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Conjugated metallothionein-carbon-doxorubicin nanotransporter for targeted breast cancer therapy.

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2018

CONJUGATED METALLOTHIONEIN-CARBON-DOXORUBICIN NANOTRANSPORTER FOR TARGETED BREAST CANCER THERAPY

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Abstract

Metalothionein (MT) is a polypeptide of molecular weight in the range of 6-10 kDa. MT typically contains 60 to 68 amino acid residues. MT is characterized by its unique content of metal ions as well as its sulfur content. Higher MT levels were observed in proliferating cells. This fact demonstrates the importance of MT in the process of cellular regulation (relationship to cancer). The most widely used drug for patients with breast cancer metastases is an anthracycline antitumor antibiotic doxorubicin (DOX). However, the clinical use of DOX is limited by dose-related heart muscle damage (cardiomyopathy), more prevalent with increasing cumulative doses. For this reason, creation of novel pharmaceutical formulations based on using alternative methods as nanocarriers for targeted drug delivery to tumour cells is a crucial task in modern pharmacology. The aim of this work was to design a nanotechnological construct. The construct is designed as two separate nanotransporters. The nanotransporter (A) is formed by an antibody-modified AgNPs particle and a carbon nanotube with encapsulated DOX (AgNPs/Ab1/MWCNT/DOX/ODN1). The nanotransporter (B) is engineered with SPION particle modified with antibody and with bound MT (SPION / Ab2 / MT / ODN2). Construct AgNPs/Ab1/MWCNT/DOX/ODN1-SPION/Ab2/MT/ODN2 is formed using an oligonucleotide anchor. Individual parts of the nanotransporter were studied using appropriate methods. The presence of MT was monitored electrochemically by Brdicka method in connection with the transfer technique (AdTSV). Characteristic MT signals RS2CO (-1.15 V), Cat1 (-1.25 V), Cat2 (-1.45 V), Cat3 (-1.75 V) were observed at accumulation time of 120s. SDS PAGE confirmed the presence of MT on SPION nanoparticles at sizes 7 to 15 kDa. The DOX signal was fluorometrically monitored (Em 590 nm, Ex 490 nm). AgNPs sizes ranged from 15 - 20 nm, and the SPION nanoparticles ranged from 20 - 50 nm. Additionally, used AgNPs nanoparticles exhibited significant antiproliferative activity (growth inhibition by 20 - 40 %) on a model culture *S. cerevisiae*. Created nanoconstruct A showed growth inhibition *for S. cerevisiae* by more than 50 %. The nanoconstruct after these various analysis shows a high potential as an anticancer drug and may be an innovative way how to deal with the breast cancer in a targeted therapy

Keywords: Nanomedicine, carbon nanomaterials; anticancer drugs, electrochemical analysis, biophysical analysis

1. INTRODUCTION

Cancer belongs worldwide to the leading cause of human death. The early detection and precise diagnosis of the onset of a disease is the most promising approach to accelerate healing processes or to improve survival of patients. There are different approaches for cancer treatment, including surgery, radiation therapy,

