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# ELEKTRON KRYO-MIKROSKOPICKÉ TECHNIKY V BIOLOGICKÉM VÝZKUMU A NANOTECHNOLOGIÍCH

# ELECTRON CRYO-MICROSCOPY TECHNIQUES IN BIOLOGICAL RESEARCH AND NANOTECHNOLOGIES

**Mgr. Veronika Mistríková**

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| °C: | Celsius |
| µm: | Micrometer |
| nm: | Nanometer |
| BSA: | Bovine serum albumine |
| $CaCl_2$: | Calcium chloride |
| CCD: | Charged coupled device |
| Cryo-TEM: | Transmission electron cryo-microscopy |
| DABCO: | 1,4-Diazabicyclo[2.2.2] octane |
| DAPI: | 4',6-Diamidino-2-phenylindole |
| $D_4^{ethyl-D}$: | Tetra(methyldimethoxy silylethyl)tetramethyl cyclotetrasiloxane |
| DODAB: | Dimethyldioctadecyl ammonium bromide |
| EM: | Electron microscopic |
| FA: | Formaldehyde |
| FS: | Freeze substitution |
| GA: | Glutaraldehyde |
| HP-: | High-pressure- |
| HPF: | High-pressure freezing |
| IEM: | Immunoelectron microscopy |
| IF: | Immunofluorescence |
| K: | Kelvin |
| KPi: | Potassium phosphate buffer |
| kV: | Kilovolt |
| $MgCl_2$: | Magnesium chloride |
| MPa: | MegaPascal |
| n.d.: | No date |
| NGS: | Normal goat serum |
| $NH_4Cl$: | Ammonium chloride |
| $NaIO_4$: | Sodium periodate |
| OA: | Oleic acid |
| PBS: | Phosphate buffered saline |
| PIPES: | 1,4-Piperazine bis [2-ethanosulfonic acid] |
| RT: | Room temperature |
| RTS: | Rapid transfer system |
| Saccharomyces cerevisiae: | S. cerevisiae |
| TEM: | Transmission electron microscope (microscopy) |
| UA: | Uranyl acetate |
| UV: | Ultraviolet |
| YPD: | Yeast extract, peptone, glucose |
| $X_{OA}$: | Mole fraction of OA |

ABSTRAKT

Příprava biologických vzorků pro transmisní elektronovou mikroskopii není triviální úkol. Vzorky musí odolat vakuu přítomném v mikroskopu, a proto je často nutné uplatnit nefyziologické postupy při jejich zpracování. Tyto postupy obvykle zahrnují fixaci na bázi aldehydů, nahrazení vody alkoholem (t.j. dehydrataci/substituci), a zalití do pryskyřice, která vytváří podporu pro následnou přípravu tenkých řezů, které pak mohou být vloženy do mikroskopu. V posledním desetiletí získala dominantní postavení v oblasti výzkumu buněčné biologie metoda kryo-fixace (vitrifikace) za pomoci ultrarychlého vysokotlakého zmrazování a následná kryo-substituce a zalití vzorků do pryskyřice při nízkých teplotách. Tímto způsobem byli úspěšně vitrifikovány různé biologické vzorky s tloušťkou až několik stovek mikrometrů do stavu, který byl srovnatelný s jejich *in vivo* strukturou. Kryo-fixace izolovaných biologických objektů (s omezenou tloušťkou do několika mikrometrů) je možná i v tenké vrstvě vitrifikované vody za pomoci imerzní kryo-fixace při normálním tlaku. V kombinaci s kryo-elektronovou mikroskopií se tato metoda stala nejefektivnejším a základním principem pro tvorbu elektron kryo-mikroskopických obrázků plně hydratovaných vzorků s velmi vysokým rozlišením na úrovni několika desetin nanometrů. Obě tyto metody jsou prezentovány v této práci. Pučící kvasinky *Saccharomyces cerevisiae* zpracované kryo-fixací za pomoci vysokotlakého zmrazování a následné kryo-substituce byly vybrány jako biologický objekt pro jemné ultrastrukturální a immunocytochemické studie s využitím klasické transmisní elektronové mikroskopie; liposomy a jiné typy vezikulárních struktur zpracované imerzní kryo-fixací byly vybrány jako nanoobjekty pro elektron kryo-mikroskopické studie.

ABSTRACT

Preparation of biological samples for transmission electron microscopy is not a trivial task. The samples must withstand a vacuum environment present inside a microscope, and it is often necessary to use non-physiological procedures for their processing. These procedures usually involve aldehyde-based fixation, replacing water with alcohol (i.e. dehydration/substitution), and embedding into a resin, which creates support for the subsequent preparation of thin sections that can be placed into the microscope. In the last decade, the method of cryo-fixation (vitrification) using ultra-fast high-pressure freezing followed by freeze substitution and low-temperature resin embedding gained a dominant position in the cell biology research. In this way, a range of biological samples with a thicknesses up to several hundreds of micrometers was successfully vitrified to a state that was closely related to their *in vivo* structures. The cryo-fixation of isolated biological objects (with a limited thickness up to several micrometers) is possible in a thin layer of vitrified water by plunge freezing at ambient pressure. In combination with electron cryo-microscopy, this method has become the most effective and fundamental principle for the high-resolution studies and image analysis of fully hydrated samples breaking the sub-nanometer limit. These methods are presented in this thesis. The budding yeast *Saccharomyces cerevisiae* processed by high-presure freezing and freeze substitition was selected as a biological object for both fine ultrastructural and immunocytochemical studies using conventional transmission electron microscopy; the liposomes and other types of vesicular structures vitrified by plunge freezing were selected as nanoobjects for electron cryo-miscroscopic studies.

# 1 INTRODUCTION

Transmission electron microscopy (TEM) has become an indispensable tool in many fields of life science research providing unique information about the biological structure-function relationships from the cellular to nearly atomic scale since its invention in 1931 by Max Knoll and Ernst Ruska (Ernst Ruska – Autobiography, n.d.). Currently, there is an enormous variety of experimental techniques of sample preparation for TEM, which allows us to look at a biological material in many different ways. Chemical fixation, dehydration and embedding do, of course, together constitute the major pathways, by which the vast majority of biological sample processing is carried out for TEM and also for some light microscopic observations (Bozzola, 2007; Glauert, 1974; Griffiths, 1993). These techniques, however, almost always involve the removal of water from the samples, which inevitably leads to changes in the native biological structure: a lot of soluble material is extracted, and further *in vivo* distribution of ions and other mobile components and subcellular structures is radically changed. Such considerations led to a much greater attention being paid to an alternative possibility for the preparation of biological samples: that of a cryo-immobilization. The catalyst for this 'revolution' was the development of new instruments (Moor & Riehle, 1968; Studer *et al*, 2001) and TEM imaging techniques (Adrian *et al*, 1984; Dubochet *et al*, 1988; Pierson *et al*, 2009).

