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The Development of New Technologies for Medicine and Agroecology by Using Spherosomes

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***Abstract*—**Article devoted to the development of technologies for medicine and agroecology by using plant organelle – spherosome. Technological method of purification and isolation of this organelle by using novel nanostructured carbon sorbent – "nanocarbosorb" ARK type are presented. Also the methods of preparation of nanocontainers based on using of spherosome with loaded isosorbide dinitrate, piroxicam or diclofenak are exhibited. We found that the spherosome could be applied for ecological aims as bioregulator and also as biosensor for determination of ammonia ions in water reservoirs at concentration range 1mM to 100mM.

***Keywords*—**Biosensor, nanocontainer, phosphatidylinositol, spherosome, vegetative reproduction.

I. Introduction

SCIENTIFIC discoveries open way for development of innovation technologies. We would like to present how our scientific investigation lead to the development of innovation technologies. In our Laboratory of enzyme structure and regulation of Aytkhozhin's Institute of molecular biology and biochemistry of the Republic of Kazakhstan structure and functions of subcellular organelle – spherosome was established. The spherosome consist of only one phospholipid – phosphatidylinositol (PI) and only one protein – glutamate dehydrogenase (GDh) [1]. While GDh may be released from spherosome only by PI specific phospholipase C there is only one opportunity to explain this phenomenon that GDh is attached to inner bilayer PI vesicle membrane by its own covalently bound glycosylphosphatidylinositol anchor which is anchored to PI membrane by mechanism which is described by Low [2]. Thus the GDh is attached to PI membrane by its own covalently bound PI anchor. Numerous molecules of GDh form the dense protein covering of the spherosome as illustrated in Fig. 1.

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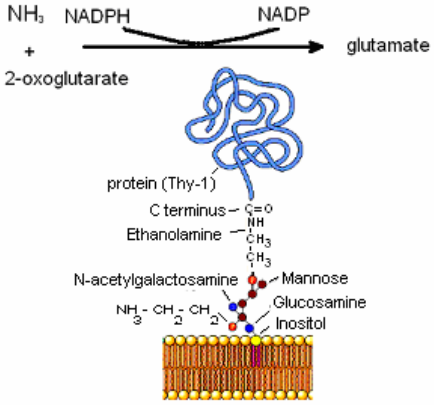


Fig. 1 The scheme of attachment of GPI protein to the PI membrane. GDh is attached to PI membrane of nanocomplex by its own covalently bound glycosylphosphatidylinositol anchor (Moran et al., 1991; Fergusson, 1999)

The molecules of GDh of the spherosome are in latent inactive state. From the structure of spherosome it may be concluded that spherosome have the next function – storage of PI and GDh in the grains.

II. Results and Discussions

A. Method of Isolation of PI

The unique structure of spherosome allows to develop the new technology. Since spherosomes contain only one type of phospholipid – PI, it is very convenient to develop the method of isolation of chromatographically pure preparation of PI. This method is protected by US patent № 4,977,091 [3]. For isolation of PI by our method it is necessary to isolate spherosomes from dry wheat seeds. First of all it is necessary to cut off the embryionic part of wheat seeds. Inembryonated seeds were milled on laboratory mill. Using sieve we took the bran from the milled flour. The fraction of bran was homogenized in porcelain mortar in 0,05 M tris-chloride buffer pH=7,4. The homogenate was centrifuged at 10000xg during 10 minutes. The supernatant was used for preparation of spherosomes.

For purification of spherosomes from supernatant we used the nanostructured carbon sorbent which was developed by supervisor professor Z.A. Mansurov in Combustion problem Institute of Al-Farabi Kazakh National University [4]. This carbon sorbent contains carbon nanostructured elements

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which provide rigid carcass of the sorbent. Using this sorbent allows us to purify spherosome which is 2-3 times faster than by gel chromatography on Sepharose. Spherosome was purified by chromatography on column with "Nanocarbosorb" ARK type which is produced by scientific-industrial technological center "Zhalyn" (Almaty, Kazakhstan). Results of purification of spherosomes are presented in Fig. 2.