phototherapy, chemotherapy and biotherapy. Although chemotherapy has advantage in its efficiency, it has important limitations such as toxic side effects, damage of healthy cells, arising of the multidrug resistance and tumor recurrence. Therefore, new strategies are developed to overcome this problem. Nanoparticles and especially carbon nanoparticles have excellent physical properties, like NIR absorption or Raman enhancement and can be used for different approaches, among others for photothermal or photodynamic therapy, effective drug or gene delivery, or for diagnostic. Carbon is the fourth most abundant element in the universe, after hydrogen, helium and oxygen. Carbon atoms can build complicated organic structures. It can exist in different forms, which range from zero-dimensional (0-D, e.g. fullerenes) to three-dimensional (3-D, e.g. graphite). An analysis of a protein by electrochemical methods is not so broaden as by others techniques such as mass spectrometry [1,2]. On the other hand there have been published a number of papers, where the authors proved that an electrochemical technique could be a suitable tool for a determination of proteins [3-7]. More than 80 years ago Prof. Rudolf Brdicka laid the foundations of an electrochemical protein determination. He discovered an electrochemical catalytic signal of proteins in the presence of cobalt solution [8]. This technique has been used as a sensitive and selective tool for distinguishing of tumour and healthy tissue in 50s of the last century [9,10]. After that the electrochemical techniques were replaced by electrophoretic methods. In the beginning of 80s of the last century the method of Prof. Brdicka has been modified [11] and used for the detection of MTs [12]. The most widely used drug for patients with breast cancer metastases is an anthracycline antitumor antibiotic doxorubicin (DOX). However, the clinical use of DOX is limited by dose-related heart muscle damage (cardiomyopathy), more prevalent with increasing cumulative doses. For this reason, creation of novel pharmaceutical formulations based on using alternative methods as nanocarriers for targeted drug delivery to tumour cells is a crucial task in modern pharmacology [13]. The aim of this work was to design a nanotechnological construct. The construct is designed as two separate nanotransporters.

2. MATERIAL AND METHODS

Carbon (5 mg) was dispersed in 4 mL of an HNO_3:H_2SO_4 (1:3) mixture. The obtained suspension was heated for 7 h at 70°C (Electrothermal, UK). Then, it was placed in an ultrasonic bath (Ultrasonic Cleaner T, VWR) for 30 min and centrifuged at 14 000 *g* for 30 min. (Centrifuge 5418 R, Eppendorf). The obtained supernatant was removed, and the sediment washed with 500 µL H_2O (18 mΩ) and centrifuged at 14 000 × *g* for 30 min. The last step was repeated three more times. After the last centrifugation, the supernatant was removed and the sediment was dispersed in 500 µL of a 200 µg/mL doxorubicin (DOX) solution. The obtained suspension was placed in an ultrasonic bath for 30 min and centrifuged at 14 000 × *g* for 30 min. The obtained supernatant was removed and the sediment (fullerene-DOX nanocomposites) was washed twice with 500 µL H_2O (18 mΩ). Beside, 250 µL of the suspension of fullerene-DOX prepared as above, before placement in an ultrasonic bath for 90 min, was additionally treated with 1 % acetic acid. Electrochemical measurements were performed with AUTOLAB Analyser connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl/3M KCl electrode and the auxiliary electrode was a graphite stick electrode. The supporting electrolyte was prepared by mixing buffer components. For smoothing and baseline correction the software NOVA. Differential pulse voltammetry (DPV) of metallothionein: The amount of MT was measured using AdTS DPV. The samples of the MT were reduced before each measurement by 1 mM tris(2-carboxyethyl)phosphine addition according to [6, 14]. AdTS DPV parameters were as follows: an initial potential of -1.2 V, an end potential -0.3 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV, E_{ads} = 0 V. All experiments were carried out at room temperature (24 °C). The DPV samples analyzed were deoxygenated prior to measurements by purging with argon (99.999 %) saturated with water for 60 s. AdTS DPV Brdicka reaction of MT: In our studies, the Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used; surface-active agent was not added. AdTS DPV Brdicka reaction parameters were as follows: an initial

potential of -0.35 V, an end potential -1.8 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV, E_{ads} = 0 V. For the analysis of SPIONs MT concentration the SDS-PAGE with silver staining has been used. Gels (7.5 %) were prepared using acrylamide stock solution 30 % (m / V) with bisacrylamide 1 % (m / V). Separating gel contained: acrylamide 7.5 % (m / V), bisacrylamide 0.5 % (m / V), 0.4 M Tris/HCl, 0.1 % (m / V) sodium dodecyl sulfate (SDS), pH 8.8. Stacking gel contained: 4.5 % acrylamide (m / V), 0.15 % bisacrylamide (m / V), 0.1 % SDS (m / V), 0.1M Tris/HCl, pH 6.8. Nanoconstructs were diluted 2:1 with a loading buffer (PLB Max). Each well contained 15 µl of the diluted solutions. Electrophoretic measuring conditions were: 120 V, 1.5 hours in a running buffer (24 mM Tris, 0.2M glycine and 3 mM SDS). After measurement the gel was stained with silver, scanned and evaluated by Colortest in the laboratory system Qinslab.