## *1.1 High-pressure freezing*

At present, high-pressure freezing (HPF) is the only practical way that allows thick biological samples, e.g., mammalian cells, tissues, larger bacteria cells, yeast, to be successfully vitrified in a state most closely related to living situation (Hess, 2007; Kang, 2010; McDonald, 2007; Murray, 2008; Studer *et al*, 2001; Vanhecke *et al*, 2008). The original concept of HPF was introduced by Hans Moor and coworkers in 1968 (Moor & Riehle, 1968), and detailed information can be found for example in McDonald *et al* (2007), McDonald (2009), Verkade (2008). In HPF, the application of pressure in the range of 200-210 MPa (MPa, MegaPascal; 1 atmosphere = 0.1013 MPa) at the moment of freezing (while the sample is cooled at a maximal speed to liquid nitrogen temperature that is -196°C [77,15 K; Kelvin = Celsius (°C) + 273.15]), changes the freezing behavior of water enough to cryo-preserve biological samples up to a thickness of about 200 μm (from the surface of the sample) with minimal or no ice crystal formation and optimum heat transfer (Studer *et al*, 2008; Vanhecke *et al*, 2008). This is approximately 10 times better depths of vitrification than it is possible to achieve by other freezing methods, e.g., the method of plunge freezing, slam freezing, jet freezing, operating at an ambient pressure (Hayat, 1989). In these cases, only very thin samples (5 - 20 μm in thickness) are vitrified. Moreover, the effective advantage of

HPF approach is that a rate of cooling required for vitrification is significantly reduced to about 5,000 K/s that is ~10 times lower than at ambient pressure (Vanhecke *et al*, 2008). Thus, the cellular water is vitrified and the original location of macromolecules remain unchanged, and they are not extracted as they can be by conventional methods of preparation based on chemical fixation. In such a way, the highest level of ultrastructural preservation is achieved (Hurbain & Sachse, 2011; McDonald *et al*, 2007; Studer *et al*, 2001; Studer *et al*, 2008).

*1.2 Freeze substitution and resin embedding*

Freeze substitution (FS) is the most common way to process high pressure (HP)-frozen samples (Fernandez-Moran, 1960). During the procedure, the vitrified water in biological sample is exchanged (substituted) by an organic solvent (FS medium), most commonly acetone. This process takes place at temperatures low enough to avoid the formation of ice crystals: the FS procedure usually starts at temperatures around -90 °C [183.15 K] and above the melting point of solvent being used (Buser & Walther, 2008; McDonald, 2007; Pierson *et al*, 2009). The timing of FS procedure varies considerably and may depend to some extent on the size, nature and hydratation of the sample being processed and the quality of cryo-fixation. Generally, diffusion at low temperatures is very slow and complete dehydration (and resin embedding) may take several days to complete (McDonald, 2007). In addition to solvent, the samples can be simultaneously stained by adding salts of heavy metals (e.g., uranyl acetate, osmium tetroxide) to FS media. These could also serve as a secondary fixatives during the FS procedure (Schwarz & Humbel, 2007). FS protocols for morphological studies generally rely on osmium-acetone mixtures, but may also contain other fixatives such as glutaraldeyde (Hawes *et al*, 2007). It should be noted that the action of fixatives differs significantly at low temperatures compared to their action at the ambient one. This is because the fixative becomes distributed throughout entire cells or tissues at temperatures around -90 °C [183.15], and when the temperature is permisive for fixation (-50 °C [223.15] for glutaraldehyde, -30 °C [243.15] for osmium tetroxide), the fixative is in place and does not need to diffuse through relatively large distances. Therefore, the proteins are not degraded, so the deleterious effect on the antigenicity is greatly reduced (McDonald, 2007; Schwarz & Humbel, 2007). Once the substitution is complete, the samples are gradually warmed-up to ambient temperature and processed further as for conventionally prepared samples. However, a better FS approach is to maintain the samples at sub-zero temperatures by infiltrating and polymerizing resins (i.e. Lowicryls) by ultraviolet (UV) light (Carlemalm *et al*, 1985). Even if the effect of low temperature polymerization on the dehydrated sample at the molecular level is difficult to predict, this approach is theoretically the best one for preserving structure under conditions compatible with immunogold labeling.

*1.3 Cryo-fixation followed by low-temperature substitution and embedding versus conventional method of sample preparation*

Cryo-fixation of biological samples as performed by HPF and followed by low temperature dehydration (FS) and embedding has several distinct advantages over conventional methods of sample preparation for TEM analysis. Unlike the duration of aldehyde-based fixation, cryo-fixation by HPF is achieved within milliseconds and it ensures simultaneous immobilization of all macromolecular components (Hurbain & Sachse, 2011; McDonald, 2007; McDonald, 2009). Chemicals, on the other hand, have a lag time in completely penetrating into cells and tissues (Glauert, 1974; Griffiths, 1993). Many protein networks are very labile and fall apart with the slightest osmotic or temperature changes and these unwanted effects are minimized with cryo-fixation. Thus, using HPF it is possible to study dynamic cellular processes, which can not be 'fixed' by conventional chemical fixation (Hayat, 1989; Pierson *et al*, 2009). Moreover, since the dehydration and embedding are performed at low temperatures, the artifacts induced both by the solvent, fixative, temperature are greatly reduced. Aggregation of macromolecules in organic solvents and changes of the hydration shells surrounding the biological molecules can occur even at low temperatures; but it is reasonable to assume that substitution at these temperatures preserves the hydration shell and redistribution of diffusible elements is minimized (Bleck *et al*, 2010; Pierson *et al*, 2009). The appearance of HP-frozen/FS samples is therefore very different from that observed after conventional fixation and embedding (Steinbrecht and Müller, 1987; Vanhecke *et al*, 2010). Moreover, the efficient and fast chemical fixation is often deficient in the samples containing air or large vacuoles (leaves), cell walls (plants, fungi) or cuticles, hydrophobic surfaces (insects, nematodes). Especially in these cases, cryo-fixation by HPF followed by FS procedure is the method of choice for notable superior ultrastructural preservation (Hess, 2007; Kang, 2010; Kolotuev *et al*, 2009; McDonald, 2007; Murray, 2008; Studer *et al*, 2001; Vanhecke *et al*, 2008). Altogether, the method of HPF followed by FS and low-temperature plastic embedding is becoming the most widely used method for TEM sample preparation.

*1.4 Transmission electron cryo-microscopy*

In early 80's, Dubochet and his colleagues demonstrated that it is possible to produce a thin layers of vitrified ice that contain an unfixed and unstained biological samples, which could then be examined directly in their native hydrated state using so-called transmission electron cryo-microscope (cryo-TEM) (Adrian *et al*, 1984; Dubochet *et al*, 1988). This method, currently known as the 'thin ice embedding method' or 'bare grid method', has become a basic principle for the high-resolution cryo-TEM studies and image analysis of different types of biological and non-biological objects up to the size of 100 nm, breaking the sub-nanometer limit (Dubochet *et al*, 1988).

