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Phage Capsid for Efficient Delivery of Cytotoxic Drugs

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***Abstract*—**Various nanomaterials can be used as a drug delivery vehicles in nanomedicine, called nanocarriers. They can either be organic or inorganic, synthetic or natural-based. Although synthetic nanocarriers are easier to produce, they can often be toxic for the organism and thus not suitable for use in treatment. From natural-based nanocarriers, the most commonly used are protein cages or viral capsids. In this work, virus bacteriophage λ was used for delivery of different cytotoxic drugs (cisplatin, carboplatin, oxaliplatin and doxorubicin). Large quantities of phage λ were obtained from phage λ-producing strain of *E. coli* cultivated in medium with 0.2% maltose. After killing of *E. coli* with chloroform and its removal by centrifugation, the phage was concentrated by ultracentrifugation at 130 000×g and 4°C for 3 h. The encapsulation of the drugs was performed by infusion method and four different concentrations of the drugs were encapsulated (200; 100; 50; 25 $\mu g \cdot mL^{-1}$). Free drug molecules were removed by filtration. The encapsulation was verified using the absorbance for doxorubicin and atomic absorption spectrometry for platinum cytostatics. The amount of encapsulated drug linearly increased with the increasing concentration of applied drug with the determination coefficient R^2=0.989 for doxorubicin; R^2=0.967 for cisplatin; R^2=0.989 for carboplatin and R^2=0.996 for oxaliplatin. The overall encapsulation efficiency was calculated as 50% for doxorubicin; 8% for cisplatin; 6% for carboplatin and 10% for oxaliplatin.

***Keywords*—**Bacteriophage λ, doxorubicin, platinum cytostatics, protein-based nanocarrier, viral capsid.

I. Introduction

THE boom of nanomedicine in recent years has led to the development of numerous new nanomaterials that can be used as nanocarriers in the drug delivery. Nanomaterials provide some unique features that are not shown in the bulk materials with the same chemical composition [1], most notably color but more importantly chemical or magnetic properties or melting temperature [2].

Use of nanocarriers can provide some useful attributes. The encapsulation of cytotoxic drug into nanocarrier can significantly decrease negative side effects that are often associated with the treatment. It also increases the efficiency of administered drug and provides ways of specific targeting just to unhealthy cells or virions [1]. In addition, the water-solubility, biocompatibility and storage life-time is increased [3], [4].

The nanocarriers can either be synthetic or natural-based [5]. The disadvantage of many synthetic nanocarriers is their toxicity in patient's body. Protein cages that can naturally be found in human body do not exhibit such disadvantage [6]. However, the release of cargo from some protein cages in target cells can be problematic. Moreover, the capsid of many viruses, including phage can serve as a special type of protein cages [7]. Viral particles are usually formed by hundreds to thousands self-assembling protein molecules forming a hollow shell for nucleic acid [8]. They can have various shapes, most often spherical, but also icosahedral or rod-like and they are genetically programed to provide morphological uniformity with the size ranging from 10 nm to 1 μm [9]. Moreover, the structure of many viruses is known down to the level of atoms, so they can easily be modified with functional groups of interest [10]. They can also be easily prepared in large amount in laboratories, are very stable and easy to store [11]. They often cause immune response in patients, which can be beneficial in cancer treatment [12]. The most frequently viruses are mammalian adenoviruses, phages MS2, M13 or Qβ or plant viruses, such as cowpea chlorotic mottle virus [9].

Phages infect bacterial cells; therefore, they are not harmful to human cells; however, they can cause immune response. The immunogenicity can be solved by coating of their surface with polyethylene glycol [13]. The targeting of phage particles to cancer cells can be solved by producing of empty phage capsids during which the targeting moieties (e.g. peptides) can be cloned into genes of phage capsid to decorate its surface [10]. Moreover, the produced capsids do not contain viral nucleic acid and are therefore not infectious to beneficial bacteria in the patient's body. The protein cage composed of viral capsid is larger than other frequently used apoferritin cage [9], [14] but its size is still small enough to benefit from passive targeting by Enhanced Permeability and Retention (EPR) effect [15], [16]. The viral particles can be easily produced in large bulks and their purification only consists of host bacteria removal by centrifugation and subsequent ultracentrifugation in sucrose gradient [17].

In this work, bacteriophage λ was used as a nanocarrier for anthracycline drug doxorubicin and platinum drugs cisplatin, oxaliplatin and carboplatin.

