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Glutathione-Sensitive Nanogels for Drug Release

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Nanogels (NGs) synthesized by pulsed electron-beam irradiation of semi-dilute poly (N-vinyl pyrrolidone) (PVP) aqueous solutions, at relatively low energy per pulse and doses within the sterilization dose range, represent a very interesting family of polymeric nanocarriers. Ionizing irradiation-induced crosslinking of PVP allows to control particle size, and surface chemistry of the polymer nanoparticles without making use of catalysts, organic solvents or surfactants, and with beneficial effects onto the purity and hence biocompatibility of the final products obtained. Furthermore, the availability of reactive functional groups, either generated by the radiation or purposely grafted via copolymerisation with suitable functional monomers enables the conjugation of therapeutics drug, that make them suitable nanocarriers for biomedical applications.
In particular, we have developed a carboxyl-functionalized nanogel variant for glutathione-mediated delivery of a chemotherapeutic agent, Doxorubicin. The drug is linked to the nanoparticles through a linker containing a cleavable disulphide bridge, aminoethyldithiopropionic acid (AEDP). *In vitro* drug release experiments have shown that glutathione can induce the release of Doxorubicin, through the reduction of the disulfide bridge. These results suggest that such redox-responsive nanoparticles can deliver doxorubicin into the nuclei of tumor cells, thus inducing inhibition of cell proliferation, and provide a favourable platform to construct nanoscalar drug delivery systems for cancer therapy.

1. Introduction

The major limitation of antitumor agents used in clinical applications is their severe toxicity, caused by the high and frequent doses to be administered, which have a very short half-life and a wide tissue distribution. Currently, a few nano-therapeutics are approved or are undergoing clinical trials, and the application of nanotechnologies is expected to extend to many more commercial products in the near future. Nanomedicines have been referred to as the “real tools” to improve delivery efficacy (Grenha, 2011).
Chemical conjugation of antitumor agents to colloidally stable polymeric nanocarriers has been recently considered a viable strategy to overcome these drawbacks, thus offering a potential mechanism to improve cancer chemotherapy (Kamada et al.,2004). Crosslinked polymeric nanoparticles or “nanogels” can increase the solubility of hydrophobic drugs, such as chemoterapeutic agents, due to the high surface-to-volume ratio stemming from their nanoscale dimensions and the presence of hydrophobic pockets or domains, that are the result of the crosslinks formed onto otherwise highly hydrophilic polymeric structures (Ricca et al., 2010). The use of nanogels can improve drug absorption by reducing the epithelial resistance to transport and, simultaneously, decrease the toxicity toward healthy cells.
Moreover, NGs have shown the capacity to carry the encapsulated drugs through the epithelium, increasing their intracellular concentration (Gonçalveset al., 2010).
In fact, the nanoparticles can spontaneously gather in the diseased region, making use of EPR (Enhanced Permeability and Retention) to achieve the purpose of passive targeting (Maeda, 2000). Another

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mechanism is the active targeting, that involves the use of peripherally conjugated targeting moieties, for enhanced delivery to a specific site, based on molecular recognition.
Controlled release mechanisms are particularly interesting for intracellular drug delivery. Indeed, different types of stimuli responsive nanogels have been designed to be sensitive to environmental changes, such as temperature, pH, ultra sound, redox, and enzyme responsive ones (Westand Otto, 2005).
However, the development of therapeutic devices based on nanoparticles in general, including nanogels, is still limited by the lack of synthetic strategies, which are simultaneously economically-viable and able to grant a good degree of control over the device properties, especially when produced at a large scale.
We have recently developed a *single-step* synthetic approach to generate either carboxyl or primary amino groups bearing poly(N-vinylpyrrolidone) (PVP) nanogels, based on high-energy irradiation of semi-dilute aqueous solutions of PVP in the presence of functional group carrying acrylic monomers. (Dispenza et al., 2012a, Dispenza et al., 2012b, Sabatino et al., 2013, Grimaldi et al., 2014).
In particular, for the purpose of the present investigation acrylic acid has been considered as grafting monomer. Polymer crosslinking, monomer grafting and sterilization are simultaneously achieved. Poly (N-vinylpyrrolidone) (PVP) has been extensively used in controlled release drug delivery due to its biocompatibility, chemical stability, and excellent aqueous solubility. Both linear and crosslinked PVP have shown *in-vivo* biocompatibility and ability to escape from the body by natural pathways and processes, depending on molecular weight or particle size (Mansour et al., 2010). We have demonstrated that our radiation-engineered base PVP and amino-functionalized nanogels are not cytotoxic or genotoxic at the cellular level. Indeed, they show good affinity for cells, as they rapidly and quantitatively bypass the cellular compartments, to accumulate in specific cell portions after few hours, being then completely released from the cells after 24 h (Rigogliuso et al., 2012, Dispenza et al*.,* 2014). They can also bind biological molecules, e.g. for active targeting, as demonstrated by conjugation experiments using Bovine Serum Albumin (BSA) as model protein (Dispenza et al., 2012b).
Carboxyl groups located on NGs arms have been conjugated to Doxorubicin, for glutathione-mediated delivery of this chemotherapeutic agent. For this purpose, the drug is linked to the nanoparticles through a linker containing a cleavable disulfide bridge, aminoethyldithiopropionic acid (AEDP). The release mechanism is based on the existence of a large difference in the redox potential between the oxidizing extracellular environment and the reducing intracellular cytosol. In particular, the cytosol concentration of Glutathione (GSH), a thiol-containing tripeptide that cleaves disulfide bounds by a redox reaction, is higher (10 mM) than the level in the extracellular environment (2 μM). Tumor cells present higher levels of cytosolic GSH, induced by oxidative stress, with respect to normal cells. (West and Otto, 2005). *In vitro* release experiments have shown that GSH is able to trigger the release of DOX through reduction of the disulfide linkage at concentrations comparable to its levels in cytosol.

