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# Interaction of hierarchical nanoporous carbons (HNCs) with chicken embryo red blood cells (RBC)

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**Abstract:** *Interaction of hierarchical nanoporous carbons (HNCs) with chicken embryo red blood cells (RBC).* The purpose of this study was to characterize toxicity of hierarchical nanoporous carbons (HNCs) on chicken embryo red blood cells (RBC), which are a perfect model to adapt the hemolysis assay in evaluation of the *in vitro* blood compatibility of nanoparticles. The samples of blood were treated with different concentration of HNCs (10, 50 and 100 μg/ml). Hemolysis assay showed that the hemolytic activity depends on the dose of HNCs. The microscope observations have shown a difference in morphology between treated and untreated RBC: changes in cell membrane shape and the occurrence of pathological erythrocytes forms.

*Key words*: hierarchical nanoporous carbon, nanoparticles, red blood cells, toxicity

## INTRODUCTION

The nanoparticles of carbon allotropes have recently been widely investigated due to their *in vitro* and *in vivo* biological activity. The results of experiments on diverse carbon nanomaterials have shown that their different behavior in living organism may be useful in all biology and medicine fields, including areas such as drug/gene delivery (Feng et al. 2011, Wu et al. 2014), bioimaging, biosensing (Shen et al. 2012), antibacterial materials (Hu et al. 2010), and cancer therapy (Farokhzad et al. 2006, Cho et al. 2008). In one of the studies appeared that carbon nanotubes are needle-like potential carriers of bioactives including drug, genes and proteins. Moreover, functionalized nanotubes are more soluble, biocompatible, and have greater potential for attaching certain molecules and targeting them into cancer cells (Torchilin 2011, Mody et al. 2014). Another promising carbon nanoparticle is graphene. Flakes or a surface of graphene may be used as bioactive molecules (Sawosz et al. 2014). Furthermore, the nanodiamonds have a great biocompatibility but they are still a foreign non-degradable material for biological organisms (Zhu et al. 2012).

The new nanoparticles synthetized in the Military University of Technology in Warsaw called hierarchical nanoporous carbons (HNCs) are carbon allotropes, which are the first nanoparticles occurring in a cubic shape. The HNCs are closed-cage nanoparticles with really thick walls such as those of a single graphenepetal. The structure of HNCs is analogous to the structure of fullerenes – other carbon nanoparticles, which

were already tested for their anticancer properties. The difference in the structure revealed that the HNCs contain mixture of magnesium and oxalic acid powders (MgO) (Dyjak et al. 2016).

The main parameters for the biocompatibility of carbon nanoparticles are hemolytic properties and interactions with red blood cells (RBC) thus they have a great potential in drug delivery systems (Mocan 2013). The erythrocyte model is much closer to the physiological condition of living cells and their results are more accredited to be the foundation of the *in vivo* study. Therefore, we decided to choose chicken erythrocytes since on the contrary to the human ones, they contain a nuclear (Zhang et al. 2014). The proper assessment of potential toxic effects of carriers drug/gene delivery is important forasmuch almost every platform in such systems requires intravenous administration. In the use of nanoparticles in the drug delivery, it is necessary to determine the toxicity of the RBC. Most of the biomedical applications of nanoparticles require intravenous administration, which enables them to interact with RBC and other immune cells. Therefore, hemocompatibility assays are of utmost importance (Pan et al. 2016).

## MATERIAL AND METHODS

### Preparation and characterization of hierarchical nanoporous carbons (HNCs)

Hierarchical nanoporous carbons (HNCs) synthetized in the Military University of Technology (Warsaw, Poland). Shape and size of HNCs were evaluated using a JEM-2000EX transmission electron microscope (TEM) at 80 keV (JEOL Ltd, Tokyo, Japan). The samples for TEM were prepared by placing hydrocolloid droplets into formvar-coated copper grids (Agar Scientific, Stansted, UK). The test was performed in triplicate. Zeta potential was measured in milli-Q water by a ZEN3500 Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Prior to application, the carbon nanoparticles were dispersed in phosphate buffered saline (PBS) to prepare the following concentrations: 10, 50, and 100 μg/ml. The solutions were then sonicated for 30 min.

