RUTHENIUM-BASED CORE-SHELL NANOPARTICLES WITH EXCEPTIONAL *IN VITRO* BIOCOMPATIBILITY

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Abstract: The current study demonstrates design preparation and characterization of biocompatible hybrid ruthenium core-shell nanoparticles (RuNPs) coated with polyvinylpyrrolidone (PVP) and polyoxyethylene stearate (POES). The resulting RuNPs were loaded with doxorubicin, as model anticancer drug. Resulting complex has an exceptional stability in physiological conditions. The cytotoxic effects of the complex were tested using cell lines representing breast and ovarian cancers and neuroblastoma. Although bare RuNPs had only negligible cytotoxicity, RuPDox caused an enhancement of doxorubicin cytotoxicity when compared to free doxorubicin. RuPDox promoted significantly increased stability of doxorubicin in human plasma and pronounced hemocompatibility assayed on human red blood cells. Results demonstrate that biocompatible RuNPs could have a great potential as versatile nanoplatform to enhance efficiency of anticancer therapy.

Key Words: biocompatibility, nanomedicine, polyvinylpyrrolidone, polyoxyethylene stearate

INTRODUCTION

Nanotechnology is a term that defines design and testing of functional units on a nanoscale (Sahoo et al. 2007). Material scientists have performed exceptional accomplishments in the design of various types of materials that can be used in nanomedical (Giner-Casares et al. 2016).

The frequent drawback of these materials is common systemic toxicity. One potent solution is encapsulation of nanoparticles into polymeric shells. (Quarta et al. 2012). Among the most promising belong biodegradable polymers, such as polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP) (Beik et al. 2016, Calvo et al. 2001, Moghimi et al. 2001).

Herein, we present hybrid organic-inorganic core-shell ruthenium nanoparticles (RuNPs) coated with PVP-POES shell for delivery of conventional cytostatic agent doxorubicin (Dox, hereinafter RuPDox for the whole complex). Ruthenium is notable for its pronounced biocompatibility, attributed to ability to mimic the binding of iron to serum transferrins (Kostova 2006). Particularly, biocompatibility and magnetic properties make RuNPs primary candidate of selection for manifold biomedical applications (Gibb et al. 1973).

Overall, we show that our designed RuPDox exhibit exceptional stability in non-target plasma environment with only negligible adsorption of plasma proteins. Moreover, RuPDox possess pH-responsive properties enabling for burst release of Dox in acidic pH present in endosomes and tumor hypoxic tissue. RuPDox cytotoxicity was tested in vitro on three types of malignat cells - ovarian, breast cancer and neuroblastoma. RuNPs are pronouncedly biocompatible, RuPDox

formulation significantly enhanced Dox intranuclear accumulation. Our results imply high potential of the use of RuNPs with PVP-POES as versatile nanoplatform to enhance efficiency of cancer treatment.

MATERIAL AND METHODS

Reductive colloidal synthesis of RuNPs capped with PVP

$RuCl_3 \cdot 2.5\ H_2O$ in water was added to a stirred solution of PVP dissolved in 80 ml of water. Black solution was stirred, $NaBH_4$ was supplemented and release of hydrogen was observed. The product was stirred overnight followed by volume reduction on Amicon 3k to final volume of 50 ml.

RuNPs coating with POES and non-covalent complexation of Dox

Equal volumes of RuNPs and 20% solution of POES were mixed and ultrasonicated. After that, the solution of RuNPs was mixed with Dox and ultrasonicated. Finally, resulting RuPDox was centrifuged to remove unbound Dox and resuspended in MilliQ water. Loading efficiency (LE) of Dox to RuNPs was analysed by UV-Vis spectroscope Infinite 200 PRO (Tecan, Männedorf, Switzerland) at λ 480 nm.

Attenuated total reflectance Fourier transform-infrared spectroscopy (ATR-FT-IR)

FT-IR spectra were collected using a Nicolet iS10 FT-IR spectrometer with diamond ATR attachment (Thermo Electron Inc., San Jose, USA).

Transmission electron microscopy (TEM), Doppler electrophoresis and quasielastic dynamic light scattering (DLS)

TEM analyses were performed using the sample deposited onto 400-mesch copper grids coated with a continuous carbon layer by Tecnai F20 TEM (FEI, Eindhoven, Netherlands). ζ-potential was evaluated using Doppler electrophoresis on Zetasizer Nano ZS90 (Malvern instruments, Malvern, UK) as well as particle size analysis by DLS.