2. Experimental

2.1 Nanocarriers preparation

Aqueous solutions of PVP K60 (Aldrich)at concentrations of 0.25% wt and 0.5%wt in the presence of acrylic acid (AA, Aldrich), at a molar ratio between PVP repetitive unit and AA 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 the linear accelerator at the ICHTJ of Warsaw (Poland), Electronika 10/10 at 4-10°C (Grimaldi et al., 2014). The formed nanogels, named P*(0.25)AA 50 and P*(0.5)AA50, have hydrodynamic diameter of 26 nm (±5 nm) and 40 nm (±16 nm), respectively, as measured by dynamic light scattering measurements at 90°. The carboxylated-PVP molecular structure of the nanogels produced was confirmed trough FT-IR (Grimaldi et al. 2014).

2.2 ECV-304 cell viability by MTT assay

ECV304 (endothelial cell vein) were seeded in a 96-well plates at a density of $1x10^4$ cell/well and maintained using suitable culture medium (MEM199, Euroclone, Celbar) supplemented with 10% fetal bovine serum (Euroclone, Celbar), 1% L-glutamine (Euroclone, Celbar), and 1% penicillin streptomycin antibiotic solution (Euroclone, Celbar) at 37°C, in a humidified atmosphere of 5% CO_2.
After 24 h from seeding, cells were incubated for a further 24 h or 48 h at different concentrations (30, 60, 120 μg/mL) of NGs suspension. Non treated cells were used as negative control and treated cells with free DOX at 5μM for 24 h or 48 h were used as positive control. Cell viability was evaluated using MTT assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.25 mg/mL) solution was added to each well; the plates were incubated for 2 h at 37°C. The insoluble formazan crystals, produced in the

mitochondrial compartment of viable cells, were dissolved using dimethyl sulfoxide (DMSO, 100µl /well). The absorbance of the purple solution, obtained from enzymatic reaction with the mitochondrial dehydrogenase of alive cell, was read at a wavelength of 490 nm using a DU-730 Life Science spectrophotometer (Beckman Coulter). The percentage of cell viability was calculated as the ratio between each sample with respect to the negative control (100% of cell viability).

2.3 Conjugation of NGs to AEDP-DOX

NGs were conjugated with AEDP and DOX through a standard 1-Ethyl-3-(3-dimethylaminopropyl/carbodiimide/N-hydroxysulfosuccinimide (EDC/Sulfo-NHS) based protocol, in two steps that was adapted to ours systems. The reactions were carried out at 25°C, in a pH 5 MES buffer under continuous stirring. Doxorubicin that was not linked to NGs-AEDP was then removed using dialysis tubing (MWCO 12 kDa) against water.

Conjugation degree was estimated by UV-visible absorption measurements with Shimadzu 2401-PC spectrofluorimeter (scan speed 40 nm/min, integration time 2 sec, bandwidth 1 nm) at room temperature.

***2.4 In vitro* release study**

NGs-AEDP-DOX 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 h), 1 ml of external buffer solutions were withdrawn and replaced with 1 ml of fresh PBS.

The amount of DOX released was evaluated by fluorescence measurements (λ_{ecc} =480 nm, λ_{em} =550 nm) using a Jasco 6500 spectrofluorimeter, equipped with a Xenon lamp (150W). Emission spectra, at the required excitation wavelength, were obtained with emission and excitation bandwidth of 1 nm and 3 nm, respectively.

