PHYSICOCHEMICAL INVESTIGATION OF STABILITY OF APOFERRITIN WITH ENCAPSULATED DOXORUBICIN

SIMONA DOSTALOVA1,2, KATERINA VASICKOVA1, DAVID HYNEK1,2, SONA KRIZKOVA1,2, LUKAS RICHTER1,2, ZBYNEK HEGER1,2, MARKETA VACULOVIKOVA1,2, MARIE STIBOROVA3, VOJTECH ADAM1,2

1 Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
2 Central European Institute of Technology
Brno University of Technology
Purkynova 123, 612 00 Brno
3 Department of Biochemistry
Charles University in Prague
Hlavova 2030/8, 128 43 Prague 2
CZECH REPUBLIC

simona.dostalova@mendelu.cz

Abstract: The many negative side effects of drugs used for treatment of highly diverse diseases, such as cancer, lead in recent years to the development of ways how to target the drug selectively to the diseased tissue while avoiding the healthy cells. Nanocarriers, made from various materials, can serve as a suitable platform for this targeted drug delivery. Herein, we evaluate the long-term stability of nanocarrier based on ubiquitous protein apoferritin with encapsulated doxorubicin. Various properties of the nanocarrier were observed over the course of 12 weeks while stored at various temperatures, such as premature drug release, optical properties of the encapsulated drug, nanocarrier size and surface zeta potential. The nanocarrier showed very good stability for up to 12 weeks with the best results observed with nanocarrier prepared in water and stored at dark at 4 °C.

Key Words: absorbance; fluorescence; nanomedicine; surface zeta potential

INTRODUCTION

With no universal cure for cancer, the cause behind the death of every 4th person in the developed world, various anti-tumour agents are used in the treatment, as well as still being developed (American Cancer Society 2016). The drawback of these conventional cancer treatments are its many negative side effects (Wagland et al. 2016). Doxorubicin (DOX), an anthracycline antibiotics discovered in 1969, is just one of the examples for this (Arcamone et al. 1969). While it is used in the treatment of diverse cancer types, both those of blood elements (leukemia, Hodgkin’s lymphoma) and solid tumours (breast, lung, sarcoma, bladder or ovaries) (Laskowska et al. 2016, Li et al. 2016, Vassilakopoulos et al. 2016), this is often at the expense of many side effects. These include less severe ones, such as nausea, diarrhoea, hair loss or hyperpigmentation (Panchuk et al. 2016). The most dangerous side effect of DOX, cardiotoxicity, exhibits in up to 10% of patients treated with this drug (Mazevet et al. 2013). The cardiomyopathy caused by a cumulative dose of DOX can lead to congestive heart failure (Holmgren et al. 2016). Patients treated with DOX and other anthracycline antibiotics are often co-treated with a cardioprotective agent dexrazoxane. However, this can lead to higher rate of secondary malignancies or even acute myelogenous leukemia (Cruz et al. 2016).

Due to the negative side effects that many drugs possess, various research groups in the recent years have been focused towards the development of nanoparticles able to transfer drugs selectively to the diseased tissue, with minimal effect on healthy tissue (Jeong et al. 2016). So far, nanocarriers made from many different materials have been researched, including both inorganic and organic. Each approach has its benefits and drawbacks. Inorganic particles are easier to manufacture, but are often toxic to the organism. Organic particles, especially those derived from molecules already found in the
body, are more suitable (Landesman-Milo and Peer 2016). One of the nanodrugs already used in clinical practice, is liposome-encapsulated DOX, sold under the name of Myocet. This drug has a very limited stability once DOX is encapsulated within the liposome – only 24 h at 2–8 °C (Alphandery et al. 2015). Therefore, there is a need for a more stable nanocarrier, providing similar toxicity to diseased tissue while protecting the healthy tissue.