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II. Materials and Methods

A. Chemicals

All chemicals of ACS (American Chemical Society) purity were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

The formulation for Luria-Bertani (LB) broth was as follows: 1% tryptone, 0.5% yeast extract and 0.5% sodium chloride. The formulation for LB bottom agar was as follows: 1% tryptone, 0.5% yeast extract, 0.5% sodium chloride and 1.5% agar bacteriological. The formulation for soft agar was as follows: 0.5% peptone, 0.3% meat extract and 0.5% agar bacteriological. The formulation for SM medium was as follows: 0.58% sodium chloride, 0.2% magnesium sulphate heptahydrate and 0.61% Trizma base pH 7.5.

The bacteriophage λ producing strain of *Escherichia coli* was a kind gift from Ljubica Svobodova (Department of Biology, Faculty of Medicine, Masaryk University in Brno, Czech Republic). The producing strain of *Escherichia coli* was cultivated in media containing 0.2% maltose.

B. Cultivation and Purification of Phage λ

Phage λ-producing strain of *Escherichia coli* was cultivated in LB broth with 0.2% maltose for 24 h at 37°C and 600 rpm. After cultivation, the culture was mixed with chloroform in 6:1 ratio and incubated for 1 h at 25°C to kill the growing *Escherichia coli*. The samples were centrifuged at 5 200×g and 4°C for 10 min to remove *Escherichia coli* and then at 10 000×g and 4°C for 6 min to remove the remaining contaminants. Next, the supernatant containing phage was ultracentrifuged at 130 000×g and 4°C for 3 h. The pellet containing phage was resuspended in PBS at a protein concentration of 15 $\mu g \cdot mL^{-1}$ and stored at 4°C.

C. Encapsulation of Drug Molecules in Phage λ

4 different drugs (doxorubicin, carboplatin, cisplatin, oxaliplatin) were encapsulated in purified phage by the infusion method. 125 µL of phage was mixed with 125 µL of drug at different concentrations (200; 100; 50; 25 and 0 $\mu g \cdot mL^{-1}$). Incubation was conducted for 2 h at 25 °C in dark. Free drug molecules were subsequently filtered using Amicon 3K (Merck-Millipore, MA, USA) for 15 min at 6000×g and 20 °C and the phage with encapsulated drug was rinsed twice with water. The volume was then made to the original volume (250 µL) with water.

D. Verification of Doxorubicin Encapsulation

The absorption and emission spectra of 50 µL of phage with encapsulated doxorubicin were measured prior and after filtration. Absorption spectra of the nanocarriers with encapsulated doxorubicin were measured from 230 to 850 nm. Emission spectra of the samples were measured with excitation at 480 nm (absorption maximum of doxorubicin) and emission from 515 to 815 nm. To evaluate the encapsulation efficiency, the absorption at 480 nm of phage with encapsulated drug prior to filtration was compared with the absorption of sample after filtration.

E. Verification of Platinum Drug Encapsulation

Platinum was determined on 280Z Agilent Technologies atomic absorption spectrometer (Agilent, USA) with electrothermal atomization and Zeeman background correction. Platinum ultrasensitive hollow cathode lamp (Agilent) was used as the radiation source (lamp current 10 mA). The spectrometer was operated at 265.9 nm resonance line with spectral bandwidth of 0.2 nm. The sample volume of 10 µl was injected into the graphite tube. The flow of argon inert gas was 300 $mL \cdot min^{-1}$. Zeeman background correction was used with field strength of 0.8 Tesla. Platinum was determined without the presence of chemical modifier.

III. Results

The encapsulation procedure (Fig. 1) was based on the infusion method. The cytotoxic drugs at 4 different concentrations were mixed with purified whole bacteriophage λ particles in 1:1 volume ratio and subsequently incubated for 2 h in dark at 25 °C and 400 rpm. The phage nanocarrier was purified from the free drug molecules by filtration of molecules smaller than 3 kDa through the centrifugal units Amicon 3K.

