Loading efficiency of doxorubicin into the micelle-like structures formed by function-spacer-lipid constructs self-assembly depends on constructs’ functional part

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Abstract. Supramolecular self-assemble systems based on neoglycolipids: Galili-Ad-CMG2-Ad-DOPE, A(type2)-Ad-CMG2-Ad-DOPE are studied here and compared with the well-studied Biotin-CMG2-Ad-DOPE, as well as with their combinations with NH2-CMG2-Ad-DOPE. They are function-spacer-lipid constructs with unique structure that allows them to form micelle-like supramers and be stable, what makes them a potential drug nanocarriers. The structural properties of the obtained supramolecular systems are studied depending on their functional part, and the loading efficiency of doxorubicin into the supramers is determined to reveal the influence of the functional part. The resulting supramers were separated from the unbound molecules by dialysis, the nanoparticles morphology were studied by atomic force microscopy, and the loading efficiency was calculated based on spectrophotometry data. The encapsulation of doxorubicin was confirmed based on changes in the size and shape of the supramers, as well as a decrease in the ratio of unbound molecules. According to the loading efficiency calculations, it was estimated that supramers formed by A(type2)-Ad-CMG2-Ad-DOPE are the most efficient nanocarriers with loading efficiency of 82 %. Supramers formed by NH2-CMG2-Ad-DOPE (no functional part) showed 1.5 times less efficiency. Finally, the least efficient carriers are supramers formed by Biotin-CMG2-Ad-DOPE (14%).

1. Introduction
Due to the non-selective action of antibiotics on healthy cells, side effects occur, which causes great harm to the body, destroying healthy cells. The properties of the tumor tissue are significantly different from the healthy one, due to this, it is possible to use point-to-point drug delivery. Targeted delivery allows to:
1) make a hit on cancer cells point, without hitting healthy ones, which will avoid many side effects;
2) reduce the dose of drugs that are toxic not only to the patient, but also to the environment [1].
Most often, liposomes, polyplexes, nanoparticles, dendrimers, magnetic nanoparticles, and graphene polymer nanoparticles are used as targeted delivery agents. Now they are actively developing multifunctional nanoplatforms for drug delivery based on various biocompatible nanomaterials with amphiphilic properties. According to data for 2017, the largest share (56%) of nanoplatforms approved...
for therapy are liposomes with various surface modifications. However, despite all the advantages of liposomes, they have the disadvantages of instability and low efficiency (<1% of the encapsulated objects achieve their goals). Which makes it necessary to continue the research [2].

In this paper, we study the properties of nanoparticles that self-assemble from neoglycolipids into micelle-like structures consisting of a hydrophobic "core" and a hydrophilic "shell". Neoglycolipids are synthetic analogues of glycolipids, which are biocompatible multivalent compounds consisting of several functional parts that provide unique surface properties and are very easy to create. They have a low critical aggregation concentration, which makes them stable in the biological environment. The critical aggregation concentration in water for Biot-CMG-DOPE is 50 µM [3,4,5,6].

Supramolecular self-assemble systems formed by the following neoglycolipids were studied: Galili-Ad-CMG2-Ad-DOPE, A(type2)-Ad-CMG2-Ad-DOPE, as well as their combinations with NH2-CMG2-Ad-DOPE, since the loading efficiency may depend on the functional part. They were compared with the well-studied function-spacer-lipid (FSL) construct Biotin-CMG2-Ad-DOPE. Steric hindrance, hydrogen bonds, and electrostatic interactions are factors that influence the loading efficiency and vary in different functional parts. Since NH2-CMG2-Ad-DOPE construct has no functional part, its addition to supramers allows to understand whether the presence of the functional "head" in these constructs increase loading efficiency. The absence of a functional part, on one hand, excludes steric hindrance, which results in formation of supramers with a larger aggregation number, but on the other hand, it does not give additional hydrogen bonds that can bind doxorubicin. By varying these constructs, it is possible to understand what effect gives a major contribution to the drug loading efficiency. Chemical structures of constructs under study are shown in the Figure 1.

For efficient drug delivery to the target cell, such nanoparticles must, first, retain their original surface properties after exposure to the biological environment, second, provide sufficient circulation time to enter the target area, and third, provide a relatively long-term binding to the target cell to perform its function [7,8].

The development of such nanotechnology platforms will significantly improve the efficiency of the diagnosis and treatment of tumor diseases, and will also be a step towards personalized medicine.
2. Experimental section
Doxorubicin solutions with concentrations of 5, 10, 20, 40, 60, 80 and 100 µg/ml were prepared to obtain the calibration curve. The absorbance was measured by spectrophotometry, and then the masses were calculated according to the Bouguer-Beer-Lambert law:

\[ I = I_0 e^{-kd} \quad (1) \]

The sample solutions (2 ml, 100 µM of Doxorubicin and each construct) in the bidistillate water were treated with ultrasound for 15 seconds, and after 10 minutes, the measurements were carried out at room temperature.

Spectrophotometry (UV/ VIS SPECTROMETER T90+ PG Instruments limited) was used to obtain calibration curve and to obtain mass of encapsulated drug in order to calculate encapsulation efficiency. The samples were measured at room temperature. The wavelength interval from 400 to 550 nm was taken. The peak of doxorubicin was at a wavelength of 500 nm. The results are presented in the Figure 3.

