Purification System and holey carbon TEM grids (Electron Microscopy Sciences, mesh size: 200) were purchased from Aurion (Wageningen, the Netherlands).

### 2.2. General procedures

<sup>1</sup>H NMR and COSY-NMR spectra were recorded in D<sub>2</sub>O on a Bruker Ascend 400 spectrometer; signals of the deuterated solvent were used as a reference.

Size exclusion chromatography (SEC) measurements were performed on a Waters Alliance e2695 separation module equipped with a Waters 2998 PDA detector, a Waters 2914 RI detector and a Polymer Laboratories Aquagel-OH 30 column. A 0.4 M sodium acetate buffer (containing 30% meOH) was used as eluent at a flow rate of 0.7 mL/min at 35 °C. Poly(ethylene glycol) standards were used for calibration.

Determination of solution pH was carried out with a Schott CG 842 laboratory pH metre fitted with a BlueLine 18 pH glass electrode, containing a temperature probe.

DLS measurements were performed on a Malvern Zetasizer Nano ZS at a 173° scattering angle, and analysed using the general-purpose analysis method provided in the Malvern Zetasizer software. The reference material was set to polystyrene latex and the temperature was set to 25 °C, unless stated otherwise. A standard Malvern glass cuvette was used to measure samples in acetone. The zeta potential was measured on a Malvern Zetasizer Nano ZS at 25 °C in milli-Q water, with polystyrene latex as a reference material and the  $F(\kappa a)$  parameter set to 1.5, according to the Smoluchowski approximation.

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