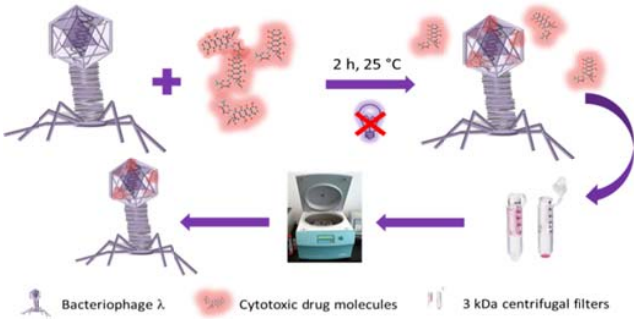
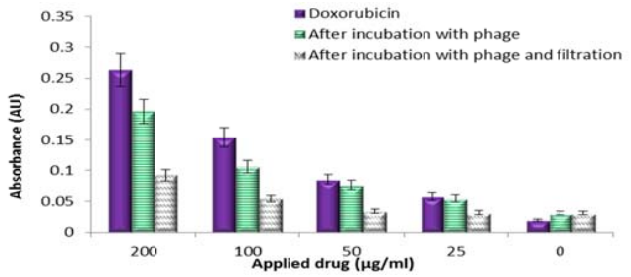
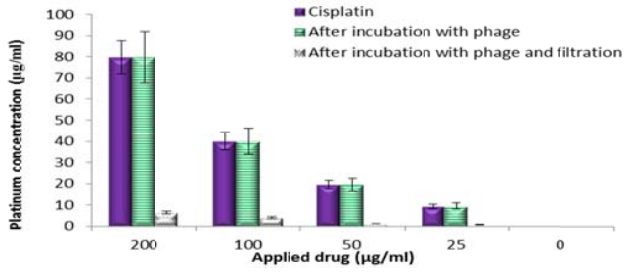


Fig. 1 The schematic procedure of used infusion method for encapsulation of drug molecules in bacteriophage λ nanocarrier. The purified phage was mixed with drug molecules for 2 h at 25°C in dark and the excess drug molecules were removed by filtration

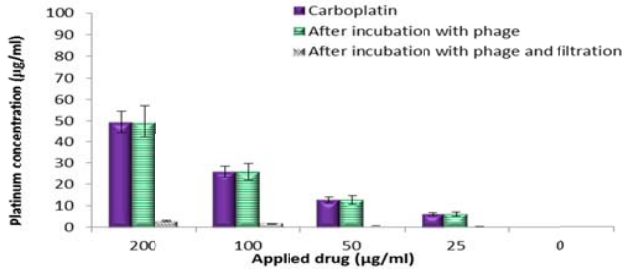
Four different cytotoxic drugs were encapsulated using the infusion method – anthracycline drug doxorubicin and platinum drugs cisplatin, oxaliplatin and carboplatin. The drugs were applied at four different concentrations – 25; 50; 100 and 200 $\mu g \cdot mL^{-1}$ with only the phage particles diluted in water as a negative control. Fig. 2 shows the results from measurement of differences in signal of free drug molecules, drug molecules after encapsulation in phage particles and after encapsulation and subsequent removal of excess drug molecules.



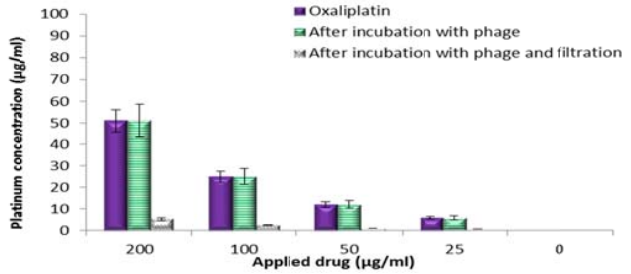
(a)



(b)



(c)



(d)

Fig. 2 The measurement of drug molecules, after encapsulation in nanocarrier and after encapsulation and removal of free drug molecules: (a) The absorbance of doxorubicin at 480 nm with 5 repetitions. (b) The platinum concentration in phage with encapsulated cisplatin measured by atomic absorption spectrometry at 265.9 nm. (c) The platinum concentration in phage with encapsulated carboplatin measured by atomic absorption spectrometry at 265.9 nm. (d) The platinum concentration in phage with encapsulated oxaliplatin measured by atomic absorption spectrometry at 265.9 nm

The verification of doxorubicin encapsulation can be performed using the absorbance maxima of doxorubicin at 480 nm where the encapsulating phage shows very low absorbance (Fig. 2 (a)). However, after encapsulation the absorbance of doxorubicin is significantly decreased in comparison with free drug (26% decrease for 200 $\mu g \cdot mL^{-1}$ of applied doxorubicin; 31% decrease for 100 $\mu g \cdot mL^{-1}$ of applied doxorubicin; 11% decrease for 50 $\mu g \cdot mL^{-1}$ of applied doxorubicin and 5% decrease for 25 $\mu g \cdot mL^{-1}$ of applied doxorubicin). Therefore, for the subsequent calculation of encapsulation efficiency, the comparison of encapsulated doxorubicin absorbance prior and post filtration was used. After filtration, a significant amount of doxorubicin was removed from the phage nanocarrier. The comparison between absorbance at 480 nm of doxorubicin encapsulated in phage λ prior and after filtration was used for the calculation of encapsulation efficiency for doxorubicin. The encapsulation efficiency was 47% for 200 $\mu g \cdot mL^{-1}$ of applied doxorubicin; 51% for 100 $\mu g \cdot mL^{-1}$ of applied doxorubicin; 45% for 50 $\mu g \cdot mL^{-1}$ of applied doxorubicin and 58% for 25 $\mu g \cdot mL^{-1}$ of applied doxorubicin. The average encapsulation efficiency of doxorubicin in phage λ nanocarrier by infusion method is 50% and the amount of encapsulated doxorubicin and thus its absorbance was linearly increasing with the increasing concentration of applied doxorubicin (with the determination coefficient R^2=0.989).