### Embryo model

Fertilized eggs (*Gallus gallus*, $n$ = 15) from Hubbard Flex Line hens were obtained from a commercial hatchery (Dembówka, Poland). After 19 days of eggs incubation (temperature 37°C, 70% humidity, turning once per hour), the embryos were immediately decapitated while blood samples were collected from the jugular vein. Blood samples were divided into the following groups: control untreated (0% hemolysis), positive control treated with 3% hydrogen peroxide (100% hemolysis), HNCs 10 μg/ml, HNCs 50 μg/ml, HNCs 100 μg/ml, hydrocolloids diluted in PBS. The samples were placed in Vacutainer tubes (BD Inc., Franklin Lakes, NJ, USA) containing ethylenediaminetetraacetic acid (EDTA), gently mixed on a rotary shaker, and incubated for 3 h at 37°C. The incubation time was based on Asharani et al. (2010). All measurements were performed eight times.

### Blood cell morphology

Blood cell morphology was investigated using light microscopy and transmission

electron microscopy (TEM). Peripheral blood smears were prepared using 5 µl of whole blood, air-dried, stained peripherally with May–Grünwald–Giemsa, and examined at a magnification of ×1.000 (Leica DM750, Leica Microsystems, Nussloch, Germany). For the TEM examination, the blood samples were fixed in 2.5% glutaraldehyde and centrifuged at 1,200 rpm. The supernatant was discarded, RBCs were dispersed in deionized water. The samples for TEM were prepared by placing hydrocolloid droplets into formvar-coated copper grids. The test was performed in triplicate.

### Hemolytic assay

The hemolysis assay was performed with embryo whole blood. After incubation, the tubes containing blood samples: control untreated (0% hemolysis), positive control treated with 3% hydrogen peroxide (100% hemolysis), HNCs 10 µg/ml, HNCs 50 µg/ml, HNCs 100 µg/ml were centrifuged for 10 min at 1,200 rpm. The absorbance of the supernatant, which includes plasma and lysed erythrocytes, was measured at 540 nm (Infinite M200, Tecan, Durham, NC, USA). Percent hemolysis was determined as compared to Shiny et al. (2014).

### Statistical analysis

Statgraphics Centurion software (StatPoint Technologies, Warrenton, VA, USA) was used for the statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. Values of $P$ below 0.05 were considered to be statistically significant.

## RESULTS AND DISCUSSION

Red blood cells from a chicken embryo are a model that allows high precision in evaluating the toxicity. Moreover, they allow to prove the hemocompatibility (Zhang et al. 2014). With increasing concentration of HNCs ($\zeta$ = –28) a higher hemolytic activity was observed (Fig. 1). In contrast to the control group it increased at 10 µg/ml to 8%, at 50 µg/ml to 30% and the percentage of hemolysis was the highest (36%) at a concentration of 100 µg/ml. Both the lack of hemolysis

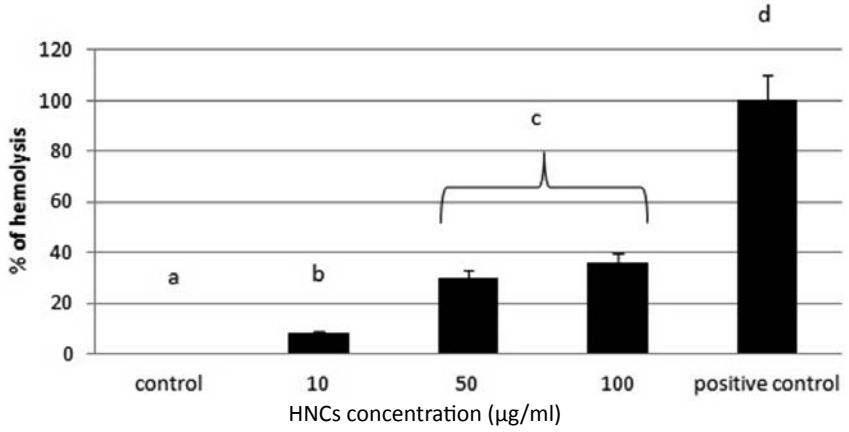


FIGURE 1. The hemolytic effect on chicken embryo blood exposed to HNCs in 10, 50 and 100 µg/ml concentration. The columns with different letters (a–d) indicate significant differences ($P$ <0.001)

