
APOFERRITIN AS A TARGETED DRUG DELIVERY SYSTEM

**Dostálová S.¹, Konečná R.¹, Blažková I.¹, Vaculovičová M.^{1,3}, Kopel P.¹,
Křížková S.^{1,3}, Vaculovič T.^{2,3}, Adam V.^{1,3}, Kizek R.^{1,3}**

¹Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic

²Department of Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, 611 37 Brno, Czech Republic

³Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, 616 00 Brno, Czech Republic

E-mail: simona1dostalova@gmail.com, kizek@sci.muni.cz

ABSTRACT

Conventional cancer treatment often effects normal cells and has many side effects. These can be addressed by the use of nanomedicine, especially its platform nanotransporters, in which cytostatic drug can be encapsulated. These nanotransporters can be dispersed in tumors through relatively large pores in tumor blood vessels but not in normal blood vessels. The nanotransporters can be coupled with antibodies, thus allowing targeted delivery of drugs. For this coupling we chose the method using linker, small peptide that interacts with Fc region of IgG antibodies, presenting the antigen binding site facing out.

The aim of this experiment was to create, characterize and test a nanotransporter based on apoferritin nanocage with encapsulated doxorubicin, modified with specific antibody. As a linker, heptapeptide HWRGWVC was used. Cysteine has known affinity towards gold, two methods of apoferritin surface modification were proposed and better results were obtained with apoferritin modified with gold nanoparticles. The presence of gold on apoferritin was proved by separation in polyacrylamide gel electrophoresis (PAGE), followed by inductively coupled plasma mass spectrometric (ICP-MS) measurement. Enzyme-linked immunosorbent assay (ELISA) was used to prove the apoferritin ability to specifically bind to target cells. Apoferritin retained its ability to open and release doxorubicin in low pH. This is very convenient, since there is lower pH in tumors due to hypoxemia.

Key words: apoferritin, doxorubicin, theranostics, antibodies, targeted delivery

Acknowledgments: Financial support from CYTORES GA ČR P301/10/0356 and DP03_2012 is highly acknowledged.

INTRODUCTION

Theranostics combines diagnostics and targeted therapy (Warner 2004). As a platform for theranostics, nanocarriers can be used (Drbohlovova, Chomoucka et al. 2013) due to their size, which allows them to get into tumor blood vessels with relatively large pores but not into normal blood vessels (Svenson 2013). They also allow delivery of multiple drugs at once, which is beneficial in such diseases as cancer (Sumer and Gao 2008).

Conventional cancer treatment is often toxic for normal cells, thus having many side effects (Sumer and Gao 2008), but these problems can be addressed by encapsulation of drug in a nanocarrier. Moreover, modification of the nanocarrier with targeting peptides or antibodies enables its specific targeting to the site of action (Bharali and Mousa 2010). This attachment to the nanocarrier surface can be realized by covalent coupling, adsorption (physical and/or hydrophobic) (Janu, Stanisavljevic et al. 2013) or based on streptavidin-biotin affinity (Pei, Cheng et al. 2001; Goldman, Balighian et al. 2002). In these methods, it is usually not possible to control the orientation of the antibody towards the nanocarrier surface and the resulting nanoparticle is relatively large (Janu, Stanisavljevic et al. 2013).

To eliminate these problems, a linker between the antibody and the nanocarrier can be used. Heptapeptide (HWRGWVC) (Janu, Stanisavljevic et al. 2013) was demonstrated as such linker. This peptide interacts with Fc region of some immunoglobulins through histidine (Yang, Gurgel et al. 2005), thus presenting the antigen binding side facing outwards (Wines, Powell et al. 2000). The cysteine on the other end of heptapeptide has known affinity towards gold (Hakkinen 2012).

As a nanocarrier, 480 kDa protein apoferritin (APO) can be employed (Blazkova, Nguyen et al. 2013). It contains a cage with internal diameter of 8 nm, in which drug can be enclosed (Uchida, Klem et al. 2007), and it can be "opened" and "closed" repeatedly via pH change (Kilic, Ozlu et al. 2012). Its surface can be modified with gold nanoparticles that have affinity toward the heptapeptide. As a drug, anthracyclin cytostatic doxorubicin (DOX) was used due to its fluorescent properties, allowing for easy visualization (Changenet-Barret, Gustavsson et al. 2013). In this study, antibody targeted, apoferritin mediated and pH triggered transport of doxorubicin was studied.

