Water-borne Polymeric Nanoparticles for Glutathione-Mediated Intracellular Delivery of Anticancer Drugs

N. Grimaldi[*], M.-A. Sabatino[*], D. Bulone[**], G. Adamo[***], S. Rigogliuso[***], G. Ghersi[***], C. Dispenza[*,**]

[*] Dipartimento di Ingegneria Chimica, Gestionale, Informatica, Meccanica, Università di Palermo, Viale delle Scienze, Edificio 6, 90128 Palermo, Italy.

[**] CNR - Istituto di Biofisica (IBF) UOS Palermo, via Ugo La Malfa 153, 90146 Palermo, Italy.

[***] Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari, Università di Palermo, Viale delle Scienze, Edificio 16, 90128 Palermo, Italy.

ABSTRACT

A new family of *water-borne*, biocompatible and carboxyl-functionalized nanogels was developed for glutathione-mediated delivery of anticancer drugs. Poly(N-vinyl-pyrrolidone)-co-acrylic acid nanogels were generated by e-beam irradiation of aqueous solutions of a crosslinkable polymer, using industrial-type linear accelerators and set-ups. Nanogels physico-chemical properties and colloidal stability, in a wide pH range, were investigated. In vitro cell studies proved that the nanogels are fully biocompatible and able to quantitatively bypass cellular membrane. An anticancer drug, doxorubicin (DOX), was linked to the carboxyl groups of NGs through a spacer containing a disulphide cleavable linkage. In vitro release studies showed that glutathione is able to trigger the release of DOX through the reduction of the S-S linkage at a concentration comparable to its levels in the cytosol.

Keywords: nanogels, e-beam radiation, disulphide linkage, doxorubicin.

Introduction

The efficacy of a cancer therapeutic drug is measured by its ability to reduce and eliminate tumors without damaging healthy tissue. However, anticancer drugs suffer some limitations related to their poor solubility in aqueous environment, non-specificity in their distribution through the body compartments and severe adverse side effects. Furthermore, during the prolonged treatment with chemotherapeutic drugs, tumor cells develop multidrug resistance (MDR) and the drugs effectiveness decreases over time [1]. Cancer-fighting therapeutic devices based on nanoparticle systems offer the possibility of overcoming the problems mentioned above. In fact, nanocarriers are able to increase the solubility of hydrophobic drugs, decrease the toxicity toward healthy cells and escape from multi-drug resistance bypassing the efflux pumps and increase the drug intracellular concentration. Nanoparticles can be formulated with appropriate size, shape, surface properties and specific chemical functionality for covalent coupling of targeting moieties. Because of these tunable physico-chemical properties, nanoparticles may combine active and passive targeting in one single platform. Upon nanoparticle devices reach the target cells, they should be able to release the drug. Stimulus-mediated release is receiving great attention for intracellular drug delivery and various stimuli-responsive nanoparticles have been developed and investigated extensively, including temperature, pH, ultra sound, redox, and enzyme responsive ones [2]. Doxorubicin is one of the most effective chemotherapeutic anticancer drugs and it is crucial for the treatment of a wide range of tumors as breast cancer, acute leukemia and malignant lymphoma [3]. We have developed a new family of *water-borne*, biocompatible and carboxyl-functionalized nanogels (NGs) for glutathione-mediated delivery of Doxorubicin. In our approach, DOX is linked to nanoparticles through a linker containing a cleavable disulphide bridge, aminoethyldithiopropionic acid (AEDP). The release mechanism is based on the existence of a large difference in the redox potential between the mildly oxidizing extracellular milieu and the reducing intracellular fluids. The reducing agent is glutathione (GSH) that is found in the blood plasma of humans with micromolar concentrations, whereas it is around 10 mM in the cytosol. The concentration level of cytosolic GSH in cells with an enhanced level of oxidative stress, as in tumor cells, is several times higher than that in normal cells [2]. In alternative to more conventional synthetic approaches, which often imply several reactive and purification steps, a

robust and economically viable manufacturing process was developed for the production of nanogels. Nanogels were generated by e-beam irradiation of semi-dilute aqueous solutions of a crosslinkable polymer and acrylic acid, using industrial-type linear accelerators and irradiation conditions typically applied for sterilization purposes in industry, by irradiating at relatively low dose per pulse, high frequency and 40 kGy of integrated dose [4-6]. In particular, poly(N-vinyl-pyrrolidone)-co-acrylic acid (PVP-co-AA) variants with controlled size and surface charge density, high colloidal stability in a wide pH range (5 - 10) and multi-arms with terminal functional groups available for further conjugation, were generated quantitatively and reproducibly. Physico-chemical properties and colloidal stability of the generated nanoparticles were investigated through DLS techniques and ζ-potential measurements. In vitro cell studies proved that the nanogels are fully biocompatible and able to quantitatively bypass cellular membrane. In vitro release experiments showed that GSH is able to trigger the release of DOX through reduction of the disulphide linkage at concentrations comparable to its levels in cytosol.