3. Results and discussion

3.1 Biological evaluation of carboxyl functionalized PVP nanogels

Biological evaluation in *vitro* was performed in order to prove the biocompatibility of the carboxyl-functionalized variants of PVP NGs selected for the purpose of this investigation. ECV304 cells were incubated with the NG systems and cell viability was performed in order to assess if these nanoparticles influence cellular metabolism.

Table 1 shows that the treatment of the cells with P*(0.25)AA50NGs at three different concentrations (30, 60, 120 µg/ml), for 24 h and 48 h, do not modify the cell viability.

Table1. Table of cells viability: ECV304 viability measured by the MTT method after treatment at different concentrations of P(0.25)AA50 nanogels for 24 and 48 h. Negative control: untreated cells. Positive control: cells treated with Doxorubicin. Absorbance data are shown as the mean of 5 replicates ± SD. The percentage of viability was calculated with respect to negative control cells assumed to be 100%.*

Sample	Absorbance (490nm)		% of Viability	
	24 h	48h	24 h	48h
Negative control	0.74 ± 0.122	1.08 ± 0.048	100%	100%
Positive control	0.48 ± 0.001	0.49 ± 0.004	66%	45%
P*(0,25)AA50 30µg/ml	0.70 ± 0.03	0.93 ± 0.087	94%	86%
P*(0,25)AA50 60µg/ml	0.71 ±0.004	0.77 ± 0.015	96%	86%
P*(0,25)AA50 120µg/ml	0.74 ± 0.006	0.9 ± 0.123	100%	83%

The small reduction of the number of alive cells is caused by the normal apoptotic process that can be observed after 48 hours from seeding. These results demonstrate that these NGs variants are biocompatible.

3.2 Incorporation and in-vitro redox-stimulated release of Doxorubicin from nanogels

NGs were conjugated with AEDP and DOX through a two steps conjugation procedure. We selected DOX as a model drug to investigate the drug release properties of the redox-responsive nanogels. Fig. 1 represents the scheme of NGs conjugation with AEDP first and with Doxorubicin later, the internalization of DOX-loaded NGs into the cell and the mechanism of release of DOX by reductive cleavage of the disulfide (S-S) bridge of AEDP, owing to the presence of GHS at its intracellular concentration. It is expected that the DOX released could then effectively reach the nucleus of the cell and induce cell death.

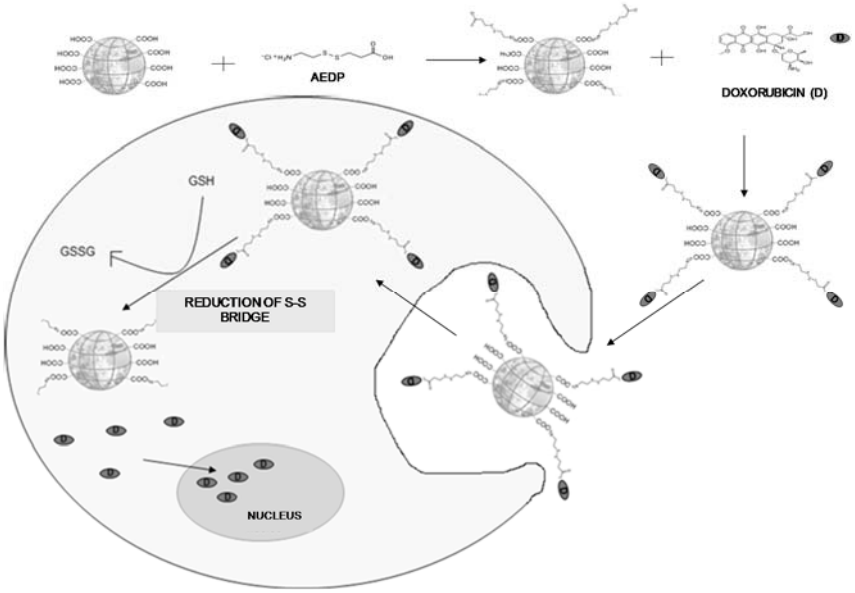

Figure 1: Scheme of NGs conjugation to DOX and controlled release mechanism proposed in cellular systems.

The conjugation degree between Doxorubicin and nanogels was estimated through UV-visible absorbance measurements. Figure 2 shows the UV-vis spectra of a carboxyl-functionalized nanogels suspension in water, before and after conjugation with DOX and the spectrum free DOX in water, as reference.
It can be observed that the NGs do not show any absorption band in the wavelength range where Doxorubicin strongly absorbs. Actually, the steady increase in absorbance approaching the low wavenumbers in the range is attributable to light scattering. Conversely, the DOX-loaded system shows the characteristic absorption band of DOX, but clearly red-shifted. This shift suggests a modification of the environment for the chromophore.
The amount of DOX conjugated to the NGs was quantified from the absorption peak at λ_{max}=486 nm, with reference to the calibration curve built using free DOX in water at different concentrations. In particular, the concentration of conjugated DOX resulted about 11 μg/ml.