Preparing a 'representative' thin vitrified sample in a controlled and reproducible manner is therefore an important step for each cryo-TEM experiment (Adrian, 1984; de Carlo, 2008; Grassucci *et al*, 2007). Typically, a drop of an aqueous suspension containing the sample particles is placed on a 'pre-treated' EM (electron microscopic) grid with perforated supporting film, mounted in a tweezers of the plunger. Excess fluid is then blotted off with filter paper and the grid is immediately plunged into a cryogen at a high velocity (Adrian *et al*, 1984). Thin film of solution, spanning the holes, is formed after blotting (de Carlo, 2008; Frederik *et al*, 2008). As the thickness is typically 50-100 nm and the hole diameter is in the range of microns, the surface-to-volume ratio is extremely high and therefore the heat extraction is very efficient (Crucifix *et al*, 2008; Dubochet *et al*, 1988). Though differnet types of cryogens can be used, plunging blotted samples into melting ethane slush kept in a container of liquid nitrogen become the most standard for this technique assuring the rate of cooling in order of $10^4$ ºC/s. Once the sample has been vitrified, it can be transferred (without rewarming) and examined in cryo-TEM at the temperatures between -180 °C [93.15] and -160 °C [113.15] (Marko & Hsieh, 2007) in order to mantain the vitreous state of ice (Dubochet *et al*, 1988; Hayles *et al*, 2010).

### 1.4.1 Low dose imaging in cryo-TEM

As known, one of the most important causes of artifacts, and sometimes misinterpretations of TEM micrographs, is the electron-beam-induced radiation damage. All biological samples are highly sensitive to electron-beam radiation. These samples suffer from degradation as a result of the impact of high-energy electrons from the focused electron beam. The concomitant dissipation of energy gives rise to chemical reactions and rearrangements that leads to the loss of structural details during the image acquisition (Chiu *et al*, 2006; Koning & Koster, 2009). This is especially true for the examination of samples embedded in a thin layer of vitrified ice. Due to the absence of protection by chemical cross-links or the stabilization by the stain molecules, the samples are more easily damaged in comparison with other preparation techniques. Special conditions are therefore necessary to substantially reduce damage of these samples and to record the high quality cryo-TEM micrographs. So far, a low dose electron imaging has been the most effective approach for overcoming these difficulties (Chiu *et al*, 2005; Chiu *et al*, 2006). In the low dose mode, the total imaging electron dose is limited to the range of 10 – 20 electrons per square Ångström depending on the temperature of the sample and the intended resolution of the study (Dubochet *et al*, 1988; Glaeser & Taylor, 1978; Chiu *et al*, 2006). Exceeding this dose generally results in a change of biological structure and 'bubbling', thus compromising high resolution imaging. Electron doses are limited not only during the actual exposure, but also during the search for suitable objects or area (Lucic *et al*, 2008).

### 1.4.2 Contrast in cryo-TEM

Though frozen-hydrated samples can contain information to the atomic resolution, they suffer from limited contrast. A poor signal-to-noise ratio of recorded images is one of the basic problems in cryo-TEM imaging (Lucic *et al*, 2008; Pierson *et al*, 2009). The contrast of sample depends on the sample itself, the defocus value of the objective lens and the thickness of ice. The ability of sample to provide amplitude contrast cannot be altered. However, the sufficient phase contrast can be obtained using an appropriate objective lens underfocus setting (Koning & Koster, 2009; Marko & Hsieh, 2007). Usually the images are recorded at a 1-3 μm underfocus in order to enhance the visibility of low-resolution structural details. With increasing thickness of ice, the background noise increases as well. It is therefore preferable to record images in areas with a thinner layer of ice, though its thickness must imperatively be superior to the particle size (Chiu *et al*, 2006).

### 1.4.3 Benefits and perspectives of cryo-TEM

Cryo-TEM is nowadays the imaging method of choice best suited for the structural and morphological analysis of a different macromolecular complexes and nanomachines at the molecular, and even nanometer level (Friedrich *et al*, 2010; Chiu *et al*, 2005; Jiang & Ludtke, 2005; Sander & Golas, 2010) because of several reasons: cryo-TEM bypasses the need for conventional fixatives, dehydration and staining, so that a biological object is observed directly and a 'close-to-native' environment is retained (Pierson *et al*, 2009). Unlike conventional TEM techniques, where the objects are flattened by adsorption onto the supporting film and covered with heavy metal staining material, cryo-TEM allows the analysis of dynamic behavior of unfixed and freely suspended samples within the layer of vitrified solution. Frozen-hydrated sample is therefore observed in the absence of stain, avoiding problems in interpretation caused by an unpredictable accumulation of staining material (Lucic *et al*, 2008). Contrary to existing techniques such as nuclear magnetic resonance spectroscopy and X-ray crystallography, cryo-TEM offers lower resolution, however this method avoids the requirement for crystallization, significantly less material is needed (10 $\mu g.ml^{-1}$ versus 10 $mg.ml^{-1}$) and works well with larger molecules and molecular complexes (Crucifix *et al*, 2008; Grassucci *et al*, 2007; Chiu *et al*, 2005). Cryo-TEM therefore found its application in a numerous scientific disciplines, including structural and molecular biology, cell biology, medicine, nanobiology and even nanotechnology research (Nicastro *et al*, 2006; Pierson *et al*, 2009; Sali *et al*, 2003).

# 2 AIMS OF THE WORK

There were several objectives in this thesis:

- to install and to establish cryo-preparation methods at the Institute of Cellular Biology and Pathology of the First Faculty of Medicine of Charles University in Prague, namely: i) the method of high-pressure freezing, freeze substitution and low-temperature embedding, and ii) the thin ice embedding method followed by electron cryo-microscopic imaging,
- to demonstrate their usability and powerfulness by applying them on particular biological and biomedical objects, which included the budding yeast *Saccharomyces cerevisiae,* and different nanovesicular structures such as the liposomes, cationic liposomes, etc.,
- to establish an optimized protocol for the immunolocalization of the nuclear and nucleolar antigens of *Saccharomyces cerevisiae,* which also involve an optimization of the preparation of the cells for both ultrastructural and immunocytochemical TEM studies with the best possible 'close-to-live' preservation.

It is well known that the preparation of yeast cells for TEM study is rather problematic, mainly because of the presence of a rigid cell wall. Chemical fixation using 'strong' fixatives may result in adequate ultrastructural preservation, but at the expense of preservation of sensitive antigens. Therefore, the use of alternative methods had to be considered. Development of new tools such as high-pressure freezers and low-temperature processing systems provided new opportunities for the processing of yeast and other biological objects for the high-resolution ultrastructural and immunocytochemical TEM analysis. We therefore aimed to establish an optimized protocol for the cryo-fixation of yeast *S. cerevisiae* followed by low-temperature dehydration, embedding and advanced TEM imaging, in order to study selected nuclear and nucleolar antigens.