Experimentals

Materials

PVP k60, acrylic acid (AA), amino-fluorescein (AF),1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC), and 2-(N-morpholino)ethanesulfonic acid (MES), glutathione (GSH), doxorubicin (DOX) were supplied by Aldrich. AEDP was supplied by VWR.

Methods

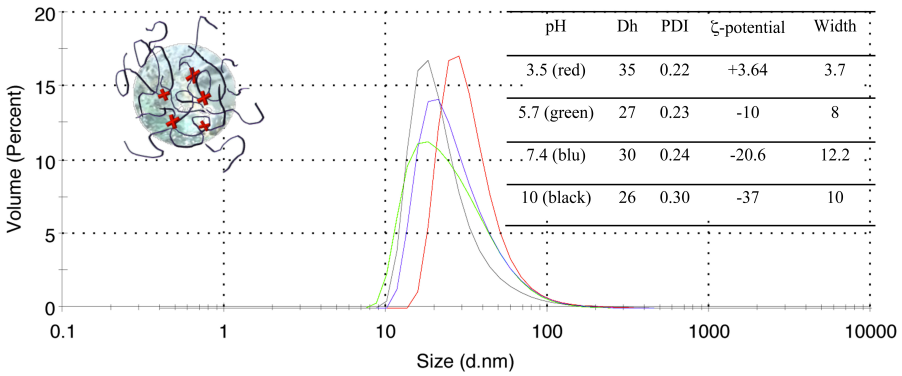
Preparation of PVP-co-AA NGs: PVP aqueous solutions at a concentration of 0.5 wt% in presence of acrylic acid (molar ratio between PVP repetitive unit and acrylic acid equal to 50) were prepared by overnight stirring, filtered with 0.22 μm pore size syringe filters, carefully deoxygenated with gaseous nitrogen and individually saturated with N_2O (N_2O ≥99.99%) prior to irradiation. Electron beam irradiation was performed using a 10 MeV linear accelerator at the ICHTJ of Warsaw (Poland). An integrated dose of 40 kGy was supplied. After irradiation samples were dialyzed (MWCO 100K Da) against distilled water for 48 h to remove eventual unreacted monomer, oligomers and low MW polymer. The yield of recovered nanogels after dialysis was determined gravimetrically. Hydrodynamic diameters (D_h) of NGs dispersions were measured by dynamic light scattering (DLS) using Brookhaven BI-9000 correlator and a 50 mW He−Ne laser (MellesGriot) tuned at λ = 632.8 nm. Particles size distributions expressed as volume % and the mean hydrodynamic diameter and PDI determined by the cumulant method are reported [8]. Surface charge density of NGs in water was measured at 25°C using a ZetaSizerNano ZS (Malvern Instruments Ltd, Malvern, UK) equipped with a He-Ne laser at a power of 4.0 mW. Mean ζ-potential values and the relative width of the distribution were reported for each sample. Both DLS and ζ-potential measurements were carried on 1 mg/ml dispersions at 20°C in water solutions of different pHs and controlled ionic strength (IS=1mM).

Cytotoxicity of nanoparticles on vein endothelial cell (ECV304) was assessed by MTT assay (Sigma). Cells were seeded in a 96-well plates at density of 1×10^4 cell/well, in complete medium MEM199. After 24 hr from seeding, cells were exposed to particle suspension at different concentrations (30 μg/ml, 60 μg/ml, 120 μg/ml), for 24 hr at 37°C. Not treated ECV304 cells were used as negative control and those treated with DOX [5 μM] for 24 hr were used as positive control. The % of cell viability was calculated as ratio between each sample with respect to the negative control (100% of viability).

Localization studies were performed on ECV 304 cells. ECV 304 were grown at a density of 5×10^3 cells/well into 12-well plates containing sterile glass coverslips in complete medium MEM199 for 24 hr. Next, the cells were incubated with 120 μg/ml of NGs, which emit green fluorescence by aminoflorescein conjugation. After different incubation times, 1 hr, 3 hr, 6 hr and 24 hr respectively, cells were extensively washed with PBS, to remove not reactive nanoparticles, and fixed with 3.7% formaldehyde for 15 minutes; then were again washed with PBS. Subsequently, the cells were stained with EtBr (1:1000) for 1 minute at room temperature. Localization was carried out by confocal microscopy analyzer (Olympus 1x70 with Melles Griot laser system).