![Calibration curve of doxorubicin](image1)

**Figure 2.** Calibration curve of doxorubicin

![Absorbance spectra of doxorubicin](image2)

**Figure 3.** Absorbance spectra of doxorubicin (left) before dialysis and (right) after dialysis.
The loading efficiency was calculated using the following equation [9-13]:

$$ EE = \frac{\text{mass of drug encapsulated}}{\text{mass of drug added}} \times 100\% \quad (2) $$

Dialysis was performed in a dialysis bag with a 10k MWCO for 4 hours with a change of solvent (Milli-Q water) every 30 minutes. For AFM imaging of the Biotin-CMG2-Ad-DOPE supramers, the sample was diluted to a suitable concentration (100 µM). The sample drop (10 µl) was applied to a freshly cleaved mica surface. After 3 minutes of incubation at room temperature, mica was rinsed to remove the unbound material and then dried in a desiccator for 24 hours. After that, AFM measurements were performed (SFC113LNMF atomic force microscope (NT-MDT), TIPSANO HA_HR ETALON cantilever). AFM images were obtained at 25°C and 35% relative humidity.

3. Results and discussion

3.1. Evaluation of supramer with highest encapsulation efficiency

Table 1 shows obtained data. Based on it, the highest loading efficiency is shown by supramers formed by A(type2)-Ad-CMG2-Ad-DOPE: 82%, and the lowest is shown by supramers formed by Biotin-CMG2-Ad-DOPE - 14%. It is worth noting that after the addition of neoglycolipids, the absorbance readings of doxorubicin significantly decreased.

| Supramer-forming construct mixed with doxorubicin | Absorbance before dialysis | Absorbance after dialysis | Drug mass before dialysis, µg | Drug mass after dialysis, µg | Encapsulation efficiency, % |
|-----------------------------------------------|-----------------------------|---------------------------|-------------------------------|-----------------------------|----------------------------|
| Galili-Ad-DOPE                               | 0.199                       | 0.166                     | 11.602                        | 7.2                         | 62                         |
| A(type2)-Ad-CMG2-Ad-DOPE                      | 0.343                       | 0.302                     | 30.8                          | 25.12                       | 82                         |
| Biotin-CMG2-Ad-DOPE                          | 0.547                       | 0.172                     | 58                            | 8                           | 14                         |
| Galili-Ad-CMG2-Ad-DOPE (50%) – NH2-CMG2-Ad-DOPE (50%) | 0.310                       | 0.207                     | 26.4                          | 12.66                       | 48                         |
| A(type2)-Ad-CMG2-Ad-DOPE (50%) – NH2-CMG2-Ad-DOPE (50%) | 0.221                       | 0.171                     | 14.536                        | 7.87                        | 54                         |
3.2. Accounting for changes in pumping intensity.

Atomic force microscopy (AFM) was used to estimate the shape and size distribution of Biotin-CMG\(_2\)-Ad-DOPE supramers. It is known that in AFM, due to the large radius of curvature of the tip of the cantilever probe, there is an expansion in the lateral size. For this reason, the AFM was used only to estimate the overall shape and measure heights, ignoring data on lateral sizes.

Before the addition of doxorubicin, most particles had heights of \(9\pm1\) nm, which is close to the size of the supramers, and after the addition, they increased to \(14\pm1\) nm.

![Figure 4](image)

**Figure 4.** (a) AFM-image of Biotin-CMG\(_2\)-Ad-DOPE after dialysis (b) cross sections of two supramers marked at (a) with height \(9\pm1\) nm.

![Figure 5](image)

**Figure 5.** (a) AFM-image of DOX+ Biotin-CMG\(_2\)-Ad-DOPE after dialysis (b) cross sections of two micelles marked at (a) with height \(14\pm1\) nm.
3.3. Results
After loading doxorubicin and dialysis, the micelle size increased from 9±1 nm to 14±1 nm, and its concentration decreased, which is confirmed by the change in light absorbance. This indicates the encapsulation of the drug. According to the spectrophotometry results, supramers formed by A(type2)-Ad-CMG2-Ad-DOPE showed highest encapsulation efficiency of 82%. Supramers formed by Biotin-CMG2-Ad-DOPE was found to have the lowest efficiency of doxorubicin uptake (14%), which may be explained by the fact that biotin possess hydrophobic properties, which creates steric hindrance and prevents drug from encapsulation.

The loading efficiency of supramers formed by constructs that have hydrophilic functional part was almost one and a half times higher than that of supramers formed by NH2-CMG2-Ad-DOPE, which lacks functional part. This means that the presence of the hydrophilic part promotes the encapsulation of doxorubicin.

4. Conclusion
In this work, we estimated drug encapsulation efficiency of supramers formed by promising constructs Galili-Ad-CMG2-Ad-DOPE, A(type2)-Ad-CMG2-Ad-DOPE, Biotin-CMG2-Ad-DOPE, as well as their combinations with NH2-CMG2-Ad-DOPE. Atomic force microscopy confirmed changes in the shapes and sizes of the supramers, and the calculation of the loading efficiency by the spectrophotometry which molecular structure gives highest encapsulation efficiency.

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