Evaluation of colloidal stability of RuNPs and RuPDox in physiological environments

To demonstrate their colloidal stability nanoparticles dispersed in the Ringer´s solution were placed in the stationary rack and kept at 25 °C.

Cell lines and culture conditions

Three human cell lines were used: *i*) the A2780 human ovarian cancer cell line, *ii*) the MDA-MB-231 - human breast cancer cell line, and *iii*) the UKF-NB-4 neuroblastoma cell line. All cell lines were purchased from Health Protection Agency Culture Collections (Salisbury, UK).

A2780 and MDA-MB-231 were cultured in RPMI-1640 with 10% foetal bovine serum (FBS), UKF-NB-4 Iscove's modified Dulbecco medium (IMDM) with 10% FBS. Media were supplemented with penicillin and streptomycin, and the cells were maintained in a humidified incubator Galaxy® 170 R (Eppendorf, Hamburg, Germany). Prior all analyses, cells were counted using Countess II FL (Thermo Fisher Scientific, Waltham, MA, USA).

Estimation of cytotoxicity

The viability was assayed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cell was incubation for 24 h at 37 °C with 5% CO_2 to ensure cell growth. After treatment, 10 µl of MTT [5 mg/ml in phosphate buffered saline (PBS)] was added to the cells and incubated. After that, MTT-containing medium was replaced by 100 µl of dimethyl sulfoxide (DMSO) and, absorbance was determined at 570 nm using Infinite 200 PRO (Tecan, Männedorf, Switzerland).

Analysis of formation of protein coronas and hemocompatibility

Plasma was isolated from whole blood. RuNPs, RuPDox and Dox were incubated in plasma. The protein coronas were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie Brilliant Blue. Gels were visualized using Azure c600 (Azure Biosystems, Dublin, CA, USA).

Hemocompatibility was assayed using human red blood cells sampled. The degree of hemolysis was determined by measuring the absorbance of the supernatant at λ 540 nm after centrifugation.

Descriptive statistics

For the statistical evaluation of the results using paired *t*-test and ANOVA. Unless noted otherwise, the threshold for significance was $p < 0.05$. For analyses Software Statistica 12 (StatSoft, Tulsa, OK, USA) was employed.

RESULTS AND DISCUSSION

Physico-chemical characterization of RuNPs and complexation with Dox

Dox is one of the most commonly used chemotherapeutic agents (Denel-Bobrowska and Marczak 2017). Several studies have shown Dox loading to different carriers such as liposomes, polymeric nanoparticles and others (Dawidczyk et al. 2017, Ding et al. 2017, Wang et al. 2017). Therefore, we have designed, prepared and tested cytotoxicity and bioavailability of hybrid organic-inorganic RuNPs loaded with Dox in this study. Reductive colloidal synthesis with Na $[BH]_4$ and PVP ends resulted in well dispersed and colloid-stable RuNPs. Further surface functionalization was performed using POES (Figure 1A).

RuPDox were tested for their LEs towards Dox. Table 1 illustrates that the highest LE (63.7%) was achieved for RuNPs coated with 20% POES. Moreover, using 20% POES, RuPDox was found to disperse readily and remained stable in dispersion for more than 24 h (Figure 1B).