3. RESULTS

In our work, a unique DOX nanotransporter was prepared to target the tumor cell. The nanotransporter (A) is formed by an antibody-modified AgNPs particle and a carbon nanotube with encapsulated DOX (AgNPs/Ab1/MWCNT/DOX/ODN1). The nanotransporter (B) is engineered with SPION particle modified with antibody and with bound MT (SPION/Ab2/MT/ODN2). Construct AgNPs/Ab1/MWCNT/DOX/ODN1-SPION/Ab2/MT/ODN2 is formed using an oligonucleotide anchor. Individual parts of the nanotransporter were studied using appropriate methods.

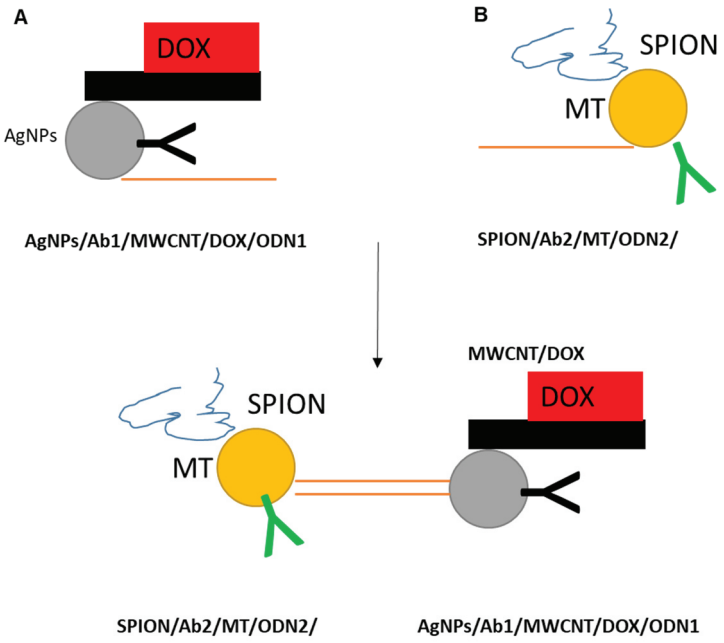

Figure 1 Schema of the proposed nanoconstruct for its detailed characterization. Part A is a dual antitumor nanotransporter. DOX is encapsulated within carbon nanomaterial (nanotube, fullerene), and then this structure is bound to AgNPs, ODN1 and model general antibody using EDC / NHS modification. Part B provides a model for the targeted protection of healthy tissue. SPION particle is modified by MT, ODN2 and antibody.

The presence of MT was monitored electrochemically by Brdicka method in connection with the transfer technique (AdTSV). It has been known more than 80 years that it is suitable to use the catalytic signal of hydrogen evolution in the presence of ammonium buffer (1 M NH_4Cl + NH_4OH) containing cobalt solution $Co(NH_3)_6Cl_3$ for the determination of the proteins containing cysteine amino acids, e. g. metallothionein (**Figure 2**).