Next, we aimed to use the technique of a thin vitreous layer for detailed morphological characterization of various nanostructures, potentially convenient to serve as carriers of biologically active molecules in the processes of targeted drug delivery. This method is able to produce very thin vitrified aqueous films with the hydrated samples embedded in them. In combination with cryo-TEM analysis it allows direct examination of frozen-hydrated samples in their near-native state with a fully preserved structure. This method is practically unique, which can be used for the analysis of one of the most intensively studied nanosystems at the present time – the liposome - without disturbing its structure and directly visualize its morphological features.

# 3 MATERIALS AND METHODS

## *3.1 Yeast strain, media and cultivation conditions*

The yeast *Saccharomyces cerevisiae* haploid strain NOY 886 (*MATα rpa*135Δ::LEU2 *ade 2-1 ura3-1 his 3-11 trp1-1 leu2-3,112 can1-100 fob1Δ::HIS3* pNOY117 [*CEN RPA135 TRPI*]) (a kind gift of Yvonne S. Osheim, University of Virginia Health System, USA) was used in this study. The yeast were grown at 26 ˚C in a standard YPD medium containing 1% yeast extract, 2% peptone, 2% D-(+)-glucose, 2% agar (for plates only) and 100 μg/ml adenine hemisulfate salt. Unsynchronized yeast cells were used in each experiment. The day prior to fixation, a single colony of yeast cells was used to inoculate 100 ml of YPD and let grow overnight with moderate shaking in the 250-mL Erlemeyer flask. The next day, the overnight culture was used to inoculate 5 ml of fresh YPD, left grown to an early log phase (OD600 ~ 0.2-0.4, corresponding to less than $10^7$ cells/mL), and then processed for the immunofluorescence (IF) or the immunoelectron (IEM) microscopy.

## *3.2 HPF and FS of S. cerevisiae for IEM*

Yeast cells were HP-frozen in a Leica EM PACT2 freezer (Leica Microsystems) equipped with the rapid transfer system (RTS) as described by McDonald & Müller-Reichert (2002). Yeast culture (5 mL) was harvested by vacuum filtration using a 15-mL suction filtration apparatus (Millipore) on 0.45 μm nitrocellulose membrane filters (Macherey Nagel), and then placed on a plate with 1% agar in YPD to prevent its dehydration during the transfer. Using a sterile toothpick, the yeast paste was scraped off the filter onto a membrane carrier (1.5 mm in diameter, 0.1 mm deep, Leica instruments) under a stereomicroscope, placed in the RTS and frozen. Time interval between the harvesting and freezing of the cells was kept under one minute. HP-frozen samples were then freeze-substituted in a Leica EM AFS2 apparatus equipped with the automatic processor Leica EM FSP (Leica Microsystems). Samples were first placed into a reagent bath loaded with one of the FS media under test (see below) pre-cooled to -90 ˚C, and processed for 24 hours. Thereafter, the temperature was gradually increased at a rate of 5 ˚C/hour (8 hours in total) to -50 ˚C, and held constant for 24 hours. All successive processing steps were performed at this temperature. After FS, the samples were washed three times with acetone and gradually infiltrated with 3:1, 1:1 and 1:3 (*v*:*v*) acetone:Lowicryl HM20 resin (Lowicryl HM20 kit, Electron Microscopy Sciences) mixtures, 2 to 3 hours for each step. The mixture was then exchanged with pure resin at least three times, 2 hours for each step. Polymerization under UV light (using a UV LED lamp integrated in the FS processor) was performed for 24 hours at -50 ˚C and for additional 24 hours at +20 ˚C (the temperature was gradually increased at a rate of 5 ˚C/hours - 14 hours in total).

The following FS media were used: (1) anhydrous acetone (EM grade, Polysciences); (2) acetone with 0.1% glutaraldehyde (GA) – prepared from a 50% aqueous GA (Electron Microscopy Sciences) stock solution; (3) acetone with 0.1% uranyl acetate (UA, Merck) - prepared from a 10% methanolic stock solution, and (4) acetone with 0.1% UA and 0.1% GA.

*3.3 Chemical fixation of S. cerevisiae for IEM*

Yeast cells were prepared as follows: 2x fixative was added directly to the yeast culture grown in YPD (1:1, *v*:*v*) to reach a final concentration of 1x fixative, i.e. 4% (w/v) formaldehyde (FA, Fluka) in 0.1 *M* PIPES (pH 6.8), 1 *mM* $CaCl_2$, 1 *mM* $MgCl_2$ and 0.1 *M* D-Sorbitol. Additionally, GA in final concentrations of 0.05% to 0.5% was used in combination with fixative mentioned above. The cells were first fixed for 5 min at room temperature (RT), pelleted and subsequently resuspended in the 1x fixative to complete the fixation for additional 1 hour. Gentle agitation with orbital shaker was applied during the fixation procedure and centrigufation at 1500g for 5 min was used for pelleting the cells. After fixation, the cells were washed twice in 0.1 *M* PIPES (pH 6.8) with 0.1 *M* D-Sorbitol and once in 0.1 M PIPES (pH 6.8) alone. The cells were then treated with 1% (w/v) $NaIO_4$ (Sigma) and 50 *mM* $NH_4Cl$ (Sigma) for 15 min as described by Mulholland & Botstein (2002). The samples were washed and dehydrated successively in a graded ethanol series (25%, 50%, 75%, and 95% ethanol) on ice in 5 min steps. Each step was followed by centrifugation at 1500g for 1 min at 4 °C. Three changes of 100% ethanol were additionally applied (5 min steps), and the final change was performed at RT. The dehydrated samples were then infiltrated with 2:1, 1:1, and 1:2 (*v*:*v*) ethanol:LR White resin mixtures and twice with pure LR White (Medium grade, kit, Electron Microscopy Sciences). Each step was carried out at RT for 1 hour using a gentle agitation with orbital shaker. The third infiltration step was performed overnight at 4 °C. Next day, the resin was exchanged for a fresh one and the samples were left to infiltrate for additional 30 min at RT. Finally, the samples were transferred into gelatin capsules (size 1, Leica instruments) containing fresh resin, allowed to settle for 15 min and polymerized at 47 °C for 2 or 3 days.