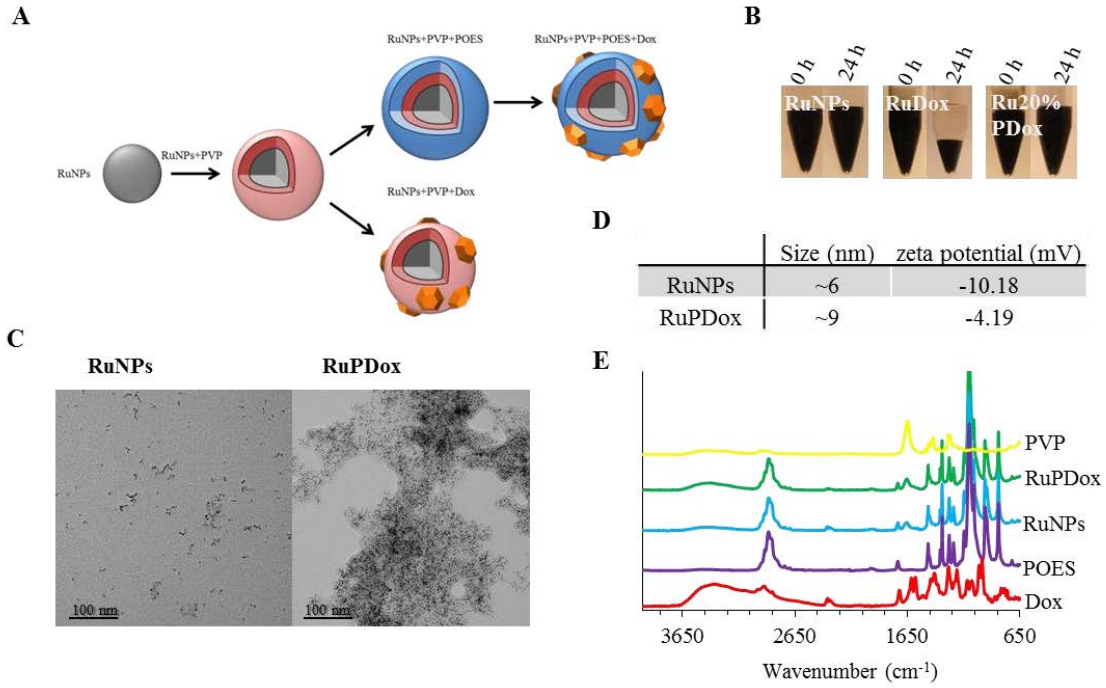
TEM micrographs showed that both RuNPs and RuPDox demonstrated relatively uniform oval-to-spherical morphology and were well dispersed (Figure 1C). The ζ-potential values of RuNPs and RuPDox in physiological conditions were -10.18 mV and -4.19 mV respectively. Although the ζ-potentials demonstrated relatively low values, nanoparticles were stable due to the presence of large molecular weight stabilizers, which shift the plane of shear to a further distance from the particle system, and thus results in a reduction in the value of ζ-potentials (Quaglia et al. 2009). The average maximum distributions of hydrodynamic diameters under physiological conditions were ~6 nm for RuNPs and ~9 nm for RuPDox (Figure 1D). The FT-IR spectra confirmed the formation of RuPDox and represented characteristic fingerprints for individual components forming RuPDox (Figure 1E).

Table 1 Analysis of Dox loading efficiency to RuNPs coated with various amount of POES.

	LE ± SD (%)
RuNPs	58.0 ± 2.6
10% POES	54.1 ± 3.4
20% POES	63.7 ± 4.0

Legend: Data are shown as means ± SD of triplicate in three independent experiments.

Figure 1 (A) Schematic illustration of synthesis of RuPDox from RuNPs by POES coating. (B) Photodocumentation of colloidal stability of synthesized RuNPs, RuDox and RuPDox with 2% POES demonstrating exceptional stability of RuPDox with 20% POES in start-point (0 h), and 24 h. (C) TEM micrographs of RuNPs (left) and RuPDox (right). (D) Hydrodynamic diameters of RuNPs and RuPDox determined by quasielastic DLS and ζ potential values determined by Doppler electrophoresis. (E) FT-IR spectra of RuPDox and individual components used for synthesis



RuNPs potentiate cytotoxicity of Dox in RuPDox formulation

Cytotoxic testing on three different types cells - breast (MDA-MB-231), ovarian cancer (A2780) and neuroblastoma (UKF-NB-4) revealed that RuNPs exhibited only negligible cytotoxic effects, while a complexation with Dox (RuPDox) resulted in a significant ($p < 0.05$) increase in cytotoxic effects in all tested cells (with the IC_{50} values between 1.2 ± 0.2 - 3.2 ± 0.2 μg/ml, all IC_{50} values are summarized in Table 1).

Generally, cytotoxicity of RuNPs is poorly known. Ramasamy and co-workers have shown that hollow mesoporous RuNPs have only slight cytotoxicity at concentrations higher than 100 μg/ml (Ramasamy et al. 2015), which is consistent with our findings and supports the low cytotoxicity of our RuNPs.