MATERIAL AND METHODS

Preparation and characterization of APODOX modified with gold

The preparation of APODOX (DOX encapsulated in APO) was according to (Blazkova, Nguyen et al. 2013). Its surface was modified with either gold nanoparticles (AuNP) or tetrachloroauric acid (HAuCl₄). Preparation of AuNPs was as follows: 0.25 ml of trisodium citrate (26.5 g/l) was added to 10 ml of 1mM tetrachloroauric acid, shaken for 1 hour at 20 °C and the resulting AuNPs had 1.4 nm in diameter. To APODOX, 25 µl 1 mM solution of AuNPs or 200 µl 1mM HAuCl₄ (followed with 3 mg of NaBH₄ and observed hydrogen evolution) was added and the mixture was shaken on Orbital Shaker (Biosan, Riga, Latvia) at 20 °C for 12 hours. Resulting product was rinsed six times with water on Amicon® Ultra -0.5 ml 3K (Merck Millipore, Billerica, MA, USA). The characterization of modified APODOX was conducted using ambient light, UV light (excitation 312 nm), and absorbance scan (230-850 nm) followed by fluorimetric measurement (excitation 480 nm, emission 515-850 nm with step 5 nm and average of 5 measurements) on fluorimetr Infinite M200 PRO (TECAN, Männedorf, Switzerland). APODOX was also run at 6 °C for 2 hours on 6 % non-denaturing PAGE with 60 mM glycine and 7 mM acetic acid pH 4 buffer system (Kilic, Ozlu et al. 2012).

Determination of gold

For the determination of gold, ICP-MS was used. Gold was determined in solution and then in mineralized polyacrylamide gel. ICP: Samples were filled up to 10 ml by ultrapure water and analyzed

by means of quadrupole ICP-MS Agilent 7500 CE (Agilent, Santa Clara, CA, USA) equipped with collision-reaction cell for suppressing polyatomic interferences. Optimization of ICP-MS parameters was performed with respect to maximum S/N ratio of signal of ^{197}Au isotope and minimum oxide formation. Mineralized sample was nebulized into ICP-MS via double-pass Scott spray chamber with Babington nebulizer. The sample uptake was 0.1 ml/s. For suppressing variation of plasma condition and sample uptake the internal standard was used – water solution containing 100 $\mu\text{g/l}$ Tl. The matrix effect was compensated using matrix-matched calibration solutions containing amount of acids as well as the mineralized samples. The concentration of Au in calibration solution was 0, 0.5, 2.0 and 10 $\mu\text{g/l}$ Au.

Enzyme-linked immunosorbent assay (ELISA)

Microtitration plate was coated with 100 ng of either goat anti-human IgG antibody (Greiner Diagnostics GmbH, Bahlingen am Kaiserstuhl, Germany) or chicken IgY antibody (HENA, Prague, Czech Republic) diluted in 0.05 M carbonate buffer pH 9.6 and incubated for 2 hours at 37 °C on Thermomixer 5355 Comfort/Compact (Eppendorf, Hamburg, Germany). Free surface of well was blocked for 1 hour at 37 °C with 50 μl of 1 % bovine serum albumin diluted in PBS. Wells were washed with 50 μl of 0.005 % PBS-T v/v.

Heptapeptide HWRGWVC (HWR peptide) was prepared according to (Janu, Stanisavljevic et al. 2013). 50 μl of APODOX (50 μg of APO) was conjugated with 0.25 μg of HWR peptide for 1 hour at 20 or 45 °C, 400 rpm. Samples were centrifuged on Amicon® Ultra - 0.5 ml 3K, at 6000 rpm and 20 °C for 15 min to remove residual HWR peptide and sample volume was adjusted to the initial volume with water. 3.5 ng of human IgG antibody was added and incubated at 20 °C for 1 hour. 50 μl of the sample was added to the previously prepared microtitration plate and incubated at 37 °C for 1 hour. Wells were washed with 50 μl of PBS and emission scan was measured. Bound APODOX was acidified with 2 μl of hydrochloric acid and emission scan was measured again.

RESULT AND DISCUSSION

Two methods were used to modify the surface of APO with gold – AuNPs and HAuCl_4 . Resulting samples and sample without modification were visualized in ambient (Fig. 1A) and UV light (Fig. 1B). The modification with HAuCl_4 probably changed the structure of doxorubicin, resulting in its blue color in UV light. There was also change in absorbance (Fig. 1C) and fluorescence (excitation 480 nm, Fig. 1D) spectra observed. On the other hand, the modification with gold nanoparticles made no visible changes in APODOX properties.

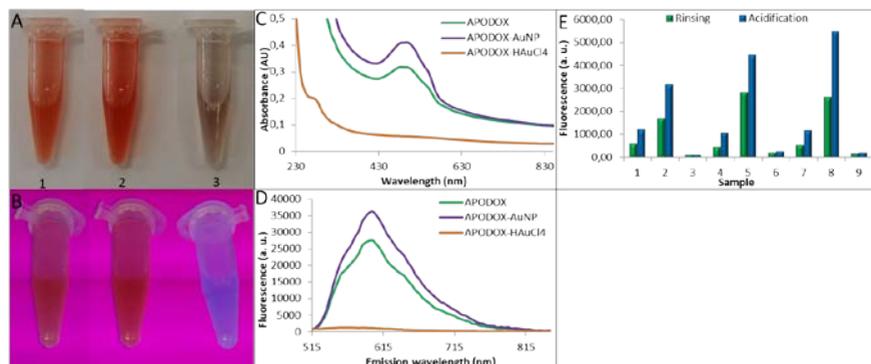


Fig. 1 Characterization of prepared APODOX. A) Visualization in ambient light: 1-APODOX, 2-APODOX-AuNP, 3-APODOX-HAuCl₄. B) Visualization in UV light. C) Absorbance scans. D) Emission scans. E) ELISA results: 1-APODOX, 2-APODOX-AuNP, 3-APODOX-HAuCl₄, 4-APODOX-HWR incubated at 20 °C, 5-APODOX-AuNP-HWR 20 °C, 6-APODOX-HAuCl₄-HWR 20 °C, 7-APODOX-HWR incubated at 45 °C, 8-APODOX-AuNP-HWR 45 °C, 9-APODOX-HAuCl₄-HWR 45 °C.