in the control group and an almost 100% hemolysis rate in the positive control group treated with 3% solution of the hydrogen peroxide, confirmed the accuracy. HNCs exhibited dose-dependent hemolytic activity towards RBC. Compared to other studies of silica nanoparticle, the size and the shape could be another factors causing induced hemolysis other than the concentration (Yu et al. 2011).

The microscope observations have shown a difference in morphology between control and treated samples with HNCs (Figs 2, 3). The membranes of RBC were disintegrated. The pictures of samples, which were treated with HNCs, are comparable to positive control. There were differentiations in the shape of cells, which were deformed. Cells also lost their biconcavity. The observation showed that the increasing level of swollen cells and other pathological forms of erythrocytes, such as echinocytes and knizocytes, depends on the degree of hemolysis (Lim et al. 2002). The most pathological forms occurred at 100 µg/ml concentration of HNCs. Moreover, all the HNCs treated groups showed the presence of ghost cells, which are the result of cell lysis. In the pictures of treated samples, the RBC had lost their typical discoid-shape and the degradation of the membranes was clearly visible. Therefore, further studies

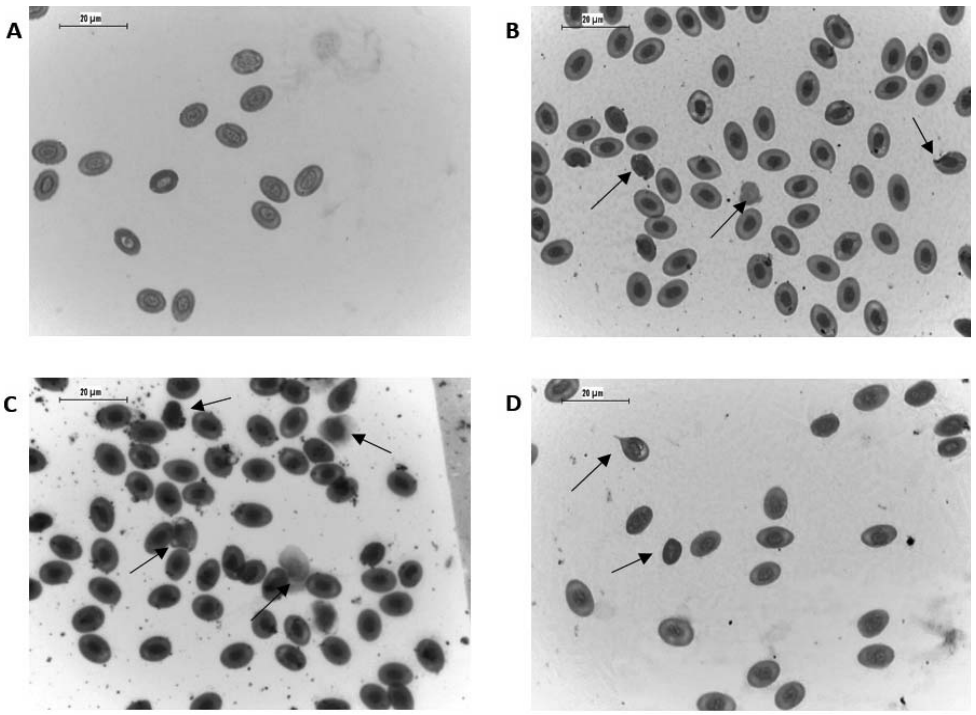


FIGURE 2. Results of blood smears stained peripherally with May–Grünwald–Giemsa from light microscope. A – control, B – sample treated with 50 µg/ml HNCs, C – sample treated with 100 µg/ml HNCs, D – sample treated with 10 µg/ml HNCs. Arrows indicate pathological forms of erythrocytes