*3.4 Microtomy, on-section immunogold labeling and staining*

Ultrathin sections (60 nm) were prepared on a Reichert Ultracut S microtome and collected on Formvar/carbon coated nickel EM grids. For the immunocytochemistry, all incubation steps were performed at RT. The sections were first pre-blocked with 5% (w/v) normal goat serum (NGS) in phosphate-buffered saline (PBS, pH 7.4) for 30 min and then incubated with primary antibody (see Subchapter 3.6) diluted in 1% (w/v) bovine serum albumine (BSA) and 0.05% Tween-20 in PBS for 1 h and 45 min. Afterwards, the grids were first washed in 0.05% Tween-20 in PBS and

then in PBS alone. Un-specific reactions were blocked with 1% NGS in PBS for 10 min before the gold-conjugated secondary antibody (see Subchapter 3.6) diluted in PBS was applied (incubation time 1 hour). Finally, the grids were washed in PBS and in double-distilled water, air-dried, and post-stained as described below, or alternatively, left unstained. In negative control experiments the incubation with primary antibody was omitted.

For quantitative evaluation of immunogold labeling, the Nop1 and Nsr1 antigens were selected due to their specific localization in the yeast nucleolus. Sets of 10 micrographs of yeast nuclei (nucleoli) at a magnification of x 7,800 were chosen randomly for each of FS media mentioned above and labeling density was determined in terms of a number of gold particles per square micrometer. For each set, an average and standard deviations were determined (Microsoft Excel). The density was evaluated for the nucleolus and for the rest of the cell section, giving us the information about the level of non-specific labeling. The measurements were performed using ImageJ software (Rasband, 1997-2009).

Sections of chemically-fixed yeasts were post-stained using 2% (w/v) aqueous UA for 5 min and lead citrate (Reynolds, 1963) for 1 min. Sections of HP-frozen/acetone- or acetone/GA-substituted cells were post-stained similarly as described above, except that 2% (w/v) UA in 70% methanol was used. Sections of HP-frozen/acetone/UA- or acetone/UA/GA-substituted cells were not post-stained. The samples were then imaged with a FEI Tecnai G20 Sphera transmission electron microscope (FEI Company) operating at 120 kV. Images were recorded with a Gatan Ultrascan$^{TM}$ 1000 slow-scan CCD (CCD; charged coupled device) camera.

*3.5 Preparation of S. cerevisiae for IF microscopy*

Yeast cells were fixed with 4% (w/v) FA in 0.1 M potassium phosphate buffer (KPi) pH 6.8 added directly to growing culture as a 2x stock. Fixation was performed for 2 hours at RT. Fixed cells were washed twice in 0.1 *M* KPi and once in 0.1 *M* KPi with 1.2 *M* D-Sortbitol (solution P). Cell walls were removed using zymolyase (ZymoResearch) diluted 1:50 in solution P at 30 °C for 10 to 20 min. Digestion of cell walls was monitored by phase contrast light microscopy using a 100x oil immersion objective. The spheroplasted cells were washed again in solution P and left to sediment for 10 min on poly-L-lysine (Sigma) treated coverslips. Permeabilization of the cells was performed as described by Pringle *et al* (1989) with 0.1% Triton X-100 in PBS for 30 min at RT or alternatively with methanol for 6 min and subsequently with acetone for 30 s at -20 °C. The cells were then incubated for 20 min with the blocking buffer (1% (w/v) BSA in PBS, pH 7.4), with the primary antibody for 1 hour (see Subchapter 3.6), washed in PBS and incubated with fluorescent secondary antibody (see Subchapter 3.6) for an additional 1 hour. Both antibodies were diluted in the blocking buffer and all steps were performed at RT. Finally, the cells were washed in PBS and

in double-distilled water, embedded in a Mowiol/DABCO mounting medium (Fluka) containing 0.4 μg/ml DAPI (4',6-diamidino-2-phenylindole) and placed onto slides. The cells were examined with the confocal microscope Leica TCS SP5 with 100x/1.4 NA oil immersion objective.

*3.6 Antibodies*

The following primary antibodies were used for IF and IEM procedure, respectively: (a) anti-fibrillarin (Abcam) diluted 1:500 for IF and 1:5 for IEM; (b) anti-Nsr1p (Abcam) diluted 1:400 for IF and 1:7 for IEM; (c) anti-Nsp1p (Abcam) diluted 1:450 for IF and 1:5 for IEM and (d) anti-α-tubulin (Sigma) diluted 1:2000 for IF and 1:50 for IEM. All used primary antibodies were mouse monoclonal, IgG isotype. As secondary antibodies donkey anti-mouse cy3-conjugated antibody diluted 1:500 for IF and goat anti-mouse 12 nm gold conjugated antibody diluted 1:10 for IEM (both from Jackson ImmunoResearch) were used.

*3.7 Nanoscopic vesicular structures*

The materials were provided by Mariusz Kepczynski research group from the Faculty of Chemistry, Jagiellonian University in Krakow. Details of their characteristics, purchase and preparation can be found in the publications No.2 and No.3 (Seznam publikací autora). Briefly, the samples under the study included the liposomes (made from egg yolk phosphatidylcholines), the cationic liposomes i.e., the liposomes covered with a dimethyldioctadecylammonium bromide (DODAB) surfactant, and the cationic liposomes covered with a novel silicone monomer tetra(methyldimethoxysilylethyl)-tetramethylcyclotetrasiloxane ($D_4^{ethyl\text{-}D}$). The monomer was introduced to the cationic liposome dispersions at pH 8.5 or 10.2. The formed structures were characterized with different methods: dynamic light scattering, zeta potential measurements (not performed in our laboratory), and cryo-TEM analysis. Next, the catanionic DODAB/oleic acid (OA) dispersions (the cationic DODAB vesicles with integrated anionic OA surfactant) were analyzed with different methods such as differential interference contrast microscopy, differential scanning calorimetry and fluorescence anisotropy technique (not performed in our laboratory). The morphology of bilayer structures formed in the dispersions was inspected using a cryo-TEM. It is worth to mention at least that the mole fractions of OA ($X_{OA}$) was equal to 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 and the total lipid content was constant and equal to 10 *mM*.

*3.8 Plunge freezing and cryo-TEM of nanovesicular structures*

First, an EM grid with perforated carbon-platinum supporting film was mounted in a tweezers of a plunger (EMS-002 Plunge Freezer, Electron Microscopy Sciences). Typically 3 to 5 μL drop of sample solution was placed onto the grid. Most of the sample was then removed by

blotting with the filter paper Whatman Grade No 1 (blotting time was about 1 - 3 s), and the grid was quickly plunged into a liquid ethane bath, cooled with liquid nitrogen to about -182 ºC. After freezing, the grid was transferred without rewarming into a FEI Tecnai G20 Sphera cryo-TEM using a Gatan single tilt cryo-transfer specimen holder and Gatan cryo-TEM transfer workstation. The images were recorded at 120 kV accelerating voltage and microscope magnification ranging from x 5,000 to x 25,000 (for the liposome vesicles) and x 5000 to x 14,500 (for the DODAB/OA dispersions) using the Gatan UltraScan™ 1000 CCD camera and low dose mode with the electron dose not exceeding 15 electrons per square Ångström. Underfocus in the range of 1.5 to 3 μm was used to obtain adequate image contrast. The applied blotting condition resulted in samples with thicknesses ranging from 100 to 300 nm.