Table 2 Summary of IC_{50} values obtained from MTT assay for tested cell lines after 24 h treatments. All values are presented as mean ± SD of six biological replicates

		Mean IC_{50} ± SD (μg/ml)		
Cell line	Time (h)	Dox	RuNPs	RuPDox
A2780	24	8.5 ± 1.2	74.2 ± 2.1	3.2 ± 0.2
MDA-MB-231	24	7.9 ± 0.7	153.5 ± 3.9	1.1 ± 0.1
UKF-NB-4	24	3.4 ± 0.2	148.0 ± 1.8	1.2 ± 0.2

Estimation of RuPDox biocompatibility

In general, high degree of biocompatibility is achieved when a tested nanomaterial interacts with the body without inducing unacceptable toxic responses.

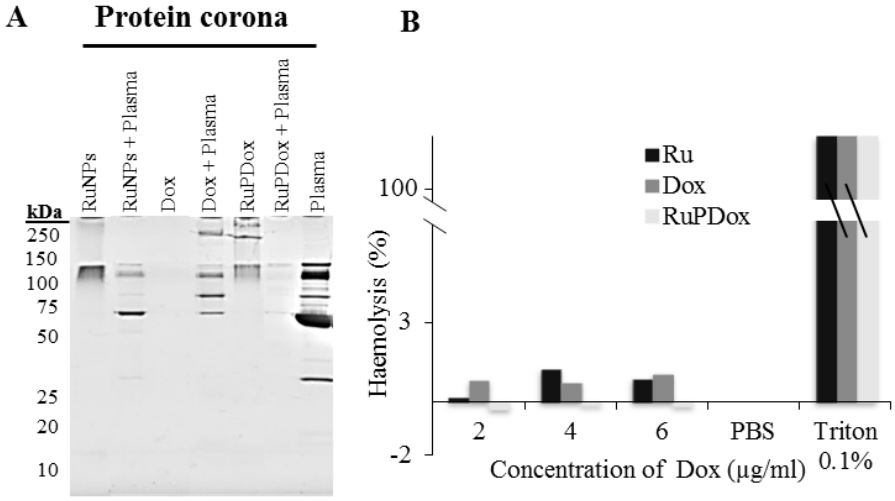
Pivotal aspect of biocompatibility is nanoparticle-blood interactions. Therefore, we firstly studied a rate of protein coronas formation, which are plasma proteins adsorbed on the surface of nanoparticles. Profiles of eluted proteins clearly demonstrate that bare RuNPs are capable to adsorb some amount of plasma proteins (Figure 2A). Moreover, we show that even free Dox is able to cause

plasma protein aggregation. Interestingly, RuPDox was shown to avoid most of unwanted interactions with plasma proteins with only small amount of protein adsorbed on the surface.

Finally, as hemolysis is often toxic effect of nanoparticles, we studied hemolysis on human RBCs. Hemotoxicity is connected with a positive surface charge, which is not present for RuNPs and RuPDox. Figure 2B demonstrates that all tested formulations caused only insignificant (max. 1%) release of hemoglobin from RBCs, which highlights exceptional hemocompatibility of RuPDox.

In general, surface sewing of the polymer produces extremely biocompatible hybrid materials. That is why the development of drug delivery systems is the most important. We have remarkably shown that RuNPs do not cause DNA fragmentation and, moreover, does not contribute to the natural genotoxic potential of free Dox, which in itself causes relatively massive DNA damage due to induction of DNA cleavage (Manjanatha et al. 2014).

Figure 2 (A) Protein corona profiles obtained after incubation of RuNPs, Dox and RuPDox with human plasma and loading onto SDS-PAGE. (B) Hemocompatibility of RuPDox assayed on human RBCs. PBS and 0.1% Triton X-100 were utilized as negative and positive controls, respectively.



CONCLUSION

In conclusion, we designed, prepared and tested cytotoxicity and biocompatibility of novel RuPDox nanoparticles. We show that hybrid organic-inorganic nanoparticles based on RuNPs must be taken into account as exceptional nanomedicine platforms. We also demonstrate that combining PVP with FDA-approved POES, such core-shell nanoparticles can act in multiple ways, which significantly enhances the Dox performance. Despite the validity of our in vitro results is limited and further in vivo experiments might be conducted, it is obvious that ruthenium-based nanomaterials have enormous potential for nanomedicine and our RuNPs with PVP-POES shell can serve as a versatile platform for complexation with distinct antiproliferative agents. Finally, based on available literature, RuNPs or ruthenium complexes are promising MRI contrast agents, hence their use will most likely enable for tracing and imaging of accumulation.

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