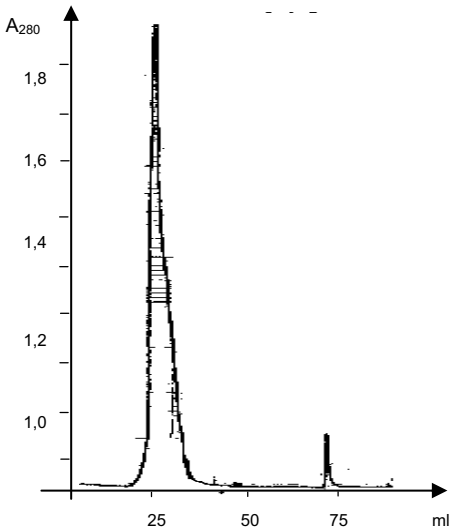


Fig. 2 Separation of cell-free extracts obtained from filling seeds of wheat. Column size is ø3cm*20. Column was filled with "Nanocarbosorb" ARK type and washed with 0,05M MES buffer, pH~7.4

The fraction of spherosomes was eluted in the first peak after chromatography on column with "Nanocarbosorb" ARK type.

For isolation of PI it was necessary to extract the fraction of spherosomes by Folch [5] reagent which consist of mixture of chloroform-methanol ratio 3:1. The extracted PI was analyzed by thin layer chromatography on two solvent systems: 1) chloroform:methanol:ammonia (35:60:5); 2) chloroform:methanol:acetic acid:water (50:25:8:4) [6]. Our method allows to obtain the homogenious PI. Our method allow to prepare PI 10 times cheaper than traditional methods of isolation of PI. Because of high price of PI preparations (price by Sigma catalogue 2006-2007, catalogue number PO639-50MG, the price per 50 mg is equal 641$) its application is very difficult. Our method open wide application of PI. First of all we tried to use our PI for preparation of PI liposomes.

B. Method of Preparation of PI Nanocontainers

The problem of delivery of medicine to diseased organ has not found satisfactory solution. The existing delivery systems have some serious disadvantages. They are not stable, easily agregate and cause danger of blocking of blood vessels and others are made from chemical substances that cause immune and allergic reaction. In this reason one of the main problem of modern medicine is to develop new generation of medicine delivering systems. In this reason we put task to make delivery systems which are made from PI. In contrast to electro neutral lecithin PI is related to negative charged phospholipid. The PI liposomes have size near 1 μm that is 10 times less than lecithin liposomes. To this reason PI liposomes can be named as nanocontainers. PI liposomes have negative charge and they push up each other and never agregate. Due to the charge the PI liposomes have more hydrophilic properties and are very stable.

To prepare PI nanocontainers dried PI was desolved in bidistilled water. Then PI solution was transfered by syringe to to the solution of 0,05M tris-chloride buffer pH=7,4 with 0,09% NaCl. After that the solution was treated ultrasonic dispergation on Ultrasonic desintegrator UD-11 type (Techpan, Poland) during 5 minutes on medium current strength. Ready nanocontainers were used for further work.

We used the property of PI nanocontainers to open in the hydrophobic solution as it is shown in Fig. 3.

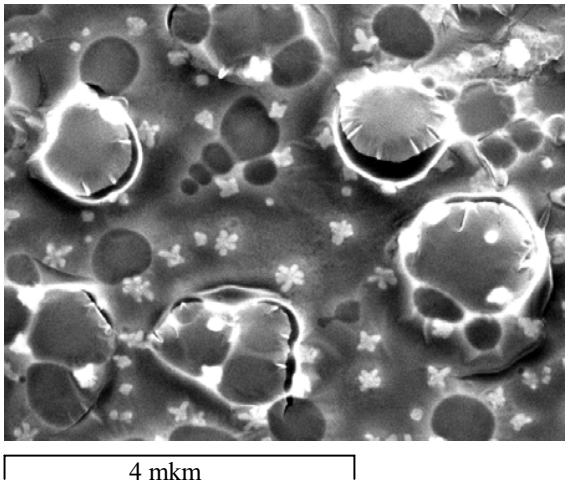


Fig. 3 Electron microscopy of opening of the nanocontainer after transfering to hydrophic solution (95% ethanol)