Diameters of individual vesicles images extracted from cryo-TEM micrographs were estimated by standard Hough transformation and their distribution was subjected to probability density function analysis, which revealed and described the characteristic populations of vesicles present in the specimen (Hlaváč & Šonka, 1992).

## RESULTS

### *4.1 The ultrastructural preservation of aldehyde-fixed yeast cells*

Typically, the FA-fixed cells exhibited irregular shapes and most of the cellular organelles showed improper preservation. The cytoplasm was retracted and distinct plasma membrane invaginations into the cytoplasm were frequently observed. The vacuoles were rather small and numerous in most cases and their contents were almost always lost. The mitochondria, Golgi cisternae, endoplasmatic reticulum and nucleus were distinguishable only due to the white profile of their membranes. Prominently, the shape of the nuclear envelope was strongly irregular and nuclear organization i.e., the presence of recognizable regions of heterochromatin and euchromatin, was almost invisible. In a majority of nucleoli that were investigated, the nucleolar subcompartments were not recognizable, though zones of lower and higher electron densities were observed.

The ultrastructural preservation in the FA/GA-fixed cells was apparently better despite the fact that the GA concentrations were kept low (from 0.05 to 0.5%) due to the requirement of antigenicity preservation. Generally, the cytoplasmic organelles showed morphology similar to those of the FA-fixed cells; however, the retraction of the cytoplasm was nearly eliminated. Preservation of the vacuolar content gradually improved with increasing concentration of GA, though without reaching an acceptable level. Nuclear shape was strongly irregular and the nucleoplasm showed a homogeneous grainy texture. As in the FA-fixed cells, the nucleolar subcompartments were hardly recognizable.

### *4.2 The ultrastructural preservation of HP-frozen/FS yeast cells*

The HP-frozen/FS yeast cells showed structural and ultrastructural preservation noticeably superior to that generally observed in aldehyde-fixed cells. The fine details of e.g., cell walls, plasma membranes, vacuolar membranes, nuclear envelopes, were very well preserved and showed smooth and continuous contours. Plasma membrane invaginations were less pronounced. The vacuoles had a spherical shapes and contents of uniform density. In the cytoplasm, the individual ribosomes, rough endoplasmatic reticulum, Golgi cisternae, mitochondria, multivesicular bodies, etc. were clearly distinguishable. Regular and smooth profiles of the nuclei were always observed. When UA was added to the FS solution it was moreover much easier to observe the double membrane profile of the nuclear envelope and its interruption by nuclear pores than it was in acetone- or acetone/GA-substituted yeast cells. The nuclear and nucleolar chromatin was always well preserved and very dense in all four types of FS media described above. In the nucleolar region, the fibrilar centers were easily distinguished as the zones of lower electron densities surrounded by electron-dense areas, in which both dense fibrils and pre-ribosomal particles were

localized. It should be mentioned that the only limitation of the procedure was problematic infiltration of lowicryl with acetone-substituted cells (gaps between the resin and the cells were commonly observed). The other cells substituted in the presence of UA and/or GA were satisfactorily infiltrated. Generally, substitution with acetone/UA FS medium yielded the best overall results of ultrastructural preservation.

*4.3 The ultrastructural immunolocalization of antigens*

The ultrastructural immunolocalization of Nop1 and Nsr1 (as nucleolar markers), Nsp1 (as a nuclear envelope marker), and α-tubulin (as a marker of microtubules) was performed on thin sections of both chemically fixed and HP-frozen/FS yeast. Each of the proteins was first specifically localized via indirect IF staining of permeabilized yeast cells. At the EM level, Nop1 was localized to the nucleoli, predominantly to the dense areas surrounding the fibrillar centers of lower electron densities in both aldehyde-fixed and HP-frozen/FS cells. The pattern of labeling was similar; however, the intensity of the labeling and localization with respect to the individual nucleolar subcomparments was more prominent on sections of cryo-fixed yeast cells. In the case of Nsr1, the gold particles were mainly distributed along and/or in the dense parts of the nucleoli of HP-frozen/FS cells. However, no labeling was detected on thin sections of aldehyde-fixed yeast cells. Nsp1 was localized inside the nuclear pore complexes, and both on their nucleoplasmic and cytoplasmic sides of HP-frozen/FS cells. No Nsp1 was detected on sections of aldehyde-fixed cells. Microtubules were specifically labeled in both aldehyde-fixed and HP-frozen/FS yeast cells sections, though in the case of alehyde-fixed yeast, the microtubules were not directly visible.

4.3.1 A quantitative evaluation of immunogold labeling of Nop1 and Nsr1

Next, we compared the efficiency of on-section immunogold labeling of Nop1 and Nsr1 between the yeast cells substituted in four FS media described above. The immunolabeling efficiency revealed to be approximately equal (within the error bar) for all used FS media giving for the nucleolus area approximately 23 and 16 gold particles/$\mu m^2$ for Nop1 and Nsr1, respectively. Although the addition of GA to FS media doubled the labeling efficiency of Nop1 (to 50 ± 12 gold particles/$\mu m^2$), in the case of Nsr1 it dropped to 8 ± 2 gold particles/$\mu m^2$. The intensity of non-specific background labeling (outside the nucleolus) was also increased in acetone/GA FS samples (and in acetone-substituted yeast) to 2 ± 5 and to 4 ± 5 gold particles/ $\mu m^2$ for Nop1 and Nsr1, respectively. In other two FS media (acetone plus UA and UA/GA) it was below 0.5 for both antibodies.

*4.4 The cryo-TEM studies of liposomes and silicone-coated liposomes*

The cryo-TEM visualization provided information regarding the morphology of the nanovesicular structures prepared in the laboratory of Dr. Kepczynski. The native (initial) liposome vesicles showed the good spherical structure with distinct bilayered phospholipid membrane surrounding an aqueous core. Although the unilamellar structures constituted the main population some multilayered liposomes were also observed. The mean size (diameter) of the liposomes observed on the cryo-TEM micrographs was about 80±33 nm. The cryo-TEM micrographs of the liposome dispersion after covering with a silicone monomer revealed a variety of different structures obtained at pH 10.2. Empty features with a thicker membrane (liposomes covered by silicone material) were present in the dispersion, but they were less numerous than the initial liposomes. A part of liposomes underwent disintegration at applied conditions with formation of the bilayer fragments, which appeared in the micrographs as a dark line and dots. The situation was quite different in a milder condition (pH 8.5). The amount of silicone-coated liposomes was much higher compared to those at pH 10.2, so under these experimental conditions, liposomes were more stable. The cryo-TEM micrograph revealed the presence of liposome bilayer fragments, which formed a kind of stack. The bilayer stacks were floating freely in the dispersion or stuck to the coated liposomes. Sparse solid particles, which were attributed to the formation of monomer droplets in the system, could be noticed. The cryo-TEM technique also enabled us to distinguish the empty and the solid objects and to calculate distribution profiles of the silicone-coated liposomes. The size of the objects was in the range of 20-180 nm and 25-200 nm for pH 10.2 and 8.5, respectively. The mean diameters were equal to 41±26 nm (pH 10.2) and 53±13 (pH 8.5). The quantitative results showed that the average diameter of covered liposomes was almost twice lower compared to these characteristic of the initial liposomes. Nevertheless, the silicone covered liposomes were stable.