The verification of platinum drug encapsulation was performed using the platinum measurement by atomic absorption spectrometry. Due to the disruption of phage particles during the measurement, no masking of detection was observed. Therefore, the comparison between the platinum concentration measured using the atomic absorption spectrometry of free platinum drugs with platinum drugs after encapsulation and subsequent removal of unbound drug molecules could be used for the calculation of encapsulation efficiency for platinum drugs. For all the applied platinum drugs, the concentration of encapsulated drug was linearly increased with the increasing concentration of applied drug with determination coefficient R^2=0.967 for cisplatin (Fig. 2 (b)), R^2=0.989 for carboplatin (Fig. 2 (c)) and R^2=0.996 for oxaliplatin (Fig. 2 (d)).

Even more significant amount of drug was removed from the phage λ nanocarrier by filtration in the case of platinum drugs. For cisplatin the encapsulation efficiency was 8% for 200 $\mu g \cdot mL^{-1}$ of applied cisplatin; 10% for 100 $\mu g \cdot mL^{-1}$ of applied cisplatin; 6% for 50 $\mu g \cdot mL^{-1}$ of applied cisplatin and 8% for 25 $\mu g \cdot mL^{-1}$ of applied cisplatin.

For carboplatin the encapsulation efficiency was 6% for 200 $\mu g \cdot mL^{-1}$ of applied carboplatin; 7% for 100 $\mu g \cdot mL^{-1}$ of applied carboplatin; 5% for 50 $\mu g \cdot mL^{-1}$ of applied carboplatin and 8% for 25 $\mu g \cdot mL^{-1}$ of applied carboplatin.

For oxaliplatin the encapsulation efficiency was 10% for 200 $\mu g \cdot mL^{-1}$ of applied oxaliplatin; 10% for 100 $\mu g \cdot mL^{-1}$ of applied oxaliplatin; 8% for 50 $\mu g \cdot mL^{-1}$ of applied oxaliplatin and 11% for 25 $\mu g \cdot mL^{-1}$ of applied oxaliplatin.

Table I shows the encapsulation efficiency of phage λ for all of the applied drugs. The encapsulation efficiency was highest for the doxorubicin drug (approximately 50% with all applied concentrations). For the platinum drugs, the

encapsulation efficiency was approximately 5× lower, with the average of 8% for cisplatin, 6% for carboplatin and 10% for oxaliplatin. Therefore, oxaliplatin showed the highest encapsulation efficiency of the tested platinum drugs but still the encapsulation efficiency was significantly lower than in the case of doxorubicin.

The difference between encapsulation efficiency for doxorubicin and platinum drugs was probably caused by the differences in their structures. The multiple aromatic circles of doxorubicin may probably be able bind to the proteins of phage λ by π-π stacking [18]. Other previous results [19] also concluded that doxorubicin is bound to the proteins of viral capsid and not the nucleic acid, with only 1% of encapsulated doxorubicin present in the DNA of bacteriophage λ.

TABLE I
THE CALCULATED ENCAPSULATION EFFICIENCY OF 15 μG·ML^{-1} OF PHAGE Λ WITH 200; 100; 50 AND 25 μG·ML^{-1} OF APPLIED DOXORUBICIN (DOX), CISPLATIN (CISPT), CARBOPLATIN (CARBOPT) AND OXALIPLATIN (OXALIPT) AS MEASURED BY ABSORBANCE AT 480 NM FOR DOXORUBICIN AND AT 265.9 NM AT ATOMIC ABSORPTION SPECTROMETRY FOR THE PLATINUM DRUGS

Applied drug concentration (μg/ml)	Encapsulation efficiency (%)			
	DOX	CisPt	CarboPt	OxaliPt
200	47	8	6	10
100	51	10	7	10
50	45	6	5	8
25	58	8	8	11
Average	50	8	6	10

IV. CONCLUSION

Bacteriophage λ was proven to be a possible protein-based nanocarrier for cytotoxic drugs. The encapsulation occurs mainly by interactions of drug molecules with the viral capsid. The highest encapsulation efficiency was observed using anthracycline cytotoxic drug doxorubicin (50%). From the tested platinum drugs, oxaliplatin had the highest encapsulation efficiency (10%), but significantly lower than doxorubicin. The difference was probably caused by better binding of doxorubicin to phage proteins through π-π stacking interactions. Phage capsid can be easily produced in large quantities and modified with targeting moieties.