And after transfering to hydrophilic solution the liposomes captivate the medicine and then they close. On the basis of the principle we developed the new effective method of loading of nanocontainer [7] which is protected by the patent of the Republic of Kazakhstan.

Nanocontainers with loaded medicine were tested in Institute of cardiology and internal deseases of Ministry of health protection of the Republic of Kazakhstan under supervision of professor Ablayuly and doctor Mansharipova. Our nanocontainers were loaded with "Isocete" - pharmaceutical names of isosorbide dinitrate which is for blocking of heart attack. Mechanism of action of "Isocete" is the same as nitroglycerol. Ointment which contains "Isocete" loaded nanocontainers was prepared. The tests were carried out on rats. The artificially caused heart attack was stopped by applying it on the skin on breasts of rats after ten minutes. Clinical tests of "Isocete" loaded nanocontainers is carried out in this institute. It is well known that the medicine related to this group habe the property to prevent heart and blood vessel spasm.

We also prepared the new anti arthritic drugs on basis PI nanocontainers. We loaded PI nanocontainers by nonsteroid anti-inflammatory medicines – piroxicam or diclofenak.

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The anti-inflammatory preparation was tested in reumatological centre of Almaty on experimental and control groups of arthritic patients under supervision of professor Seisenbayev A.S.. Each group consists of thirty persons comparable by sex, age, stage and activity of diseases processes. The patients of experimental group were applied with the ointment containing our loaded PI nanocontainers. The patients of control group were applied with the standard anti-inflammatory ointment of piroxicam or diclofenak.

The tested preparation was rendered 2-3 times day on sick joints. After 3 weeks essential reduction of a local painful syndrome was marked by 76.6% of patients of experimental group and 50 % - in control group.

Thus the obtained our anti-inflammatory preparation on basis on PI nanocontainers shows the better treatment results than standard drug. Our preparation has the next advantages in comparison with standard drug:

- the quantity of piroxicam or diclofenak reduces more than ten times than standard drug under the same or better therapeutic effect;
- more quicker and more deeper penetration of medicine into the tissues of joints and more longer prolongation of the action of the medicines;
-all this led to significant reduction of toxic effects of medicines containing in our PI nanocontainers.

Also we prepared the nanocontainers ,loaded by medicine for treatment of eye disease in the Institute of eye diseases (Almaty, Kazakhstan) under supervision of Dzhumatayeva Z.A. The tests showed that our nanocontainers loaded by vasoprostan – one of prostaglandins as vasodilator show good therapeutical effect for treatment of glaucoma.

The next tests were carried out in of Facial-mandible clinique under supervision of Doctor Myrzakulova U.R. Ointment with vasoprostan was used for treatment of saliva glands inflammatory processes. After four days of the ointment application a good therapeutical effect was achieved. Another advantages of vasoprostan are that it is used efficiently which makes treatment course cheaper, it has not irritating effect on alimentary canal, it is painlessness and more precise addressing.

Thus we developed the new generation of drug delivery systems namely PI nanocontainers. These nanocontainers have obvious advantages on contrast to other drug delivery systems: they do not block blood vessels, have no toxic effect, have a good therapeutic effect and medicine action is prolonged. Using of nanocontainers allow to make effective treatment without toxic damaging effect on other organs.

The second aspect of application of spherosomes is its application as bioregulator. As it was already mentioned spherosomes contain numerous GDh molecules. As known previously GDh is calcium bounded protein. It is well known that calcium binding proteins often are effective bioregulators.