Recently, we developed nanocarrier based on the ubiquitous protein apoferritin (APO), a 12-nm self-assembled hollow cage whose function is to store and transport iron ions in an organism. We loaded APO with DOX (Blazkova et al. 2013, Tmejova et al. 2013) and modified its surface with antibodies targeted to an antigen specific for prostate cancer. This targeted nanocarrier retained toxicity for prostate cancer cells similar to that of free DOX, while 43% of healthy cells were spared from the toxic effects of free DOX (Dostalova et al. 2016). In this work, we decided to evaluate the stability of APO nanocarrier over the course of 3 months. The stability was investigated using the fluorescence spectrometry for evaluation of prematurely released DOX from APO structure and changes in size and zeta potential of the whole nanocarrier.

MATERIAL AND METHODS

Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

Encapsulation of DOX into APO and its storage

9600 µL of mg/mL DOX was added to 960 µL of 50 mg/mL horse spleen APO and 4800 µL of water. 120 µL of 1 M hydrochloric acid was added to decrease the solution pH and dissociate the APO. The solution was mixed for 15 min. 120 µL of 1 M sodium hydroxide was added to increase the pH and encapsulate the DOX inside APO (creating APODOX). The solution was mixed for 15 min and divided into 2 equal parts. The parts were diafiltrated three times with water or phosphate buffered saline (PBS, pH 7.4; 0.137 M NaCl + 0.0027 M KCl + 0.0014 M KH2PO4 + 0.0043 M Na2HPO4), respectively, using Amicon® Ultra - 0.5 mL 3K (Merck Millipore, Billerica, MA, USA) at 6000 g for 15 min and filled to 24000 µL with the same solvent used for diafiltration. The samples were divided into 300 µL aliquots and stored for 12 weeks at -20; 4; 20 and 37 °C and dark with the 4 and 20 °C stored also under ambient light for 12 h every day.

Characterization of nanocarrier changes during storage

Every week of storage, aliquots from all storage conditions were collected and prematurely released drug molecules were removed by diafiltration with the respective solvent using Amicon® Ultra - 0.5 mL 3K at 6000 g for 15 min. The amount of released drug was determined by measurement of free DOX fluorescence compared with fluorescence of the whole sample. The fluorescence measurement was performed using Tecan Infinite 200 PRO (Tecan, Männedorf, Switzerland) with excitation wavelength of 480 nm and emission wavelengths of 515–815 nm. The encapsulated drug was evaluated by absorbance measurement using Tecan Infinite 200 PRO with wavelengths of 230–850 nm.

The average size of the nanocarrier was determined by quasielastic dynamic light scattering with Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). The nanocarrier prior to removal of released drug and diluted 100× with distilled water was placed into polystyrene latex cell and measured at a detector angle of 173°, wavelength of 633 nm and temperature of 25 °C with the refractive index of dispersive phase 1.45 and 1.333 for the dispersive environment. For each measurement, disposable cuvettes type ZEN0040, were used, containing 50 µL of sample. The equilibration time was 120 s. The measurements were performed in hexaplicates.

The surface zeta potential of the nanocarrier diluted 20× was measured using the Zetasizer Nano ZS instrument. For each measurement, disposable cells DTS1070 were employed. The number of runs varied between 20 and 40 and calculations considered the diminishing of particles concentration based Smoluchowsky model, with a F(κa) of 1.50 and an equilibrating time of 120 s.
RESULTS AND DISCUSSION

Experiment layout

The aim of this work was the evaluation of the stability of natural APO nanocarrier with encapsulated DOX (APODOX). The drug encapsulation mechanism of APO is based on its structural responsiveness to the surrounding environment (pH in the case of horse spleen APO). In neutral pH, APO forms a 12-nm icosahedral hollow cage made of 24 protein subunits (Haussler 2003). When acidified, it disassembles into its subunits and these can be mixed with any drug of choice. After adjustment of the pH back to neutral, the subunits spontaneously reassemble to again form the icosahedral structure, with the drug encapsulated within the hollow cavity. This physical entrapment allows for encapsulation of any drug large enough to not leak through the small pores in APO shell (Kim et al. 2011).

