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Details of Laboratory Work for Medical Students

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Abstract

In the modern world of pandemics, a special place is occupied by the problems of education in the field of medicine. In developed countries, medical education remains combined. Practical training of students in the laboratories of the university is very important. The article describes a biochemistry laboratory class protocol.

This protocol is described in order to create an opportunity for students to apply by doing the theoretical concepts underlying biomolecules and vesicles properties, together with the principles of centrifugation and colorimetric methodologies. The aims, objectives, methodology of teaching/learning, and assessment for this laboratory class are indicated. The proposed protocol for a laboratory class described us creates the opportunity for undergraduate students to perform experiments, to reason, and to discuss some related biochemical concepts, namely protein characterization and properties of specific staining reactions; enzyme quantification by enzymatic reaction; composition and biochemical properties of exovesicles; amphiphilic biomolecules properties; and principles and applications of centrifugation methods. This work was supported by Portuguese Ministry of Science and Higher Education (MCES).

KEY WORDS: medical teaching, medical learning, laboratory task

Introduction

Membrane cell exovesiculation is a physiological process that occurs in several situations, including apoptosis, erythrocyte aging, and storage of blood samples [1]. Different types of endogenous cellular membrane stimulus promote exovesiculation [2,3,4]. It was verified in vitro that changes in either pH or ATP, as well as the presence in incubation medium of amphiphilic compounds, induce the release of exovesicles from erythrocytes [5,6,7]. In these experimental conditions, the presence of the enzyme acetylcholinesterase (AChE) 11 is considered as a marker for the exovesiculation process [8,9].

We have proposed a method to simultaneously induce the release of the erythrocyte exovesicles, enriched in AChE and cholesterol, and label them with the fluorescent membrane probes used to induce the vesiculation: 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), or 4-heptadecyl-7-hydroxycoumarin (C17-HC) [9]. The exovesicles obtained by this method are not visible in the buffer suspensions. In the present work, we found that it is possible to visualize the exovesicle aggregates without an expensive apparatus, by the coloration of either: a) the exovesicle membrane proteins, using Coomassie blue [10]; b) the specific presence of AChE, using an adaptation of the Ellman's reagent enzyme assay [11]; or c) the phospholipids content, by phospholipase D digestion followed by phosphatidic acid coloration [12]. The importance of these coloration processes is strengthened by the fact that the most valuable method to physically evaluate the presence of the exovesicles (together with their size and shape) is light scattering spectroscopy [9], which requires an equipment not readily available in most of institutions.

The aim of the present work is to describe a biochemistry laboratory class protocol to visualize erythrocyte exovesicles, by colorimetric assays focused on their different components, based on the principles and application of centrifugation and colorimetric methods especially appropriate for undergraduate students. This is an original method that complements our recent research works [9]. Working in an innovative area of research proves stimulating for the students and encourages them to develop new solutions for practical problems.

Good teacher training is essential for quality student learning. The teacher must know all the nuances of laboratory research. For this the teacher (instuction) should have special preparation.

Previous experimental preparation. Reagents.

The fluorescent probes DPH, TMA-DPH, and C17-HC were purchased from Molecular Probes (Eugene, OR). Acetylthiocholine iodide (ASCh), 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB, Ellman's reagent), 2,4-diaminophenol, and tetrahydrofuran were obtained from Sigma-Aldrich (St. Louis, MO). Coomassie brilliant blue R 250, dibutyl phtalate, dimethyl phtalate, acetone, N,N-dimethylformamide, ammonium molybdate, sodium disulfite, NaH_2PO_4, and Na_2HPO_4 were obtained from Merck (Darmstadt, Germany). The enzyme mixture used for the phospholipid's identification (phospholipase D 400 U/liter, choline oxidase 2,200 U/liter, peroxidase 3,600 U/liter, 4-aminophenazone 0.24 mM, and diclorophenol 2.1 mM, in Tris-buffer, pH 7.6, 50 mM) was obtained from Spinreact (Sant Esteves de Bas, Spain).

Solutions.

The following aqueous solutions are needed for the present experimental work: ASCh 37.5 mM, DTNB 10 mM, Coomassie blue 1 mM, ammonium molybdate 5%, 2,4-diaminophenol (amidol) reagent (2,4-diaminophenol 10 mg/ml in sodium disulfite 0.25 g/ml), and phosphates buffer, pH 7.4, 155 mM. As the three fluorescent probes are not soluble in water, their stock solutions are prepared in organic solvents: DPH 1 mM in acetone, TMA-DPH 0.5 mM in N,N-dimethylformamide, and C17-HC 10 mM in tetrahydrofuran.

Blood Samples.

Human venous blood samples were collected with anticoagulant (10 IU of heparin/ml of blood) from healthy donors, with their previous informed consent, following our protocol with the Portuguese Blood Institute. Freshly collected whole blood samples were centrifuged for 10 min at 1,000 x g in a Sorvall TC6 centrifuge (Du Pont, Bad Nauheim, Germany). Erythrocytes were isolated by plasma and buffy-coat removal, resuspended in phosphate buffer, pH 7.4, 155 mM, and divided in aliquots.

Erythrocyte Exovesicles Isolation.

