Decreased immune response after PASylation of stealth ferritin nanocarriers

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Abstract: The effects on immunocompatibility after performed surface modifications (PEGylation and PASylation) of natural nanocarriers based on ferritin (FRT) were tested in this work. Potential cytostatic drug ellipticine (Elli) was encapsulated into FRT, leading to formation of FRTElli. The main goals of prepared PEG-FRTElli, PAS-10-FRTElli and PAS-20-FRTElli were to achieve decreased *in vitro* macrophage uptake and decreased *in vivo* complement activation. Macrophage uptake was studied *via* flow cytometry and complement activation *via* western blot. According to performed experiments PAS-10 modification was the only one that was sufficient to ensure both aspects.

Key Words: apoferritin, immune response, nanomedicine, PASylation

INTRODUCTION

Even though there is a large number of drugs that can be used in cancer treatment, the goal is to reach selective death of malignant cells while reducing toxicity to healthy cells. There are several biological barriers to provide effective drug delivery directly to malignant tissue such as renal, hepatic, or immune clearance. Herein, we focused on reducing the immune clearance. The fact is that only 5% of nanomaterial doses reach their intended target, which happens, among other reasons, due to non-specific recognition and uptake of nanoconstructs by macrophages (Bae and Park 2011). Macrophages are leukocytic cells involved in the detection, phagocytosis and destruction of bacteria and other harmful organisms (Gustafson et al. 2015). On the macrophages surface, receptors for complement proteins are located (van Lookeren Campagne et al. 2007). The system of complement proteins represent an important part of the innate immunity, acting as first-line defence against pathogenic infections (Noris and Remuzzi 2013). After intravenous administration, nanomaterials interact immediately with the immune system and activate the complement proteins (Halamoda-Kenzaoui and Bremer-Hoffmann 2018). Complement proteins can be activated *via* antibodies binding to antigen pathway, mannose binding to lectin pathway or by direct binding of the pathogen to the complement protein. All three pathways lead to the inflammatory process, macrophages accumulation in the liver and spleen or hypersensitivity reaction (Wang et al. 2017, van den Hoven et al. 2013).

Therefore, there is a huge need for ″stealth″ nanomaterials with a pronounced immuno-compatibility. Surface-related properties of nanomaterials are becoming more relevant than the category of a nanomaterial since they determine the protein corona composition and interaction with other plasma proteins and complement proteins (Moghimi and Simberg 2017). Surface modifications of nano-materials represent promising way to significantly improve the immunocompatibility of nanomaterial, e.g., decrease complement activation or reduce uptake by macrophages. PEGylation, as the first clinically approved modification of a drug, was shown to exhibit phenomenon called “accelerated blood

clearance" occurred due to production of specific anti-PEG IgM, followed by increased uptake of nanoparticles by the macrophages (Yang et al. 2013, Yang and Lai 2015). PASylation, a more biological alternative to PEGylation, is comprised of small residues of amino acids Proline (P), Alanine (A) and Serine (S) (Binder and Skerra 2017). Horse spleen FRT was chosen as a biocompatible organic nanocarrier. Its structure is strongly dependent on the pH value of the surrounding environment, thus it enables physical entrapment of cytostatic drug within the internal 8-nm cavity (Kim et al. 2011).

MATERIAL AND METHODS

Chemicals

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

Encapsulation of Elli into FRT

The stock solution of Elli with concentration of 1 mg/ml was prepared by dissolving Elli in 1 M HCl and deionized water in ratio 1 : 80. For each sample, 200 µl of 1 mg/mL Elli was added to 100 µl of deionized water and 20 µl of 50 mg/ml horse spleen 22*L*/2*H* FRT and gently mixed for 15 min. FRT structure was reassembled by 0.66 µl of 1 M sodium hydroxide solution and the samples were mixed for further 15 min. To filter out non-encapsulated Elli, solution exchange was performed 3× (centrifugation at 6000 rcf and 4 °C for 15 min). The concentration of encapsulated Elli was evaluated *via* absorbance measurement at 420 nm using Tecan Infinite 200 PRO (Männedorf, Switzerland).