There was gold concentration measured in these solutions by ICP-MS. Results show highest concentration in sample with HAuCl₄ (130.9 μ M). The gold concentration in sample with AuNP was 12.75 μ M. In non-modified APODOX it was below the detection limit. To demonstrate, that gold is bound to the surface of APODOX, PAGE was conducted, samples of gel were mineralized and gold was measured again by ICP-MS. In non-modified APODOX, the concentration was below 0.03 ng/g of gel, with AuNPs 1.98 ng/g of gel and with HAuCl₄ 12.42 ng/g of gel.

Results from ELISA show highest affinity of APODOX modified by AuNP and conjugated with HWR peptide at 45 °C. All of the nanotransporters had the ability to open and release encapsulated doxorubicin after acidification, which is accompanied by significant increase in fluorescence at the emission maximum at 575 nm (Fig. 1E).

CONCLUSIONS

Cancer treatment is often toxic to normal cells and causes numerous side effects. To eliminate these, cytostatic drug can be encapsulated in suitable nanotransporter. These nanotransporters are targeted to the site of action by coupling with targeting peptides or antibodies. To ensure the right orientation of targeting ligand, an appropriate linker is used. The aim of this experiment was to create, characterize and test a nanotransporter based on apoferritin nanocage with encapsulated doxorubicin, modified with specific antibody. Two ways of apoferritin surface modification with gold were compared, better results were achieved with modification with gold nanoparticles than gold(III) chloride hydrate. HWR peptide has a higher affinity towards gold with higher temperature during incubation. The resulting nanotransporter was able to specifically bind to target cells, while retaining the ability to open and release doxorubicin in low pH.

REFERENCES

- Bharali, D. J. and S. A. Mousa (2010). "Emerging nanomedicines for early cancer detection and improved treatment: Current perspective and future promise." *Pharmacology & Therapeutics* **128**(2): 324-335.
- Blazkova, I., V. H. Nguyen, et al. (2013). "Apoferitin modified magnetic particles as doxorubicin carriers for anticancer drug delivery." *Int. J. Mol. Sci.* **14**(7): 13391-13402.
- Drbohlovova, J., J. Chomoucka, et al. (2013). "Nanocarriers for anticancer drugs - new trends in nanomedicine." *Current Drug Metabolism* **14**(5): 547-564.
- Goldman, E. R., E. D. Balighian, et al. (2002). "Avidin: A natural bridge for quantum dot-antibody conjugates." *Journal of the American Chemical Society* **124**(22): 6378-6382.
- Hakkinen, H. (2012). "The gold-sulfur interface at the nanoscale." *Nature Chemistry* **4**(6): 443-455.
- Changenet-Barret, P., T. Gustavsson, et al. (2013). "Unravelling molecular mechanisms in the fluorescence spectra of doxorubicin in aqueous solution by femtosecond fluorescence spectroscopy." *Physical Chemistry Chemical Physics* **15**(8): 2937-2944.
- Janu, L., M. Stanisavljevic, et al. (2013). "Electrophoretic study of peptide mediated quantum dot-human immunoglobulin bioconjugation." *ELECTROPHORESIS*: n/a-n/a.
- Kilic, M. A., E. Ozlu, et al. (2012). "A Novel Protein-Based Anticancer Drug Encapsulating Nanosphere: Apoferitin-Doxorubicin Complex." *Journal of Biomedical Nanotechnology* **8**(3): 508-514.
- Pei, R. J., Z. L. Cheng, et al. (2001). "Amplification of antigen-antibody interactions based on biotin labeled protein-streptavidin network complex using impedance spectroscopy." *Biosensors & Bioelectronics* **16**(6): 355-361.
- Sumer, B. and J. M. Gao (2008). "Theranostic nanomedicine for cancer." *Nanomedicine* **3**(2): 137-140.
- Svenson, S. (2013). "Theranostics: are we there yet?" *Molecular Pharmaceutics* **10**(3): 848-856.
- Uchida, M., M. T. Klem, et al. (2007). "Biological containers: Protein cages as multifunctional nanoplatforms." *Advanced Materials* **19**(8): 1025-1042.
- Warner, S. (2004). "Diagnostics plus therapy = theranostics." *Scientist* **18**(16): 38-39.
- Wines, B. D., M. S. Powell, et al. (2000). "The IgG Fc contains distinct Fc receptor (FcR) binding sites: The leukocyte receptors Fc gamma RI and Fc gamma RIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A." *Journal of Immunology* **164**(10): 5313-5318.
- Yang, H., P. V. Gurgel, et al. (2005). "Hexamer peptide affinity resins that bind the Fc region of human immunoglobulin G." *Journal of Peptide Research* **66**: 120-137.