As previously described [9], erythrocyte suspension aliquots were incubated for 30 min, at room temperature, with each of the three fluorescent probes. The final total concentrations of DPH, TMA-DPH, and C17-HC were 0.22 μM in 0.037% hematocrit, 5.4 μM in 0.01% hematocrit, and 0.11 mM in 0.01% hematocrit, respectively. These values were optimized for the fluorescence measurements in erythrocytes according to the membrane/water partition coefficients [13] and fluorescence quantum yields of each probe. As the fluorescence probes reach equilibrium between the aqueous and lipid phases, and the unincorporated probes do not fluoresce [14], there was no need for a washing procedure to be done. The exovesicles were obtained on the supernatants of centrifugations (10 min, 1,000 x g) carried out 1 (t1), 24 (t24), and 48 h (t48) after the initial incubation. If the equipment is available, the exovesicles in the supernatants should be concentrated to ~2/3 of the initial volume (≈3 h at 30°C and 240 g in a micro test tubes Eppendorf concentrator model 5301, Hamburg, Germany).

Note

We believe that even during a pandemic, the required number of laboratory sessions cannot be reduced.

These procedures, referred as “previous experimental preparation by the instructor,” can be partially or totally carried out by the students, depending on their background and experience, and on the laboratory class time available.

Experimental Procedure for Lab Class.

Both phthalate esters (dibutyl phtalate and dimethyl phtalate) and their mixtures are not miscible with water. These mixtures, used to separate erythrocytes according to their density [15], were adapted by us to the identification of the erythrocyte exovesicles and prepared following the proportions presented. It must be kept in mind that the exovesicles

suspensions are blood-derived products. Thus, proper care and handling procedures must be followed during their manipulation. Fifty-microliter aliquots of exovesicle suspensions were added to each one of the five micro test tubes, containing 1 ml of the phtalate esters mixtures. After gentle homogenization, the micro test tubes were centrifuged at room temperature for 1 min at 8,500 x g in a Heraeus Sepatech Biofuge 15 (Osterode, Germany). The process was carried out for the different exovesicles suspensions under evaluation.

Protein Coloration with Coomassie Blue.

Following an adaptation of the Bradford method [10], 10 μl of Coomassie blue 1 mM were added to each micro test tube containing the exovesicles in phtalate esters gradient medium. After gentle shaking, the tubes were centrifuged again for 2 min at 8,500.

AChE Coloration with Ellman's Reagent.

Following an adaptation of the Ellman method [11], 15 μl of DTNB 10 mM and 10 μl of ASCh 37.5 mM were added to another set of micro test tubes containing the exovesicles in phtalate esters gradient medium. After gentle shaking, the tubes were incubated at 37°C during 20 min and centrifuged at room temperature for 2 min at 8,500.

Phospholipids Digestion and Phosphatidic Acid Coloration.

Ten microliters of the enzyme mixture containing phospholipase D (to hydrolyze the phospholipids to phosphatidic acid), 10 μl of ammonium molybdate 5%, and 10 μl of 2,4-diaminophenol (amidol) reagent (for coloration) were added to a last set of micro test tubes containing the exovesicles in phtalate esters gradient medium. After gentle shaking, the tubes were centrifuged again for 2 min at 8,500.

Results and discussion

We got colorless (unstained), blue (when stained with Coomassie blue), or yellow (when stained with Ellman's reagent or with the mixture used for phospholipid coloration) spheres at the top of the micro test tubes, in the colorless bulk of the phthalates gradient media.

These spheres are aggregates of exovesicles, which proteins are blue-stained in the presence of Coomassie blue. This coloration is due to the formation of complexes as a consequence of the dye binding to the proteins. The unbound form of Coomassie is red, with an absorption maximum at a wavelength (λ) of 465 nm. Upon protein binding, the dye shows a blue shift (λ = 595 nm), as indicated in the Bradford's method for protein quantification [10].

The yellow spheres were obtained after the addition of ASCh, which is hydrolyzed to thiocholine and acetate by the erythrocyte exovesicles AChE. The reaction of the thiol group of thiocholine with DTNB generates the yellow anion 5-thio-2-nitro-benzoic acid. These two coupled reactions were described by Ellman et al. as the principle of a rapid colorimetric method for AChE activity determination [11] and it is still largely used as a gold standard for this enzyme activity quantification [16].

The method used for phospholipid identification leads also to a yellow coloration of

the spheres. Initially, the phospholipids are enzymatically hydrolyzed by phospholipase D to phosphatidic acid. Despite the fact that the hydrolysis can also be achieved by acid treatment (e.g. with perchloric acid), this would also lead to a partial degradation of the spheres. After the hydrolysis, the phosphate group of the phosphatidic acid forms an oxidized phosphomolybdate complex upon reaction with molybdate. The reduction of this complex by the 2,4-diaminophenol reagent origins the characteristic coloration, commonly used for phospholipid quantification [12].

Further Perspectives.

This is an original method that complements our recent research works [9]. Working in an innovative area of research proves stimulating for the students and encourages them to develop new solutions for practical problems. After this laboratory class, students are invited to reach for physiological and biotechnological exovesicles applications [17,18,19]. The information obtained by them can be used for analysis and discussion on a further tutorial class.

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