Surface modification with PAS sequences

25 µl of 1.3 nm gold nanoparticles were added to 321 µl FRTElli solution and the samples were mixed for 14 h to allow adsorption of Au nanoparticles to the charged amino acid residues on the surface of FRTElli nanoparticles (leading to FRTElli-Au). Solution exchange was performed to remove unbound Au nanoparticles. 3 µl of 1.25 mg/ml PAS-10 (ASPAAPAPASC) or PAS-20 (ASPAAPAPASPAAPAPSAPAC) was added to FRTElli-Au and the samples were incubated for 1 h at 45 ºC to allow binding of cysteine to gold. Then, solution exchange was performed to remove unbound molecules of PAS peptides.

Surface modification with PEG

50 µl of 10 mM methoxy PEG maleimide in PBS (phosphate buffered saline, pH 7.4: 0.137 M NaCl + 0.0027 M KCl + 0.0014 M KH_2PO_4 + 0.0043 M Na_2HPO_4) and 629 µl of PBS was added to FRTElli and mixed for 1 h. To remove unbound PEG, the sample was 5× diafiltrated using Amicon® Ultra 0-5 mL 50K Merck Millipore (Billerica, MA, USA) at 6000 rcf and 4 °C for 15 min.

Evaluation of macrophages uptake

Macrophage uptake was performed with murine macrophage cell line derived from Abelson murine leukemia virus-induced tumor RAW 264.7. A suspension of 300 000 RAW 264.7 cells was seeded into 12-well plate. After 24 h incubation, the cells were treated with 20 µM Elli in the form of free Elli, FRTElli, PEG-FRTElli, PAS-10-FRTElli, and PAS-20-FRTElli for 30 min. The cells were washed with phosphate buffered saline (PBS, pH 7.4), scraped and centrifuged at 1000 rcf for 10 min. Then, the cells were resuspended in 3% fetal bovine serum (FBS) in PBS solution. Macrophage uptake was analyzed *via* fluorescence using 488-nm laser and 533/30 filter on the BD Accuri C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The flow rate was 35 µl/min and a minimum of 100 000 cells was analyzed in each group.

In vivo activation of complement (C3)

Twelve female nude athymic BALB/c nu/nu mice were used for *in vivo* studies. The use of the animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The experiments were performed with the approval of the Ethics Commission at the Faculty of Medicine, Masaryk University, Brno, Czech Republic. The mice stayed in individually ventilated cages at 12/12 h light/dark cycle and provided *ad libitum* with standard diet and water.

