

SPECIFICATION

Method for Loading Exosomes with Small Non-Coding RNA

The invention relates to molecular biology, in particular to methods for loading (filling) exosomes with small non-coding RNA, so as to enable their subsequent targeted delivery to individual tissues of living organisms.

Known is a method for loading exosomes with anti-cyanidins [Patent WO2014134132 A1 Milk-derived microvesicle compositions and related methods], where exosomes are obtained from cow's milk, further processed with microRNA, as a result of which exosomes can be used as microRNA carriers from the perspective of their use in treating various illnesses. This method has the following disadvantages: 1) exosomes from cow's milk are used in the known method, which makes it impossible to achieve a highly effective delivery to individual tissues of humans. Due to differences in protein sequences, cow exosomes may be perceived by the human organism as foreign, and not penetrate into several organs and tissues effectively enough in comparison to human exosomes. 2) the known method provides no data about the maximum effectiveness of loading microRNA molecules, and given the potential for its continued therapeutic use and rather high cost of synthesizing these molecules, an effective load may be a significant factor for the practical application of microRNA loaded in exosomes.

The technical effect achieved through realizing the claimed method is the possibility of maximally loading exosomes obtained from the biological fluids of a human with small non-coding microRNA, which subsequently makes it possible to use the loaded exosomes as carrier constructs for delivering therapeutic agents into the tumor cells of living organisms.

The aforementioned technical result is achieved by mixing exosomes obtained from the urine or peripheral venous blood of a human and a small non-coding RNA into a phosphate-buffered saline (PBS) in a ratio per 1 ml of solution of $1 \cdot 10^{10}$ to $5 \cdot 10^{10}$ exosomes and $10 \cdot 10^{12}$ to $50 \cdot 10^{12}$ copies of small non-coding RNA; the obtained mixture is then placed in a thermostat at 35-40°C for 14-18 hours.

After incubating the substances, an ultracentrifugation stage is required in order to separate the exosomes with the loaded substance, which form deposits on the bottom of the test tube, from the loaded free substance, which remains in solution in the ultracentrifugation process (supernatant).

Ultracentrifugation preferably takes place at 100000 g for 2 hours, which ensures a maximum deposition of the loaded exosomes contained in the solution.

Example 1

The exosomes preliminarily obtained by method of gradient ultracentrifugation on saccharose from a 100 ml volume of human urine were mixed in a quantity of $10 \cdot 10^{10}$ and $80 \cdot 10^{12}$ copies of small non-coding RNA hsa-mir-34a in 8 ml of PBS. The obtained mixture was divided into 8 samples of 1 ml each: the first sample was placed in a thermostat at 40°C for 10 hours, the second sample was placed in a thermostat at 40°C for 12 hours, the third sample was placed in a thermostat at 40°C for 14 hours, the fourth sample was placed in a thermostat at 40°C for 16 hours, the fifth sample was placed in a thermostat at 40°C for 18 hours, the sixth sample was placed in a thermostat at 40°C for 20 hours, the seventh sample was placed in a thermostat at 40°C for 22 hours, and the eighth sample was placed in a thermostat at 40°C for 24 hours. Upon expiration of the corresponding incubation period, the samples were subjected to ultracentrifugation at 100000 g for 2 hours, after which the obtained deposit was

divided into 1 ml PBS, 3 freezing-defrosting cycles were performed in order to destroy the exosomes and obtain a free molecule marker (small non-coding RNA hsa-mir-34a) with the subsequent PCR staging in real time. As a result, the concentration of small non-coding RNA hsa-mir-34a was the highest in the third, fourth and fifth samples. As clearly evident from table 1, the concentration of loaded microRNA smoothly rises, reaching maximum values in the third, fourth and fifth samples, and then smoothly tapers, which shows that the most effective incubation period lies within an interval of 14-18 hours.

Table 1: Influence of incubation time on the effectiveness of loading microRNA into exosomes

Sample and incubation time	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8
Concentration of small non-coding RNA hsa-mir-34a (copies/ml)	1.89 *10 ¹²	2.12 *10 ¹²	2.84 *10 ¹²	2.97 *10 ¹²	2.87 *10 ¹²	2.51 *10 ¹²	2.38 *10 ¹²	2.05 *10 ¹²

Example 2

The exosomes preliminarily obtained by method of gradient ultracentrifugation on saccharose from a 100 ml volume of human urine were mixed in a quantity of $2 \cdot 10^9$ and $10 \cdot 10^{12}$ copies of small non-coding RNA hsa-mir-122 in 1 ml of PBS (Sample No. 1). Also obtained were samples with an exosome concentration of $1 \cdot 10^{10}$ (Sample No. 2), an exosome concentration of $5 \cdot 10^{10}$ (Sample No. 3), and an exosome concentration of $25 \cdot 10^{10}$ (Sample No. 4). The obtained samples were placed in a thermostat at 40°C for 16 hours. Upon expiration of the corresponding incubation period, the

samples were subjected to ultracentrifugation at 100000 g for 2 hours, after which the obtained deposit was divided into 1 ml PBS, 3 freezing-defrosting cycles were performed in order to destroy the exosomes and obtain a free molecule marker (small non-coding RNA hsa-mir-122) with the subsequent PCR staging in real time. As a result, the concentration of small non-coding RNA hsa-mir-122 was the highest in the second and third samples. As clearly evident from table 2, the concentration of loaded microRNA smoothly rises, reaching a maximum in the second and third samples, and then smoothly tapers, which shows that the optimal concentration of exosomes lies within an interval of $1 \cdot 10^{10}/\text{ml}$ to $5 \cdot 10^{10}/\text{ml}$.