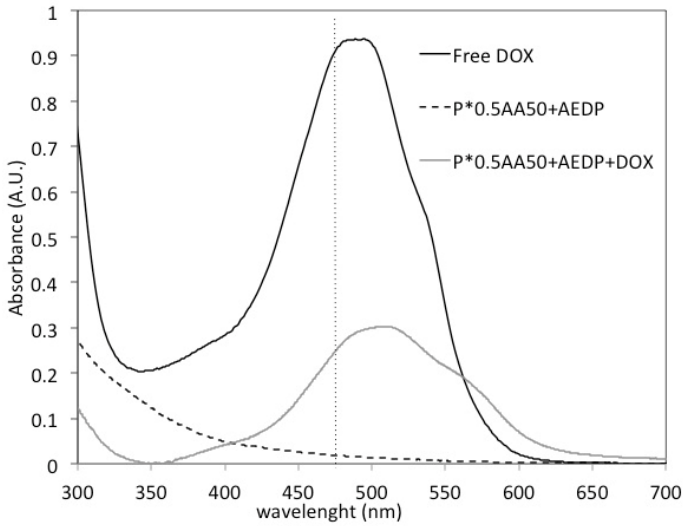

Figure 2. UV-vis spectra of a carboxyl-functionalized NG suspension in water before and after conjugation with DOX (11μg/ml) and of free DOX in water (62,5μg/ml).

In vitro drug release studies were performed by incubating DOX-loaded NGs suspensions filling a dialysis tube at 37°C in a PBS buffer containing 10 mM and in the same buffer with no GSH, as control. The amount of DOX released in the receiving phase was quantified through fluorescence measurements.

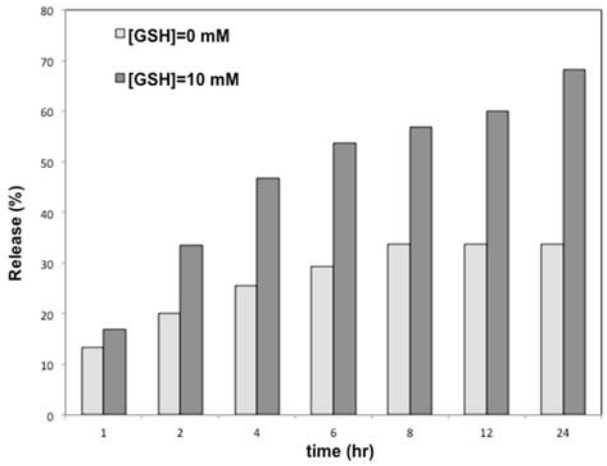

Figure 3. Percentage of doxorubicin released by the NGs as function of the time, at 37°C and in PBS buffer, with and without glutathione.

The release profiles built up from the fluorimetric reading at λ_{em}=550 nm (λ_{ecc}=480 nm)for both systems as function of the time are shown in Figure 3. The amount of DOX released is reported as the molar ratio between the DOX released at the time and the DOX linked to nanoparticles, estimated from the fluorescence reading at the time t=0 h. It can be observed that both systems release the DOX, but in the presence of GSH the amount of DOX released is substantially higher, of about 70% vs. 34%, and the release is faster. No burst effects are evident. In absence of GSH, the DOX released is likely the one that was physically entrapped in the nanogels. In fact, due to its hydrophobicity, this molecule can be favourably hosted in the nanogels hydrophobic domains. Indeed, even after prolonged observation a significant percentage of this drug is not released. Conversely, in the presence of GSH the percentage of released drug is significantly higher and the release rate is faster.

4. Conclusions

DOX-loaded PVP nanogels were prepared by conjugating carboxyl-modified PVP NGs with Doxorubicin, a well-known chemotherapeutic agent. The nanogels were synthetized by pulsed electron-beam irradiation of semi-dilute poly(N-vinyl pyrrolidone)/acrylic acid aqueous solutions. Nanogels showed a good biocompatibility against ECV304 cells, properties that make them suitable as biomedical nanocarriers. Due to the presence of carboxyl groups on the surface of the nanoparticles, it was possible to functionalize them by the conjugation with the drug. The controlled release mechanism proposed relies on the cleavage of the disulfide bridge present in the spacer interposed between the particle and the drug. *In vitro* release studies showed that glutathione is able to trigger the release of DOX at a concentration comparable to its levels in cytosol (10 mM).

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