Preparation of AEDP-DOX-P(0.5)AA50 NGs:* NGs were conjugated with AEDP and DOX by a two steps process. A standard EDC/Sulfo-NHS based protocol was adapted to our systems [7]. Both steps were carried out at 25°C , in pH 5 MES buffer, in excess of ligands with respect to the carboxyl groups and under continuous stirring. AEDP-DOX-P*(0.5)AA50 NGs were then purified through prolonged dialysis against water.

Reduction-Triggered DOX release: DOX-AEDP-P*(0.5)AA50 NGs were placed into a dialysis tubing (MWCO 12 kDa) with 10 mM of GSH or without, as control, and immersed in 20 ml of PBS (pH 7.4). The systems were kept at 37°C under shaking (200 rpm). At predetermined time intervals (1, 2, 4, 6, 8, 12, 24 hr), 1 ml of external buffer solutions were withdrawn and replaced with 1 ml of fresh PBS.



pH	Dh	PDI	ζ-potential	Width
3.5 (red)	35	0.22	+3.64	3.7
5.7 (green)	27	0.23	-10	8
7.4 (blu)	30	0.24	-20.6	12.2
10 (black)	26	0.30	-37	10

Figure 1. Particles size distributions in water at different pH obtained by DLS measurements. In inset table, the mean hydrodynamic diameter and PDI for each distribution shown and mean ζ-potential and relative width for each system are reported.

The amount of DOX linked to NGs and the amount released were determined by fluorescence measurements (λ_{ecc} 480 nm, λ_{em} 550 nm) using a Jasco 6500 spectrofluorimeter.

Results and discussion

Water borne PVP-co-AA NGs were generated through e-beam irradiation, starting from a semi-diluted polymer aqueous solution in presence of acrylic acid. The yield of process was about 90% [9]. Nanogels variants with different particles size, surface properties and degree of functionalization were obtained [9]. In this work only a brief description of the basic physico-chemical properties of the system selected for the conjugation with AEDP and DOX is reported. Size, surface properties and colloidal stability in a wide pH range (5-10) are very important parameters for intracellular delivery of nanoparticles and were investigated through DLS measurements and ζ-potentials determinations. As shown in Figure 1, the particle size distributions of P*(0.5)AA50 NGs in water solution of different pHs and equal ionic strength (IS=1 mM) display a monomodal distributions for all the systems, with an average diameter varying from 35 nm to 26 nm and low PDI values (0.23-0.3). The hydrodynamic diameter is fairly invariant in a wide pH range (5-10), only at pH 3.5, it is slightly bigger due to the onset of aggregation phenomena, which become faster at pH<3.5. At this pH, below the pKa of acrylic acid (pKa=4.75), a great proportion of carboxyl groups present on nanogels multi-arms becomes protonated, thus ζ-potential value is about 0 mV. At pH>3.5, the ζ-potential values become negative and their absolute values increase with the pH due to progressive dissociation of carboxyl groups. Since carboxyl groups are preferentially located on the "multi-branched arms" of NGs, their degree of dissociation does not affect the NGs' hydrodynamic size. Carboxyl groups located on NGs arms are easily accessible to covalent conjugation with fluorescent probes, small peptides, such as folic acid, and oligonucleotides. The availability, different positions and accessibility of functional groups on NGs allow performing multiple conjugations on the same nanoparticles (data here not shown for brevity). The chemical structure of the produced NGs was characterized through different spectroscopic techniques and it closely resembles that of the linear PVP [9]. P*(0.5)AA50 NGs were thought as devices for therapeutically purposes, therefore it is necessary to check on their biocompatibility by *in vitro* assays before proposing them for any biomedical application. The biocompatibility of P*(0.5)AA50 NGs was evaluated using MTT viability assay. The Figure 2 shows the relative viability of ECV304 cells after 24 hr of incubation with NGs dispersions at different concentrations. It is evident that, in the presence of NGs, cells viability is similar to that of the negative control (untreated cells). Differently, DOX-treated cells (positive control) show a lower cells viability. P*(0.5)AA50 NGs are not cytotoxic.

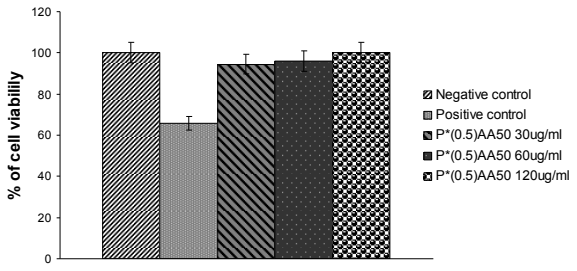


Figure 2. Evaluation of cell viability using MTT analysis.