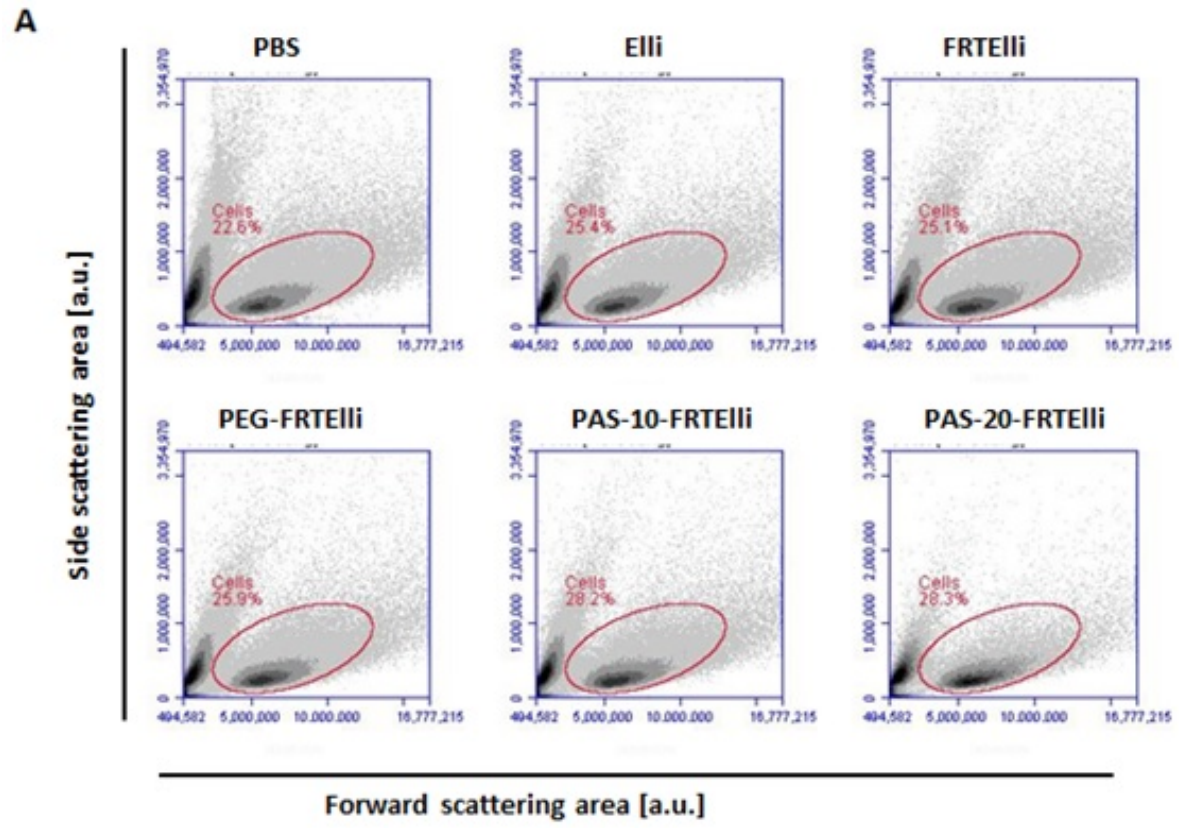
The treatment of mice was carried out *i.v.* as two applications (100 µg dose of Elli for each mouse, either free or in the form of FRTElli or PAS-10-FRTElli). The control group received 100 µl of PBS. After 1 week, the mice were euthanized by intraperitoneal injection of 1% Narkamon + 2% Rometar, 5 µl/g of body weight, followed by intracardiac blood collection in ethylendiaminetetraacetic acid-treated tubes. Plasma was isolated after blood collection by centrifugation (1000 rcf, 20 ºC, 10 min)