*4.5 The cryo-TEM studies of DODAB/OA dispersions*

The effect of $X_{OA}$ on the morphology of structures formed in the DODAB/OA dispersion was examined with cryo-TEM. In a typical cryo-TEM micrograph of the extruded DODAB dispersion ($X_{OA}$ = 0) the vesicle population did not showed ideally spherical vesicle structures as known for liposomes or the cationic vesicles. Instead, a shape diversity of the vesicular structures with angular or ellipsoidal geometries and sizes between 100 and 200 nm was revealed. On the contrary, when $X_{OA}$ = 1, the uni- and bilamellar vesicles of predominantly spherical shape dominated in dispersion. The diameter of vesicles was close to 100 nm. In the case of the system where $X_{OA}$ = 0.2, the structures appeared as faceted vesicles with highly deformed wrinkled walls. There was a population of bilamellar vesicles and not all structures were completely closed. The

sizes of the features were similar to those for $X_{OA}$ = 0, i.e., between 100 to 200 nm. In the dispersions with $X_{OA}$ = 0.4 and 0.6, the lipids were organized into large planar membranes, whose dimensions were in the order of tens or hundreds of micrometers. Such objects were too large for the cryo-TEM analysis. Therefore, these DODAB/OA dispersions were shortly sonicated in order to obtain smaller fragments of the lipid bilayer. Thereafter, the mutually overlapping bilayer flakes were observed in the lipid dispersion at $X_{OA}$ = 0.4. Interestingly, the fragments of lipid membranes contained features that had lower contrast. These sites might be holes in the membrane. The cryo-TEM analysis of the sample with $X_{OA}$ = 0.6 confirmed the presence of the planar membranes. In that case no holes were observed. With the further increase of OA content in the membrane up to $X_{OA}$ = 0.8, unilamellar vesicles were formed in the DODAB/OA dispersion. Those vesicles possess predominantly spherical shape and their sizes were close to 100 nm.

# 5 DISCUSSION

The work presented here was devoted to the utilization of cryo-preparation methods in the processing of samples for conventional TEM and cryo-TEM image analysis. We described and compared different protocols for the processing of the yeast *S. cerevisiae* for fine ultrastructural and immunocytochemical TEM analysis. An approach using an aldehyde-based fixation, alcohol dehydration, LR White resin embedding, ultrathin sectioning and heavy metal post-staining was compared with that of cryo-fixation by high-pressure freezing, freeze substitution and low-temperature Lowicryl HM20 embedding, ultrathin sectioning and (or not) post-staining, in order to emphasize the importance and power of presented low-temperature processing methods.

As known, yeast cells are particularly difficult to preserve for TEM analysis, mainly due to the presence of a thick cell wall that acts as a barrier against diffusion of fixatives (Mulholland & Botstein, 2002; Wright, 2000). Especially when yeasts are proccesed in a classical way, which includes conventional aldehyde-based fixation and dehydration at RT, significant ultrastructural artifacts are observed in TEM; as a result the preservation of antigens is significantly influenced in a negative way. As we showed, the immunoreactivity of two antigens under the study (namely, Nsr1 and Nsp1) was not preserved in those samples, though Nop1 and α-tubulin were localized. However, it was not an easy task to correlate the antigen localization with the subcellular arrangement in these cells. Fine ultrastructural details were not of high-resolution, especially in the case of nuclei and nucleoli. It was not so suprising, since the TEM micrographs of aldehyde-fixed *S. cerevisiae* cells previously allowed to describe the nucleolus only as a strongly electron-dense region of the nucleus (Melese & Xue, 1995; Trumtel *et al*, 2000).

On the other hand, the cryo-preparation techniques are currently on the way to replace chemical fixation as a standard approach (Hurbain & Sachse, 2011). Among these methods, the cryo-fixation by HPF followed by low-temperature dehydration (FS) and low-temperature embedding is believed to be the method that preserves high-resolution details of cellular structure in a state closely related to the living situation (Moor & Riehle, 1968). The available evidence suggests that even the piece of an uncryo-protected tissue up to about 200 μm (600 μm theoretically) can be effectively vitrified by this approach (McDonald, 2007; McDonald, 2009; Studer *et al*, 2008). As presented in this work, we implemented HP-cryo-fixation followed by FS and embedding into Lowicryl HM20 with the objective to formulate a 'standard protocol', which can be applied to yeast *S. cerevisiae*, and/or alternatively to a wide variety of other samples, yielding optimal preservation of both ultrastructure and antigenicity. We analyzed FS media of different composition, i.e, acetone, acetone/UA, acetone/GA and acetone/UA/GA, for their positive/negative impact on the preservation of cell morfology and immunoreactivity. We compared the appearance

of the TEM images of *S. cerevisiae* thin sections prepared by conventional method with the TEM images of HP-frozen/FS cells. Differences remained considerably significant, though the primary goal to preserve mainly the antigenicity restricted the choice of reagents used for substitution and resin embedding. Both types of preparation showed almost the same details in the sense that the cell constituents seen with one method were visible also with the other, e.g., organelles, membranes, nuclei. However, the higher magnification view of ultrastructural details of HP-frozen/FS cells were greatly different from that observed in aldehyde-fixed cells. Beyond the notable superior high level of preservation of the ultrastuctural details, the sensitivity of immunocytochemical localization of antigens on sections was unambiquosly increased contrary to samples processed in a conventional manner: Nop1, α-Tubulin and Nsp1 antigens were clearly immunolocalized on sections of these cells. Moreover, the immunocytochemical detection of *S. cerevisiae* Nsr1 protein was reported for the first time. Altogether, HPF followed by FS in acetone with 0.1% UA gave the most satisfactory results from all tested media – both for the ultrastructural preservation, and also for immunodetection of tested antigens. However, since the 'clear' sub-nucleolar compartmentalization was never observed in any of HP-frozen/FS samples, our findings are in agreement with the bipartite model of *S. cerevisiae* nucleolar organization (Thiry & Lafontaine, 2005). Undeniably, the cryo-fixation preserved cells in a 'close-to-native' state and following low temperature processing significantly minimized the extraction of material, whereas chemical fixation and dehydration could not take place without extensive intra- and inter-molecular cross-linking and aggregation (Dubochet & Sartori Blanc, 2001). Therefore, the images generated using HPF can consequently serve as a 'gold standard', by which all other sample preparation methods for yeast or other biological objects can be judged (Dubochet *et al*, 1983).