Table 2: Influence of exosome concentration on the effectiveness of loading microRNA

Sample and exosome concentration	No. 1 $2 \cdot 10^9/\text{ml}$	No. 2 $1 \cdot 10^{10}/\text{ml}$	No. 3 $5 \cdot 10^{10}/\text{ml}$	No. 4 $25 \cdot 10^{10}/\text{ml}$
Concentration of small non-coding RNA hsa-mir-122 (copies/ml)	1.37 $\cdot 10^{12}$	2.79 $\cdot 10^{12}$	2.97 $\cdot 10^{12}$	2.24 $\cdot 10^{12}$

Example 3

The exosomes preliminarily obtained by method of gradient ultracentrifugation on saccharose from a 100 ml volume of human urine were mixed in a quantity of $30 \cdot 10^{10}$ and $60 \cdot 10^{12}$ copies of small non-coding RNA hsa-mir-16 in 6 ml of PBS. The obtained mixture was divided into 6 samples of 1 ml each: the first sample was placed in a thermostat at 25°C for 16 hours, the second sample was placed in a thermostat at 30°C

for 16 hours, the third sample was placed in a thermostat at 35°C for 16 hours, the fourth sample was placed in a thermostat at 40°C for 16 hours, the fifth sample was placed in a thermostat at 45°C for 16 hours, and the sixth sample was placed in a thermostat at 50°C for 16 hours. Upon expiration of the corresponding incubation period, the samples were subjected to ultracentrifugation at 100000 g for 2 hours, after which the obtained deposit was divided into 1 ml PBS, 3 freezing-defrosting cycles were performed in order to destroy the exosomes and obtain a free molecule marker (small non-coding RNA hsa-mir-16) with the subsequent PCR staging in real time. As a result, the concentration of small non-coding RNA hsa-mir-16 was the highest in the third and fourth samples. As clearly evident from table 3, the concentration of loaded microRNA smoothly rises, reaching a maximum in the third and fourth samples, and then smoothly tapers, which shows that the most effective incubation temperature lies within an interval of 35-40°C.

Table 3: Influence of incubation temperature on the effectiveness of loading microRNA into exosomes

Sample and incubation time	No. 1 25°C	No. 2 30°C	No. 3 35°C	No. 4 40°C	No. 5 45°C	No. 6 50°C
Concentration of small non-coding RNA hsa-mir-16 (copies/ml)	0.78 *10 ¹²	1.88 *10 ¹²	2.79 *10 ¹²	2.84 *10 ¹²	2.11 *10 ¹²	1.91 *10 ¹²

Example 4

The exosomes preliminarily obtained by method of gradient ultracentrifugation on saccharose from a 100 ml volume of human urine were mixed in a quantity of 5*10¹⁰ and 1*10¹² copies of small non-coding RNA hsa-mir-122 in 1 ml of PBS

(Sample No. 1). Also obtained were samples with a small non-coding RNA concentration of 10×10^{12} (Sample No. 2), a small non-coding RNA concentration of 50×10^{10} (Sample No. 3), and a small non-coding RNA concentration of 100×10^{12} (Sample No. 4). The obtained samples were placed in a thermostat at 40°C for 16 hours. Upon expiration of the corresponding incubation period, the samples were subjected to ultracentrifugation at 100000 g for 2 hours, after which the obtained deposit was divided into 1 ml PBS, 3 freezing-defrosting cycles were performed in order to destroy the exosomes and obtain a free molecule marker (small non-coding RNA hsa-mir-122) with the subsequent PCR staging in real time. As a result, the percentage of small non-coding RNA hsa-mir-122 loaded into the exosomes was the highest in the second and third samples. As clearly evident from table 1, the percentage of loaded microRNA smoothly rises, reaching a maximum in the second and third samples, and then smoothly tapers, which shows that the optimal concentration of small non-coding RNA lies within an interval of $10 \times 10^{12}/\text{ml}$ to $50 \times 10^{12}/\text{ml}$.

Table 4: Influence of small non-coding microRNA concentration on loading effectiveness

Sample and small non-coding RNA concentration	No. 1	No. 2	No. 3	No. 4
	$1 \times 10^{12}/\text{ml}$	$10 \times 10^{12}/\text{ml}$	$50 \times 10^{12}/\text{ml}$	$100 \times 10^{12}/\text{ml}$
% of small non-coding RNA hsa-mir-122 loaded into exosomes	25%	28%	30%	16%

Let it be noted that use can be made of any type of small non-coding RNA, with any nucleotide sequence, without influencing the loading quality, and that a specific microRNA is selected only based upon the set tasks.

CLAIM

1. A method for loading exosomes with small non-coding RNA, in which exosomes obtained from the urine or peripheral venous blood of a human and a small non-coding RNA are mixed into a phosphate-buffered saline (PBS) in a ratio per 1 ml of solution of $1 \cdot 10^{10}$ to $5 \cdot 10^{10}$ exosomes and $10 \cdot 10^{12}$ to $50 \cdot 10^{12}$ copies of small non-coding RNA; the obtained mixture is then placed in a thermostat at 35–40°C for 14–18 hours.