Scientific Report

Project: Engineered glycopeptide-based micro/nanomotors for anti-tumoral co-drug release
Acronym: GlyPepTum
Code: PN-III-P1-1.1-PD-2019-0271, **No**. 149/2020

Stage no. 3 / 2022

Design and synthesis of new polymersomes with stomatocyte shape – advanced studies. Therapeutic effect of the glycopeptide-based micro/nanomotors

- **Summary of the scientific report**

In the third stage, according to the project plan, advanced study regarding the design and synthesis of new polymersomes with stomatocyte shape were realized and also the therapeutic effect of the glycopeptide-based micro/nanomotors was study after loading with two anti-tumoral drugs – dacarbazine (DTIC) and vinblastine (VCL) - for melanoma therapy.
A series of magnetic polymersomes were prepared based on MAC-pLeu (maleoyl-chitosan-g-poli(leucine)), MAC-p(Leu-co-CBZ-Lys)$_{1:0.5}$ (maleoyl-chitosan-g-poli(leucine-co-CBZ-lysine) and MAC-p(Leu-co-CBZ-Lys)$_{1:0.75}$ and Gox-coated magnetic particles in various glycopeptide/MNP ratios: 3:1, 4:1, 5:1. The systems with glycopeptide/MNP ratio: 3:1 were selected for the loading of antitumor drugs as a result of the optimal colloidal stability in the simulated tumor environment and in that of healthy tissues.
The magnetic polymersomes loaded with DTIC and VLC presented a release kinetics of up to 48 hours.
The biological properties of the magnetic polymersomes systems loaded with dacarbazine indicated that all the studied systems (MAC-pLeu, MAC-p(Leu-co-CBZ-Lys)$_{1:0.5}$ and MAC-p(Leu -co-CBZ-Lys)$_{1:0.75}$) have an extraordinary potential in melanoma therapy, also having a reduced toxicity on normal cells compared to that of the simple drug.

Design and synthesis of new polymersomes with stomatocyte shape – advanced studies
Preparation of the polymerosome encapsulating enzyme-conjugated MNPs.Influence of preparation conditions on the capacity to self-assembled in polymersomes with stomatocyte shape
The ability to self-assemble of polymersomes based on polysaccharides grafted with peptides was optimized by investigating the influence of the Leu/CBZ-Lys ratio, the glycopeptide concentration and the glycopeptide/MNP mass ratio to ensure not only the encapsulation of therapeutic agents in the glycopeptide matrix, but also to maintain its stability when magnetic nanoparticles conjugated with enzymes are included.

Influence of glycopeptide concentration

The effect of the concentration of the MAC-pLeu(1:1) compound on the size of the particles formed by dialysis was investigated at the following concentrations: 0.10, 0.50, 1 and 2 mg/mL. The values of the average dimensions of the nanoparticles showed significant changes with the decrease of the concentration the glycopeptide used in the preparation of the polymersomes as a result of a shielding effect on the surface charge. This variation was attributed to the different conformations adopted by the glycopeptide macromolecular chains in solution.

Influence of the ratio between the glycopeptide and the GOx-coated magnetic nanoparticles on the colloidal stability of the magnetic polymersomes

Based on the results obtained from the study of the influence of glycopeptide concentration on the size and colloidal stability of simple polymersomes, the MAC-pLeu(1:1)_1 (c=1mg/ml w/v) batch was chosen for subsequent use in the preparation of magnetic polymersomes. Starting from the optimal concentration of 1mg/ml, three glycopeptide/MNP mass ratios were prepared for each chitosan derivative: MAC-pLeu, MAC-p(Leu-co-CBZ)$_{1:0.5}$ and MAC-p(Leu-co -CBZ) $_{1:0.75}$. The systems formed by the two components showed particle sizes ranging from 194 nm to 336 nm, suggesting that the glycopeptides and MNPs were assembled into polymersomes in aqueous buffer solution.

Influence of glycopeptide composition on the colloidal stability of magnetic polymers

For magnetic nanoparticle/glycopeptides systems, those containing MAC-p(Leu-co-CBZ-Lys)$_{1:0.5}$ or MAC-p(Leu-co-CBZ-Lys)$_{1:0.75}$ showed higher average size values (290 nm-336 nm) as a result of the introduction of another amino acid (CBZ-Lys) in the formation of the peptide chain, but also a lower zeta potential (+7.26 mV - +7.68 mV) compared to samples based on MAC-pLeu+MNP (+19.0 mV). A possible explanation would be that with the increase in the percentage of CBZ-Lys, which has a hydrophobic character induced by the CBZ (carbobenzyloxy) radical, a shielding of the surface charges occurs and thus a decrease in the ZP values is recorded.

Based on the conclusions obtained from the colloidal stability studies that can be influenced by the composition of the glycopeptides but also by the ratio between glycopeptides and magnetic particles, the ratio 3:1 glycopeptides/MNPs was selected for the subsequent characterizations.