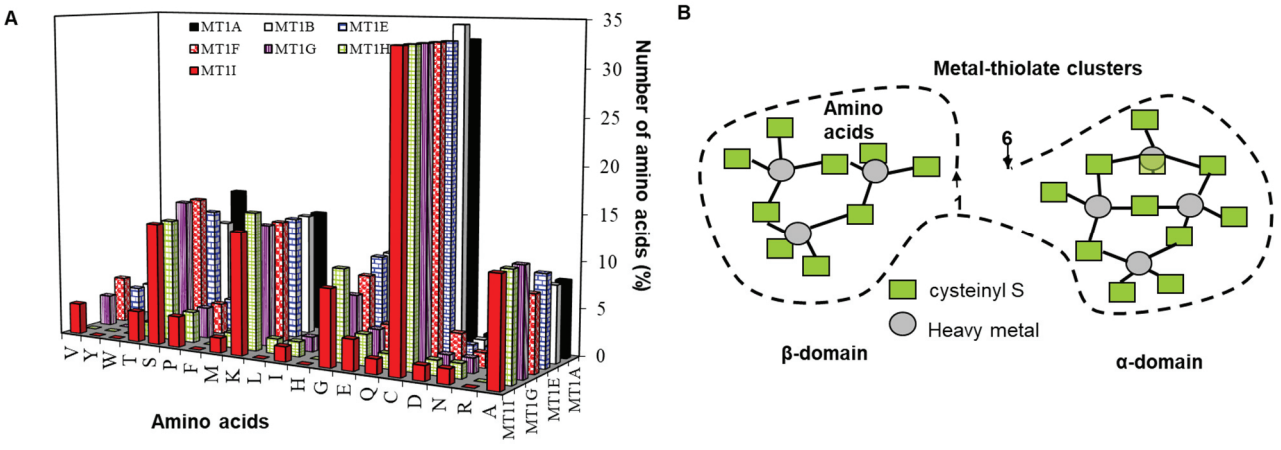

Figure 2 Content of aminoacids in human metallothioneins of MT1 class according to **Ex**pert **P**rotein **A**nalysis **Sy**stem - ExPASy **(**A**)**. (**B**) Model of Metal-thiolate clusters, according to [6].