Unlike cryo-immobilization of complex samples by HPF, other types of rapid freezing methods operating at an ambient pressure can be successfuly used for the cryofixation of, e.g., suspensions of cells and microorganisms, organelles, tissue cultures, in a thickness range of about 5-20 μm (Hayat, 1989; Pierson *et al*, 2009). The method of plunge-freezing, for example, is currently widely used in combination with cryo-TEM imaging to study a range of macromolecular complexes and other types of biological nanomachines (Adrian *et al*, 1984; Dubochet *et al*, 1988; Nicastro *et al*, 2006; Pierson *et al*, 2009; Sali *et al*, 2003).

Here, we presented the use of the technique of a thin vitreous layer performed by plunge freezing for detailed morphological characterization of various nanostructures. Nanoscopic vesicular structures have been the subject of considerable interest in different field of science over the last decades (Jin *et al*, 2010; Schwendener, 2007). Among them, the liposomes have been the most intensively studied nanosystems due to their unique properties such as non-toxicity, biodegradability, as well as simplicity of preparation and control over the composition and size.

Currently, they are frequently studied as possible vehicles (nanocarriers) for the delivery of therapeutic compounds, such as drugs or enzymes (Schwendener, 2007; Takeuchi *et al*, 2000; Takeuchi *et al*, 2001; Yaroslavov *et al*, 2008). Likewise, the DODAB vesicles (the cationic liposomes) may be used as nucleic acid carrier systems for gene transfection (Barreleiro, 2002; Li *et al*, 2008) or as vehicles for drug delivery (Pacheco & Carmona-Ribeiro, 2003; Shi *et al*, 2002). Unfortunately, the major problem with the liposomes is their low stability during storage, which is due to their strong tendency for degradation, aggregation, and fusion leading to uncontrolled leakage of the entrapped compounds (Takeuchi *et al*, 2001). Contrary to the liposomes, the morphology of structures, which are formed in the DODAB dispersions at RT, strongly depends on the method of preparation, which is not an easy task (Feitosa, 1997; Lopes *et al*, 2008). There have been a considerable number of attempts to overcome those problems (Haidar *et al*, 2008; Takeuchi *et al*, 2000). However, conventional TEM, as well as atomic force microscopy and scanning electron microscopy can not unambiguously answer questions about their morphology due to the destructive effects related to particles immobilization (adsorption on a support) or that of staining agents (Jin *et al*, 2010). Here, we presented the results of the first studies on successful stabilization of liposomes by covering their surface with a thin silicone layer using a novel silicone precursor. Next, we reported on the properties of bilayers composed of DODAB and OA at various molar ratios. The formed structures were clearly characterized with different methods; however, their detailed morphology could be evaluated only by cryo-TEM. The method of plunge freezing was used to produce thin vitrified aqueous films with the hydrated vesicles embedded in them. As the vesicles were directly imagined in a vitreous hydrated state, the perturbation of the objects was limited. We are confident that the samples were observed in their *in vivo* conformation; thus we were able to clearly characterize their morphology and distribution profiles. As shown, this method is practically unique and provides invaluable information neccessary for the development of these nanocarriers as well as other types of nanocarriers in a near future.

# 6 SUMMARY

The aim of this work was to develop a new protocol for the successful preparation of the yeast *Saccharomyces cerevisiae* for both fine ultrastructural and immunocytochemical TEM studies. Described methods covered: i) conventional aldehyde-based fixation procedure, ii) cryo-fixation by HPF, FS in four different media and low-temperature embedding into Lowicryl HM20, iii) thin sectioning, iv) immunolabeling using antibodies recognizing nucleolar (Nsr1 and Nop1), nuclear (Nsp1) and α-tubulin antigens, v) post-staining, and vi) TEM analysis. Each step of fixation and embedding protocol was assessed for its potential to retain both the morphology and antigenicity. As presented, sensitive antigens, which have been altered by conventional way of sample processing, were preserved after cryo-fixation followed by our FS protocol. In addition, the ultrastructural preservation of these samples was incomparable to that obtained with conventional processing method. Even though that only *S. cerevisiae* were investigated, we are confident that similar results can be obtained with other biological objects. In addition, the protocol allows variable alterations according to the desired field of study.

We also tried to address the question of the utilization and advantage of technique of cryo-TEM in the study of different vesicular structures (the liposomes, silicone-covered liposomes and DODAB/OA vesicles) in the nanometer range scale. We described how the method of plunge freezing followed by cryo-TEM imaging helps to elucidate data that cannot be obtained by alternative methods that could be used to characterize these vesicles, including analysis by conventional TEM. The cryo-TEM was used for both a visualization of the formation of objects in solution and for their morphological characterization. As the method allows direct imaging of hydrated samples in the most natural environment currently possible, limited perturbation of the objects was observed. The vitreous state of samples was maintained throughout the procedure.

Altogether, we wish to emphasize the importance of the methods presented here. Currently, the biological samples prepared by cryo-methods (HPF followed by FS) possess the highest level of ultrastructural preservation; and therefore these are the methods of choice for fine ultrastructural and immunocytochemical TEM analysis. For a high-resolution analysis of isolated macromolecular complexes or other types of biological nanomachines, the rapid freezing in a vitreous layer of water is currently the only technique that retains native state of the object before cryo-TEM imaging. In structural biology is the imaging of biological sample in its most native state a fundamental requirement. The development of currently presented methodologies is ongoing, and a number of improvements can be expected in a near future. We are therefore certain to gain invaluable cell biological insights. Unambiguously, these methods have a great potential to open new applications in a biomedical research.

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1. publikace *in extenso*, které jsou podkladem disertace

a) s impact faktorem

No.1 Mistrikova V, Bednar J: *Saccharomyces cerevisiae* nuclear and nucleolar antigen preservation for immunoelectron microscopy. Folia Biol (Praha) 2010; 56(3):97-109
Impact factor (2010): 0.729

No.2 Lewandowska J, Kepczynski M, Bednar J, Rzad E, Moravcikova V, Jachimska B, Nowakowska M: Silicone-stabilized liposomes. Colloid Polym Sci 2010, 288(1): 37-45
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Impact factor (2010): 2.861

b) bez impact faktoru: -

1. publikace *in extenso* bez vztahu k tématu disertace

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Moravčíková V, Bednár J.: Lokalizace rDNA transkripčních míst u *Saccharomyces cerevisiae*. In: Sborník 9. Studentská Vědecká Konference, konaná 21. května 2008, Galén, ISBN 978-80-7262-592-5, str. 22-23

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