Characterization of structural and morphological properties of the new micro/nanomotors

The FT-IR analysis of the simple polymersomes showed absorption bands characteristic of the chitosan derivative modified with maleic anhydride (MAC): valence vibrations of the –OH group superimposed with the valence vibrations of the N-H bond – range 3700 – 3000 cm^{-1}, vibrations of valence of C-H at 2920 cm^{-1}; the stretching vibration of the carbonyl group C=O 1706 cm^{-1} and deformation vibrations of the C-O bond - in the range 1084 - 1018 cm^{-1}. The peaks characteristic of the stretching vibration for amide I and amide II in the polypeptides are observed between 1635-1631 cm^{-1} and 1518-1514 cm^{-1} and are most often associated with the secondary structures of the polypeptides formed through inter - and intrachain hydrogen bonds

In the case of magnetic polymersomes, the absorption bands characteristic of the stretching vibration of amide I appear at 1655 cm^{-1} and respectively 1651 cm^{-1} in the corresponding FT-IR spectra, confirming the presence of glycopeptides in the composition of new systems. Absorption bands characteristic of the Fe-O bond are observed in all magnetic polymersomes spectra at 521 cm^{-1} for the MAC-pLeu+MNP (3:1)

and MAC-p(Leu-co-CBZ-Lys)$_{1:0.5}$+MNP systems (3:1)) and 523 cm^{-1} for MAC-p(Leu-co-CBZ-Lys)$_{1:0.75}$+MNP nanoparticles (3:1)).

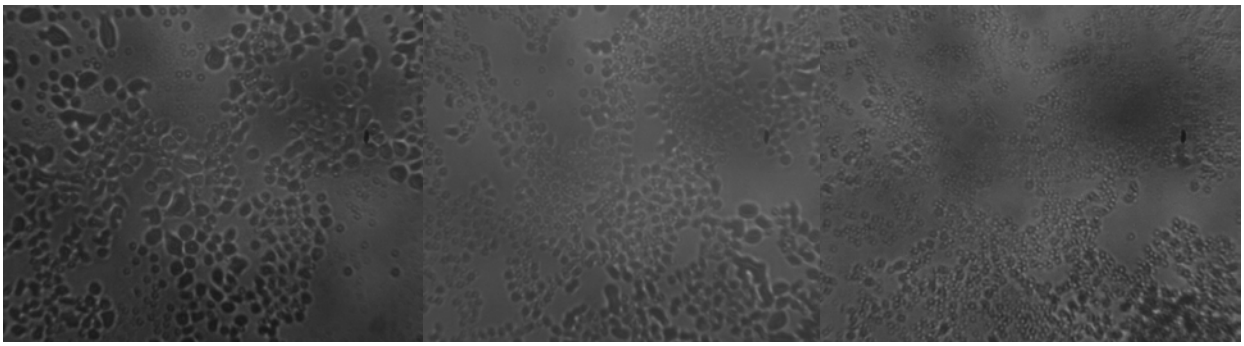
The size and morphology of the magnetic polymersomes were observed by the STEM technique. The images obtained showed that the three synthesized systems (MAC-pLeu+MNP(3:1), MAC-p(Leu-co-CBZ-Lys)$_{1:0.5}$+MNP(3:1) and MAC-p(Leu- co-CBZ-Lys)$_{1:0.75}$+MNP(3:1)) have a spherical shape and present a uniform size distribution. Although it is difficult to directly observe the glycopeptide layer on MNPs core from STEM images, the dispersion behavior in aqueous medium of the GOx-immobilized MNPs has improved after coating.

In regard to their thermostability, all glycopeptides presented below 100^{o}C a weight loss of 3.57–8.03%, which derives from water desorption. The thermal decomposition of MAC-pLeu, MAC-p(Leu-co-CBZ-Lys)$_{1:0.5}$ and MAC-p(Leu-co-CBZ-Lys)$_{1:0.75}$ took place in 3 stages, an increase was observed the gradual increase in thermal stability due to the introduction of CBZ-Lys sequences into the glycopeptide structure.

Therapeutic effect of the glycopeptide-based micro/nanomotors

Micro/nanomotors behaviour in the presence of simulated biological fluids

Propulsion and response to environmental stimuli were tested in buffer solutions with a pH corresponding to the tumor environment (pH 6.5). The autonomous movement of the micro/nanomotor was observed and recorded at room temperature through an optical microscope coupled with specific objectives (100x/160 mm) (see Figure 1).



a b c

Figure 1. Selection of microscopy images recorded for the systems: a) MAC-pLeu+MNP(3:1), b) MAC-p(Leu-co-CBZ-Lys)$_{1:0.5}$+MNP(3:1) and c) MAC -p(Leu-co-CBZ-Lys)$_{1:0.75}$+MNP(3:1).

In vitro experiments of drug loading and release

The controlled release of anticancer drugs was studied by the dialysis method at two pHs that simulate the physiological values of the tumor extracellular microenvironment and normal tissues (pH 6.5 and pH 7.4, respectively).

The release rate of DTIC from the magnetic polymersomes increased as the pH decreased from pH 7.4 to 6.5. It was found that approximately 40.6%, 78.5% and 93.6% of DTIC loaded in polymersomes was released in 24 hours in the simulated tumor microenvironment. It is most likely that the drug-polymersome interactions were reduced by the repulsive electrostatic forces between the positively

charged DTIC molecules and the glycopeptide network. Thus, DTIC molecules can be released from magnetic polymersomes at a controlled rate due to the acidic microenvironment of cancer tissues, acidic intracellular lysosomes or endosomes in cancer cells.