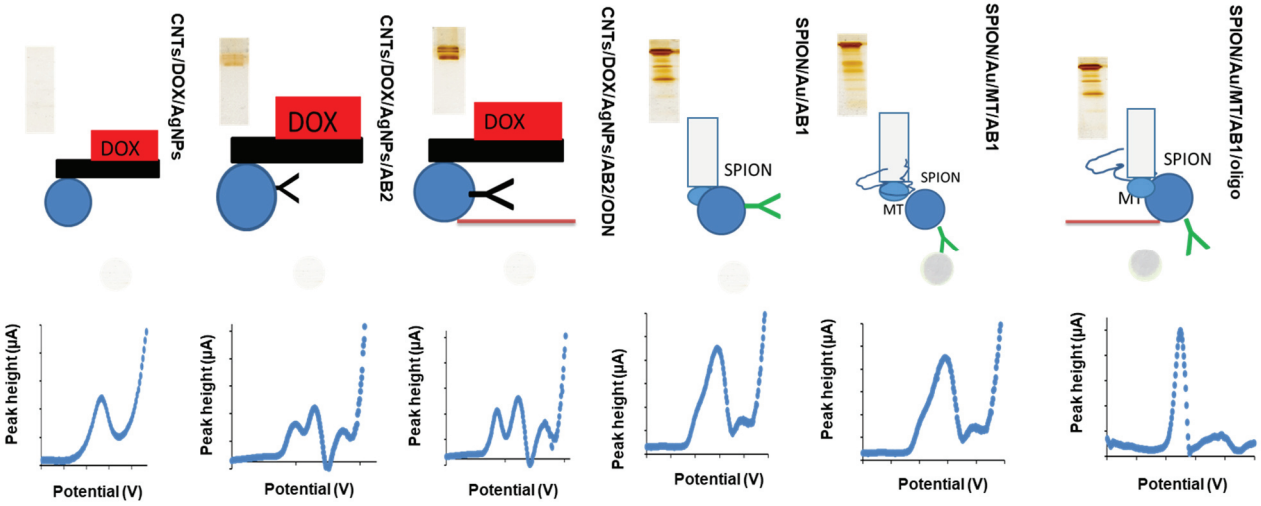

Figure 3 Detailed characterization of prepared nanoconstructs by means of electrophoresis, dot-blot analysis and electrochemical detection of proteins. On the NC45 nitrocellulose membrane (pore size 0.45μm, Serva, Germany) was pipetted 2 μl of antibody. The membrane was allowed to dry. Then blocked by 1 % BSA (bovine serum albumin) dissolve in PBS (Phosphate buffered saline) on a Multi-Rotator, Multi RS-60 (BioSan) for 40 minutes. Then the membrane was insered into a sample solution and again strirred on a rotator shaker for 1 hour. The membrane was then washed three times with PBS-T (Phosphate buffered saline with 0.05 % Tween 20) and then inserted into the gold-labeled antibody and stirred for 1 hour on rotator shaker. In the end the membrane was washed with PBS-T, allowed to dry and scaned for another evaluation. Other experimental details is in Material and Methods section.

A number of authors have concerned with explanations of the processes, which proceed on the surface of working mercury electrode during Brdicka reaction. We observed during modified Brdicka reaction by AdTS DPV analysis four MT signals - Co_1, RS_2Co, Cat1 and Cat2. Signals of Cat1 a Cat2 correspond to the reduction of hydrogen at the mercury electrode [15]. Another signal, which is appeared at the potential about -1.0 V, relates with the reduction of the RS_2Co complex [15]. In addition the signal called Co_1 could result from reduction of $[Co(H_2O)_6]^{2+}$ [15]. It clearly follows from the figure that character of the mentioned MT signals

change with different MT amount. Signal Co_1 decreased and shifted to more negative potential with decreasing MT amount. The signal is almost un-detectable at MT amount under 0.6 pmol. The others mentioned MT signals of Brdicka reaction (RS_2Co, Cat1 and Cat2) are getting well-developed and separated with decreasing MT concentration. In addition RS_2Co and Cat signals decreased and slowly shifted to more positive potential according to decreasing MT concentration. Moreover, SDS PAGE confirmed the presence of MT on SPION nanoparticles at band sizes 7 to 15 kDa (see in **Figure 3**). Additionally, dot blot technique was used to confirm the presence of the bound antibody. Thus, it was clearly demonstrated that AgNPs were modified by antibody 1 and SPION (20-50 nm) were modified with antibody 2. MWCNTs were chemically modified for DOX binding according to established procedures. The DOX signal was fluorometrically monitored (Em 590 nm, Ex 490 nm). The amount of DOX bound to MWCNTs was around 50-60 %. Another part of the nanoconstruct were AgNPs (15-20 nm) that show antiproliferative effects. Additionally, used AgNPs nanoparticles exhibited significant antiproliferative activity (growth inhibition by 20-40 %) on a model culture *S. cerevisiae*. Created nanoconstruct A showed growth inhibition *for S. cerevisiae* by more than 50 %. The nanoconstruct after these various analysis shows a high potential as an anticancer drug and may be an innovative way how to deal with the breast cancer in a targeted therapy.

4. CONCLUSION

A unique universal nanotransporter, DOX and AgNPs, was developed to target the tumor cell. The nanotransporter (A) was formed by an antibody-modified AgNPs particle and a carbon nanotube with encapsulated DOX (AgNPs/Ab1/MWCNT/DOX/ODN1). The nanotransporter (B) was engineered with SPION particle modified with antibody and with bound MT (SPION / Ab2 / MT / ODN2). Construct AgNPs/Ab1/MWCNT/DOX/ODN1-SPION/Ab2/MT/ODN2 was formed using an oligonucleotide anchor. Individual parts of the nanotransporter were studied using appropriate methods. In the next work, the nanotransporter will be treated with suitable target antibodies and tested on suitable cell lines.

ACKNOWLEDGEMENTS

This work has been supported by the NANOCSDOXO (306/2018/FaF) and the Grant ST.D170 from Wroclaw Medical University and the international collaboration project of The European Technology Platform for Nanomedicine.

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