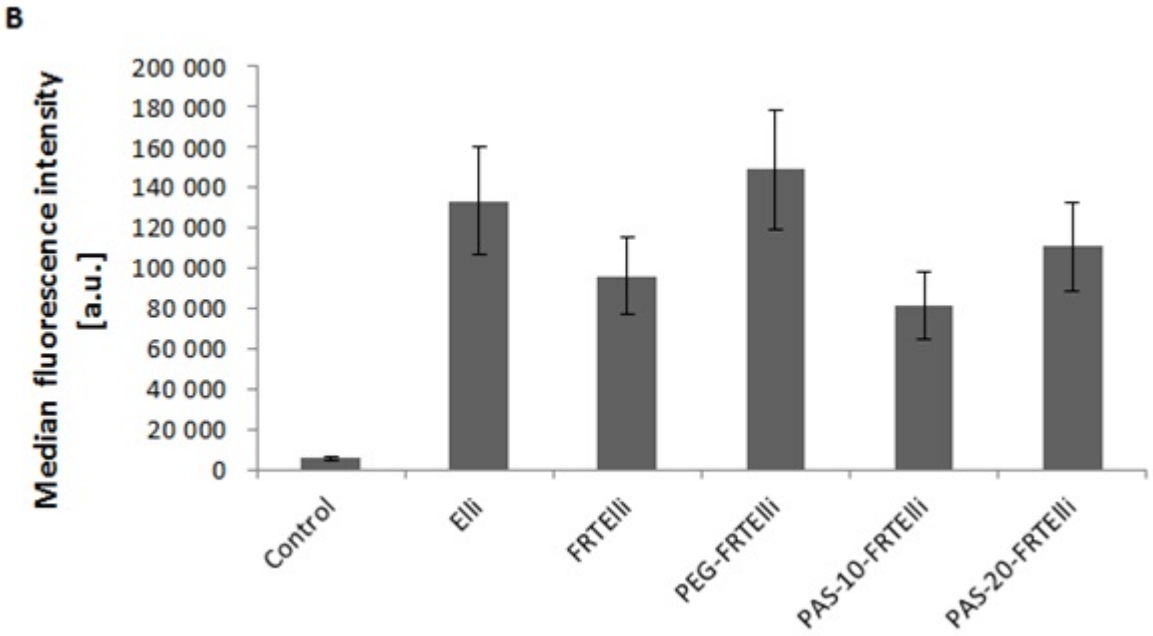
Electrophoresis (200 V, 4 ºC, 35 min) of the plasma samples was done under non-reducing and non-denaturing conditions in 7.5% sodium dodecylsulphate gel. Proteins were electrotransferred onto the Immobilon®-FL transfer membrane (EMD Millipore Corporation, Burlington, MA, USA) and the non-specific binding was blocked with 5% (*w*/*v*) skimmed milk powder for 1 h at 20 ºC. Complement C3 monoclonal antibody (LF-MA0132, Thermo Fisher Scientific, USA, dilution 1:1 000) was used as primary antibody, incubation was performed at 20 ºC for 1 h. After washing, membrane was incubated with secondary antibody, which was labeled with horseradish peroxidase (p0260, Dako, Santa Clara, CA, USA, dilution 1:5 000) for 1 h at 20 ºC. Clarity Western ECL Blotting Substrate (Bio-Rad) was used in order to develop the signals and chemiluminescence was visualized *via* Azure c600 (Azure Biosystems).

RESULTS AND DISCUSSION

Figure 1A showed cell distribution in each sample, population of live cells is shown in red circle. The macrophage uptake (Figure 1B) was expressed as median fluorescence intensity of Elli. Obtained data (Figure 1B) showed that free Elli enters into macrophages. Importantly, the highest macrophages uptake was detected for PEG-FRTElli, which point out at possible immunogenicity of PEG (Ganson et al. 2006, Judge et al. 2006). On the other hand, the lowest activation of immune response was detected for PAS-10-FRTElli. Modification with PAS-10 sequences was the only one that caused the decrease of macrophage uptake compared to unmodified FRTElli.

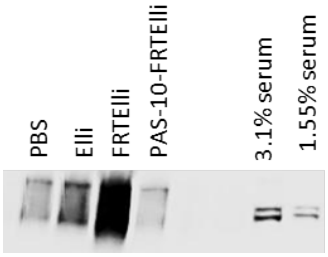
Figure 1 Macrophage uptake via flow cytometry (A: forward scatter vs. side scatter, B: macrophage uptake)





The lowest *in vivo* activation of C3 complement protein (Figure 2) was achieved after treatment with PAS-10-modified FRTElli (81.9% ± 4.2% decrease of activation compared to FRTElli). Group treated with free Elli caused also lower activation than in unmodified FRTElli (74.8% ± 3.5% decrease of activation compared to FRTElli). Results were confirmed by three independent replicates. These findings correspond with *in vitro* macrophage uptake, that PAS sequences are able to decrease the immune response.

Figure 2 Western blot analysis of complement (C3) activation performed on Elli, FRTElli, PAS-10-FRTElli treated samples, including positive (serum) and negative (PBS) control



CONCLUSION

Three types of FRTElli surface modifications were tested (modification with PAS-10, PAS-20 sequences and PEGylation) in order to decrease immune response after intravenous delivery. Modifications with PEG and PAS-20 sequences did not appear as suitable due to the increase in macrophage uptake. On the other hand, modification with PAS-10 sequences led to decrease macrophage uptake and also decreased *in vivo* complement activation. The *in vitro* and *in vivo* experiments presented in this work serve as suitable platform to carrying out additional preclinical studies of PAS-10-FRTElli.

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