Figure 1 shows the layout of the experiment in this work. The APODOX was prepared in two solvents – water and PBS. These were aliquoted and kept for 12 weeks at various temperatures at dark (-20; 4; 20 and 37 °C). The samples at 4 and 20 °C were also simultaneously kept under ambient light to evaluate the influence of light on the stability of the nanocarrier. Every week, aliquots from the different conditions were collected and chosen measurements aimed to help evaluate the stability were performed.

Properties of nanocarrier at the start of the experiment

<table>
<thead>
<tr>
<th>Solvent</th>
<th>water</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premature DOX release (%)</td>
<td>6.4</td>
<td>6.2</td>
</tr>
<tr>
<td>Encapsulated DOX absorbance at 480 nm (AU)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Encapsulated DOX fluorescence (a. u.)</td>
<td>24905</td>
<td>31137</td>
</tr>
<tr>
<td>Nanocarrier size (nm)</td>
<td>12/43.8</td>
<td>12/68.1/342</td>
</tr>
<tr>
<td>Surface nanocarrier zeta potential (mV)</td>
<td>27.9</td>
<td>29.5</td>
</tr>
</tbody>
</table>
The properties of freshly prepared nanocarrier in the two solvents were evaluated at the start of the experiment (see Table 1). One of the most undesirable properties of a nanocarrier is the premature release of its cargo, whether in patient’s organism or during its storage. The undesired cargo release can lead to increased toxicity for healthy cells (Yang et al. 2015). Due to the nature of APO drug encapsulation; there is low premature release of the drug molecules from the nanocarrier (6.4% for nanocarrier prepared in water and 6.2% for nanocarrier prepared in PBS, respectively). This is probably caused by the fact that some drug molecules may be adsorbed to the surface of nanocarrier instead of encapsulated in its cavity (Konecna et al. 2014).

Due to the structure of doxorubicin, it is possible to easily detect it using its absorbance maximum at 480 nm and its emission maximum at 600 nm (Konecna et al. 2014). The amount of encapsulated DOX in APO prepared in water and PBS was comparable, with 0.2 AU absorbance at 480 nm in both cases, which revealed encapsulation efficiency of 68%. However, the nanocarrier prepared in PBS exhibited higher fluorescence.

Other very important properties of the nanocarrier are its size and surface zeta potential. Both of these properties are indicative of the nanocarrier ability to enter individual cells (Stewart et al. 2016). The size of majority of APODOX particles prepared in water was 12 nm, with occasional aggregates of 43.8 nm and a zeta potential of -27.9 mV which shows the stability of freshly prepared particles. While the size of majority of APODOX particles prepared in PBS was also 12 nm, there was higher amount of 43.8-nm aggregates and occasionally even aggregates of 342 nm. The zeta potential was also indicative of the nanocarrier stability (-29.5 mV).

**Stability evaluation of the nanocarrier**

*Table 2 Changes observed in APODOX nanocarrier stored for 12 weeks in water. (-) ... lower value of observed parameters compared with freshly prepared samples. (+) ... higher value of parameters compared with freshly prepared samples.*

<table>
<thead>
<tr>
<th>Temperature of storage (°C)</th>
<th>-20</th>
<th>4</th>
<th>20</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored at dark/under light</td>
<td>Dark</td>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>Change in premature DOX release (%)</td>
<td>-2.3</td>
<td>-2.0</td>
<td>-2.3</td>
<td>-3.6</td>
</tr>
<tr>
<td>Change in encapsulated DOX absorbance at 480 nm (%)</td>
<td>+5.6</td>
<td>+18.1</td>
<td>+11.1</td>
<td>+32.2</td>
</tr>
<tr>
<td>Change in encapsulated DOX fluorescence (%)</td>
<td>+9.2</td>
<td>+16.0</td>
<td>+3.4</td>
<td>+3.3</td>
</tr>
<tr>
<td>Change in nanocarrier size (%)</td>
<td>+303.8</td>
<td>+25.0</td>
<td>+151.8</td>
<td>+67.5</td>
</tr>
<tr>
<td>Change in nanocarrier surface zeta potential (%)</td>
<td>-5.5</td>
<td>-4.4</td>
<td>-12.4</td>
<td>-26.1</td>
</tr>
</tbody>
</table>