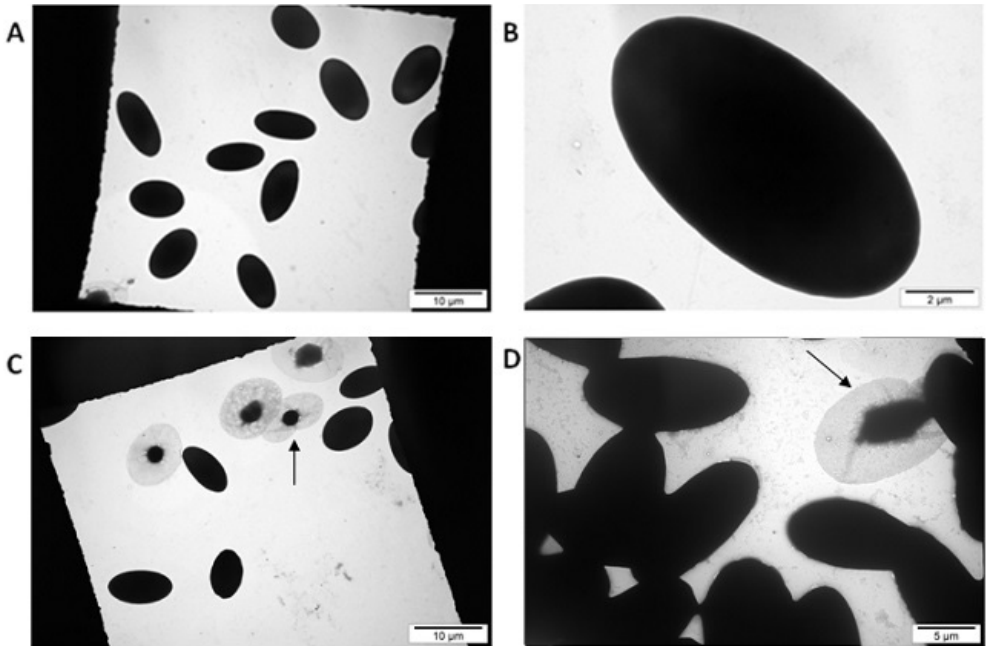


FIGURE 3. Pictures from TEM. A, B – control, C, D – samples treated with 50 µg/ml HNCs. Arrows indicate ghost cells

should concentrate on reducing the dose and finding other than intravenous ways of administration of the nanoparticles.

## CONCLUSION

Treatment with HNCs damages the membrane in RBC differentiation: alters the shape and causes the occurrence of pathological forms such as echinocytes and knizocytes. Cell lysis results in the presence of multiple ghost cells. In the conducted study, HNCs exhibited dose--dependent hemolytic activity towards RBC.

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**Streszczenie:** *Interakcja hierarchicznych nanoporowatych nanocząstek węglowych (HNCs) z czerwonymi krwinkami zarodka kury.* Celem tego badania było określenie toksyczności hierarchicznych nanoporowatych nanocząstek węglowych (HNCs) wobec krwinek czerwonych, pozyskanych z zarodka kury (RBC). Są one idealnym modelem do oceny testu hemolizy w badaniach zgodności nanocząstek w warunkach *in vitro*. Do próbek krwi zarodka kurzego zostały dodane HNCs w różnych stężeniach (10, 50 i 100 µg/ml). Test hemolizy wykazał, że aktywność hemolityczna zależy od dawki HNCs. Podczas obserwacji mikroskopowych zaobserwowano różnice w morfologii między traktowanymi HNCs a nietraktowanymi RBC. Stwierdzono zmianę kształtu błony komórkowej krwinek i występowanie patologicznych form erytrocytów.

*Słowa kluczowe*: hierarchiczne nanoporowate nanocząstki węglowe, nanocząstki, czerwone krwinki, toksyczność

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