NGs movements through the cell membrane and their internalization was investigated by confocal microscope analysis using a fluorescent variant of P*(0.5)AA50 NGs. We analyzed nanogels distribution in cell compartments at different times. As shown in Figure 3, after 1 (A) and 3 hr (B) of incubation, the amount of nanoparticles internalized slowly increases; whereas, after 6 hr (C) the nanogels are cytoplasmatic compartmentalized in the perinuclear area.

After 24 hr (D) nanogels concentration inside cells decreases, probably due to excretion as waste products in the extracellular environment. NGs showed a good affinity for cells, as they rapidly and quantitatively bypass the cellular membranes, being then released from the cells. P*(0.5) AA50 NGs were conjugated with AEDP and DOX through a two steps conjugation procedure. The choice to link the drug by a spacer with a cleavable linkage, over the physical encapsulation or the direct bond to NGs, was driven by the need to achieve a great control of drug release at the target site. Upon reaching the target tumor cells, the "drug" conjugated NGs should be internalized by cells and the release triggered by the stimulus. For DOX-AEDP-P*(0.5)AA50 NGs the stimulus is a redox agent, glutathione.

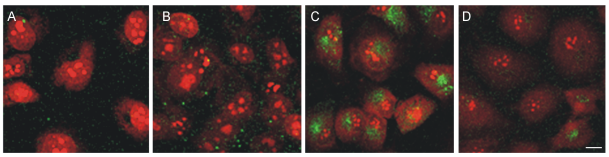


Figure 3. Localization assay by confocal microscopy analysis of a fluorescent variant of P*(0.5)AA50 NGs, incubated on ECV304 cell respectively for: A. 1 hr; B. 3 hr; C. 6 hr and D. 24 hr. Cell were stained with Ethidium Bromide. (Red) Magnification 40X; bar 10 mm.

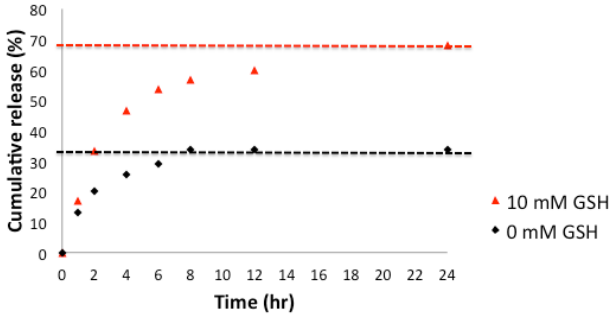


Figure 4. In vitro release of DOX from DOX-AEDP-P*(0.5)AA50 NGs in PBS (pH 7.4) at 37 °C with or without GSH.

The responsiveness of redox-sensitive NGs conjugated with AEDP and Doxo to the GSH stimulus was studied in vitro. The drug release studies were performed with 10 mM of GSH or without, as negative control. The amount of DOX released was quantified through fluorescence measurements. The release profiles for both systems are shown in Figure 4. The % of DOX release is reported as the molar ratio between the DOX released at the time t and the DOX linked to nanoparticles at the time t=0 hr. The total amount of DOX linked to the nanogels, after the purification step, was determined through fluorescence measurements. It can be observed that both systems release the DOX, but in the presence of GSH the amount of DOX released is substantially higher, about 70% vs. 34%, and the release is faster. No burst effects are evident. In absence of GSH, it is likely released the DOX which is not covalent linked to the nanoparticles. In fact, due to its hydrophobicity this molecule can be favorably hosted in the nanogels interiors. In vitro drug release tests demonstrate that the disulphide bridge can be broken under a reductive molecule stimulus, such as the GSH. These results prompted us to evaluate their performance into biological systems.

Conclusions

A new type of redox-responsive NGs was synthetized. The synthetic approach developed ensures the quantitative production of functionalized nanogels with tailored size and surface properties. Nanogels show high colloidal stability, biocompatibility, ability to bypass cell membranes and preferentially accumulate near the nuclei within 6 hr, being then progressively released by cells. The carboxyl groups located on the nanogels multi-branched arms can be used for further conjugation to generate DOX-AEDP-P*(0.5)AA50 redox-responsive NG variants. In vitro release studies showed that glutathione is able to trigger the release of DOX through reduction of the AEDP's S-S linkage at a concentration comparable to its levels in cytosol (10 mM). The combination of a single-step, robust and economically viable manufacturing process with all the favorable properties of the produced NGs make these nanoparticles very interesting candidates as smart nanocarriers of drugs at specific tumor sites. Studies on the GSH mediated intracellular drug release against cells are on going.

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