The measurements from each individual week were averaged to show the influence of individual storage conditions on the nanocarrier. Table 2 shows the changes observed in nanocarrier prepared in water. Even though the undesired premature release of DOX from the APO structure was very low in the freshly prepared samples, it was even further lowered in all the stored samples. The best results were obtained with APODOX stored at 37 °C. The observed absorbance and fluorescence of the encapsulated DOX was higher in all stored samples compared with the freshly prepared samples. However, this did not correlate with the percentage of prematurely released DOX. Significant differences in nanocarrier size in samples stored at various conditions were observed. The samples stored at -20 °C showed a formation of aggregates of up to 300% larger size than were observed in freshly prepared samples. The most stable size throughout the storage was observed in samples stored at 4 °C and dark with only 25% increase of size. The formation of aggregates, combined with different amount of prematurely released drug can explain the changes of DOX absorbance and fluorescence. The surface zeta potential was less negative in all observed samples, showing lower stability of the nanocarrier. The best results were observed in samples stored at 4 °C and at dark.
Table 3 Changes observed in APODOX nanocarrier stored for 12 weeks in PBS. (-) ... lower value of observed parameters compared with freshly prepared samples. (+) ... higher value of parameters compared with freshly prepared samples.

<table>
<thead>
<tr>
<th>Temperature of storage (°C)</th>
<th>-20</th>
<th>4</th>
<th>20</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored at dark/under light</td>
<td>Dark</td>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>Change in premature DOX release (%)</td>
<td>+1.1</td>
<td>-0.4</td>
<td>+0.4</td>
<td>-0.5</td>
</tr>
<tr>
<td>Change in encapsulated DOX absorbance at 480 nm (%)</td>
<td>+11.3</td>
<td>+21.2</td>
<td>+26.8</td>
<td>+28.6</td>
</tr>
<tr>
<td>Change in encapsulated DOX fluorescence (%)</td>
<td>+8.1</td>
<td>+1.7</td>
<td>+0.5</td>
<td>-10.8</td>
</tr>
<tr>
<td>Change in nanocarrier size (%)</td>
<td>+125.7</td>
<td>+362.0</td>
<td>+318.3</td>
<td>+405.2</td>
</tr>
<tr>
<td>Change in nanocarrier surface zeta potential (%)</td>
<td>-9.6</td>
<td>-4.8</td>
<td>-4.2</td>
<td>-27.2</td>
</tr>
</tbody>
</table>

Table 3 shows the changes observed in nanocarrier prepared in PBS. In contrast to the results obtained with nanocarrier stored in water, the samples stored in PBS showed slightly higher percentage of premature drug release than was observed in freshly prepared samples. The only storage conditions in which the premature release was slightly lower were 4 and 20 °C at dark. The observed absorbance and fluorescence of the encapsulated DOX was also higher in all stored samples compared with the freshly prepared samples. However, as the percentage of prematurely released drug was in many cases increased, these results did not correlate. Significant differences in nanocarrier size in samples stored at various conditions were observed, even more so than in the samples in water. The largest aggregates were observed in samples stored at 20 °C (486.5% larger than in freshly prepared samples for storage under light and 405.2% for storage at dark). The best results were obtained for samples stored at -20 °C with aggregates of up to 125.7% larger size than those in freshly prepared samples. The surface zeta potential was less negative in most observed samples, showing lower stability of the nanocarrier, with the exception of samples stored at 37 °C. It can be concluded that the observed stability of nanocarrier kept in water was higher than that stored in PBS.

CONCLUSION

The experiment presented in this work dealt with the evaluation of stability of a nanocarrier for anti-cancer drugs, based on naturally occurring and versatile protein apoferritin. Overall, the presented nanocarrier showed high stability for up to 12 weeks with the optimal results were obtained with nanocarrier stored in water at 4 °C and dark.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency of Mendel University in Brno (IP_28/2016) and the Grant Agency of the Czech Republic (NANOCHEMO GA CR 14-18344S). The authors wish to express their thanks to Mgr. Michal Horak for perfect technical assistance.

REFERENCES