We tested the stimulatory effects of calcium binded spherosomes. The treatment of cutsteams of *Hippophae rhaminoides L.* by 0.01% solution of Ca2+ bounded spherosome causes the intensive formation of roots, whereas the treatment of the cutsteams by solution of spherosome without Ca2+ has not any effect as it shown from Fig. 4.

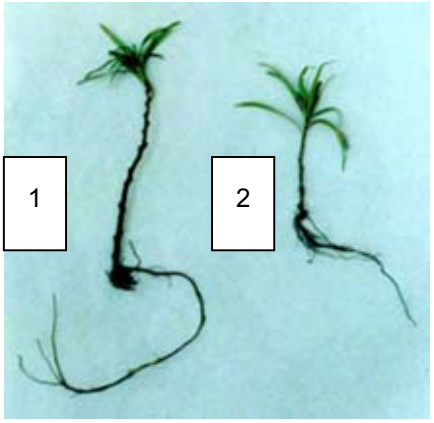


Fig. 4 Effect of 0.01% Ca2+ bounded bioregulator solution on morphogenic effects on sea-buckthorn. [latin - Hippophae rhamnoides L.]. 1 – experimental stem; 2 – control stem

The treatment by Ca2+-spherosome of callus culture from *Triticum aestivum* caused the formation of primary roots and steam (Fig. 5).

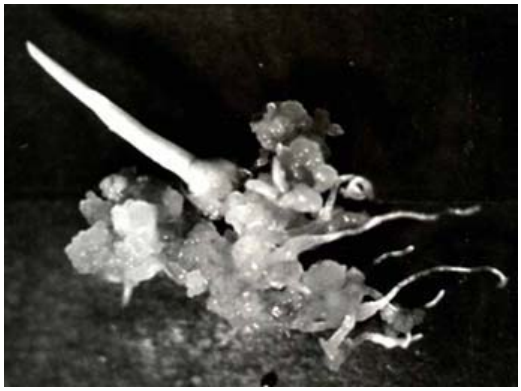


Fig. 5 Effect of Ca2+-spherosome on formation of primary roots and coleoptiles of wheat callus culture (*Triticum aestivum*)

Thus we established that spherosomes as whole organelle show high biostimulatory effects which may be very usefull for vegetative reproduction of tree plants and for wide application in cell cutures engineering [8]. For the first time it was shown that biostimulator is not bioorganic substance but protein-lipid complex – spherosome.

We found the third aspect of spherosome application for ecological aims.

It is well known that anthropogenic pollution by waste waters causes increase in the contents of ammonia ions in natural water reservoirs – lakes and rivers, whereas in unpolluted reservoirs ions of ammonia are absolutely absent [9]. Thus the presence of ammonia ions is the evidence of pollution of water reservoirs. Therefore highly sensitive and accurate method for the determination of ammonia ions concentration is necessary for ecological monitoring of natural waters. Existing methods have low sensitivity and are not precise. They allow determining the ammonia ions only in concentration range from 1mM to 100mM in the water. Therefore for earlier prevention of water pollution there is

high necessity for the development of new biosensor with high sensitivity to ammonia.

Using chromatography on nanostructured carbon sorbent we had isolated spherosome from filling grains of wheat and maize. It was very surprising that this spherosome from filling cereal grains shows activity of Nicotinamide adenine dinucleotide phosphate-GDh (NADP-GDh) without any treatment. Thus the whole body of spherosome shows its activity without disturbing its integrity. This makes the spherosomes from filling cereal grains very convenient for using it as a biosensor. The main feature of nanocomplex is its high sensitivity to ammonia ions. Linear response concentration for spherosomes from filling cereal grains is from 0.5μM to 10μM ammonia ions. Due to these properties the spherosomes may be very useful as nanobiosensor for ecological monitoring of pollution by sewer waters of natural reservoirs – lakes and rivers. Also this nanosensor can be applied for determination of ammonia ions, NADPH and 2-oxoglutarate in biological liquids for clinical diagnostic [10].

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