The release process of VBL loaded in the magnetic polymersomes took place for at least 48 hours, and the release rate was faster in the buffer solution of pH 6.5 compared to the phosphate buffer with a pH of 7.4. This can be attributed to the type of core-shell structure, and the presence of the polymer provides more functional groups for a better interaction with the drug molecules. Therefore, results of co-drug release tests of two antitumor drugs showed that magnetic polymersomes based on glycopeptides and MNPs have a release behavior dependent on the environmental pH and composition of the glycopeptide for both drugs.

Biodegradability assessment

The degradation of nanoparticles was studied in the presence of lysozyme, the enzyme that cleaves 1,4-β-glycosidic bonds and depolymerizes the macromolecular chain of chitosan into oligomers. The biodegradation was evaluated at 37 °C by the DLS technique, inside a glass cuvette, to measure in situ the changes that D_h of the magnetic polymersomes undergoes over time.

The size of the nanoparticles initially increases in the first hours as a result of the swelling of the magnetic polymersomes in the buffer solution (glycopeptide-induced sensitivity), then decreases as a result of the start of the degradation process (after 180 min for MAC-pLeu and MAC-p(Leu-b-CBZ - Lys)$_{1:0.5}$ and 14 days for MAC-p(Leu-b-CBZ-Lys)$_{1:0.75}$). From this point, in the case of the MAC-pLeu system, the size of the polymersomes suddenly increases due to the formation of less stable oligomer chains, which tend to aggregate. The degradation of the nanoparticles in each system seems to be faster for those nanoparticles on the surface of the aggregate, which means that the degradation process does not occur at the same speed for all systems. Therefore, the degradation kinetics is probably related to the aggregation phenomenon that occurs between the split polymer chains and which is dependent on the glycopeptide composition.

Toxicity of the components of the new micro/nanomotors on cancerous cells

The results showed that MEP (MAC-p(Leu)+MNP(3:1)), MEP0.5 (MAC-p(Leu- co-CBZ-Lys)$_{1:0.5}$+MNP(3:1)) si MEP0.75 (MAC-p(Leu- co-CBZ-Lys)$_{1:0.75}$+MNP(3:1) does not affect the viability of normal fibroblasts at the tested concentrations. Nanoparticles loaded with DTIC were not cytotoxic for normal fibroblasts at the tested concentrations, but they determined cytotoxic effects on malignant melanoma cells at certain concentrations. Also, NPs loaded with DTIC determined stronger cytotoxic effects on malignant melanoma cells compared to free DTIC at the same concentration.

In vivo anti-tumoral effect of the new micro/nanomotors

In the case of hemocompatibility testing, when red blood cells came into contact with MAC-pLeu, a minor hemolysis occurred (3.21±0.05%), but without significance compared to the negative control group, which indicates that this substance has a good hemocompatibility *in vitro*.

Incubation of the erythrocyte suspension with MEP produced only a weak hemolysis of them (2.83±0.03%), without notable differences compared to the negative control group, an element that suggests its good hemocompatibility *in vitro*.

The measurement of the activity of liver enzymes did not detect significant differences regarding the values of ALT, AST and LDH in the blood in the groups that received MAC-pLeu and MEP, compared to the group with distilled water, 24 hours and 7 days after administration tested substances.
The subcutaneous injection of MAC-pLeu and MEP did not produce substantial variations in the plasma levels of urea and creatinine compared to the control group, at both time points in the experiment.
No statistically significant changes were noted in the activity of serum complement and in the phagocytosis capacity of PMN (figurative elements of blood) from the peripheral blood between the animals in the groups with MAC-pLeu, respectively MEP and the animals that received distilled water at 24 hours and at 7 days. The use of MAC-pLeu and MEP was not accompanied by obvious variations in the serum values of IL-6 and TNFα compared to the control group, at both times of the determinations.
The determination of serum complement fractions did not show significant differences between C3 and C4 values in the animals treated with MAC-pLeu, MEP and the animals that received distilled water after 24 hours and 7 days in the experiment.

Dissemination of the results

Scientific results – Research Articles:

-1 article published in an open access journal: *Alina Gabriela Rusu, Aurica P Chiriac, Loredana Elena Nita, Vera Balan, Alexandru Mihail Serban, Alexandra Croitoriu, Synthesis and Comparative Studies of Glucose Oxidase Immobilized on Fe3O4 Magnetic Nanoparticles Using Different Coupling Agents, Nanomaterials 2022, 14(12), 2445, IF = 5.719.*

Scientific results – International Conferences:

-1 participation at international conferences with poster presentations: *Synthesis and characterization of dacarbazine loaded chitosan polymersomes, Alina Gabriela Rusu, Aurica P. Chiriac, Loredana Elena Nita, Vera Balan, Polymers 2022 - New Trends in Polymers Science: Health of the Planet, Health of the People", Torino, Italia, 24-27 May 2022 (poster).*