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REFERENCES

[1] Park, K. Facing the truth about nanotechnology in drug delivery. *Acs Nano*, 2013, vol. 7, no. 9, pp. 7442-7447. ISSN 1936-0851.
[2] Leonard, B. *Cancer nanotechnology: going small for big advances: using nanotechnology to advance cancer diagnosis, prevention and treatment*. DIANE Publishing Company, 2009. pp. ISBN 9781437912722.
[3] Gu, F. X. et al. Targeted nanoparticles for cancer therapy. *Nano Today*, 2007, vol. 2, no. 3, pp. 14-21. ISSN 1748-0132.
[4] Chomoucka, J. et al. Magnetic nanoparticles and targeted drug delivering. *Pharmacological Research*, 2010, vol. 62, no. 2, pp. 144-149. ISSN 1043-6618.
[5] Peer, D. et al. Nanocarriers as an emerging platform for cancer therapy. *Nature Nanotechnology*, 2007, vol. 2, no. 12, pp. 751-760. ISSN 1748-3387.
[6] Sawant, R. M. et al. "SMART" drug delivery systems: Double-targeted pH-responsive pharmaceutical nanocarriers. *Bioconjugate Chemistry*, 2006, vol. 17, no. 4, pp. 943-949. ISSN 1043-1802.
[7] Dostalova, S. et al. Delivery of doxorubicin using protein nanocarriers. *J. Metallomics Nanotech.*, 2014, vol. 1, no. 4, pp. 34-38. ISSN 2336-3940.
[8] Dostalova, S. et al. Viral drug nanocarriers. *J. Metallomics Nanotech.*, 2014, vol. 1, no. 1, pp. 30-35. ISSN 2336-3940.
[9] Ma, Y. J. et al. Virus-based nanocarriers for drug delivery. *Advanced Drug Delivery Reviews*, 2012, vol. 64, no. 9, pp. 811-825. ISSN 0169-409X.
[10] Steinmetz, N. F. Viral Nanoparticles in Drug Delivery and Imaging. *Molecular Pharmaceutics*, 2013, vol. 10, no. 1, pp. 1-2. ISSN 1543-8384.
[11] Lankes, H. A. et al. In vivo gene delivery and expression by bacteriophage lambda vectors. *Journal of Applied Microbiology*, 2007, vol. 102, no. 5, pp. 1337-1349. ISSN 1364-5072.
[12] Koprowski, H. et al. Human anti-idiotype antibodies in cancer-patients - is the modulation of the immune-response beneficial for the patient. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 1984, vol. 81, no. 1, pp. 216-219. ISSN 0027-8424.
[13] Kaiser, C. R. et al. Biodistribution studies of protein cage nanoparticles demonstrate broad tissue distribution and rapid clearance in vivo. *International Journal of Nanomedicine*, 2007, vol. 2, no. 4, pp. 715-733. ISSN 1176-9114.
[14] Uchida, M. et al. Biological containers: Protein cages as multifunctional nanoplatforms. *Advanced Materials*, 2007, vol. 19, no. 8, pp. 1025-1042. ISSN 0935-9648.
[15] Duncan, R. A Sat, Y. N. Tumour targeting by enhanced permeability and retention (EPR) effect. *Annals of Oncology*, 1998, vol. 9, pp. 39-39. ISSN 0923-7534.
[16] Maeda, H. Macromolecular therapeutics in cancer treatment: The EPR effect and beyond. *Journal of Controlled Release*, 2012, vol. 164, no. 2, pp. 138-144. ISSN 0168-3659.
[17] Yang, Q. A Catalano, C. E. Biochemical characterization of bacteriophage lambda genome packaging in vitro. *Virology*, 2003, vol. 305, no. 2, pp. 276-287. ISSN 0042-6822.
[18] Wang, Z. M. et al. Structural studies of several clinically important oncology drugs in complex with human serum albumin. *Biochimica Et Biophysica Acta-General Subjects*, 2013, vol. 1830, no. 12, pp. 5356-5374. ISSN 0304-4165.
[19] Dostalova, S. et al. Bacteriophage λ as a doxorubicin nanocarrier *J. Metallomics Nanotech.*, 2014, vol. 1, no. 3, pp. 64-68. ISSN 2336-3940.