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PRINCIPLES OF MEMBRANE PERMEABILIZATION OF CELLS AND ORGANELLES

BACHELOR THESIS

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PRINCIPY PERMEABILIZACE BUNĚČNÝCH MEMBRÁN A ORGANEL

BAKALÁŘSKÁ PRÁCE

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1)Prepare a review of methods usable for permeabilization of cells and organelles. In the Introduction part, focus on the overview of biomolecules occurring in the cell and organelles membranes. Then, describe various principles for controlled permeabilization of the membranes using different approaches, e.g. using lysis, detergents, toxins, ultrasound, etc. The aim of the bachelor thesis could be also seen in comparison of different methods and description of their dis-/advantages.
2)Use medical and scientific databases for preparation of the bachelor thesis, e.g. ScienceDirect, HighWire, NCBI Pubmed, etc.

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DECLARATION OF THE AUTHOR

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ANNOTATION

This thesis deals with the cellular and organellar membrane permeabilization and its occurence in physiological cell death and some pathophysiological processes with closer attention to cancer. Laboratory induced membrane permeabilization has found its importance within the development of treatment and better understanding of mechanisms staying behind certain diseases. Methods and their principles used to effectively disrupt the phospholipid bilayers in case of laboratory analysis has been described. Based on the increasing knowledge in the field of molecular biology, better treatment and early diagnostics of diseases can be achieved. This thesis helps to understand principles staying behind the cellular and organellar membrane permeabilization and analytical approaches used within the framework of evolving molecular analysis and cellular treatment.

KEYWORDS

Permeabilization, cell membranes, organellar membranes, cell lysis, cancer, apoptosis

ANOTACE

V rámci této bakalářské práce je rozebrána permeabilizace buněčných membrán a organel a výskyt tohoto jevu v rámci apoptózy a určitých patofyziologií, jako je rakovina. Představeny byly i některé metody umožňující úmyslně rozrušit buněčné membrány, aby bylo možné lépe porozumět těmto mechanismům na buněčné úrovni a umožnit tak vyvinutí vhodné léčby. S rostoucí znalostí molekulární biologie roste efektivita léčby a zlepšují se možnosti včasné diagnostiky různých onemocnění. Tato bakalářská práce přibližuje tématiku membránové permeabilizace a principy metod přispívající k vyvíjející se molekulární analýze a buněčnému zacházení.

KLÍČOVÁ SLOVA

Permeabilizace, buněčné membrány, membrány organel, buněčná lýze, rakovina, apoptoza

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LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
ATP	Adenosine triphosphate
ANT	Adenine nucleotide translocator
CypD	Cyclophilin D
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EndoG	Endonuclease G
ER	Endoplasmic reticulum
IM	Inner membrane
IMS	Intermembrane space
LMP	Lysozomal membrane permeabilization
MIMP	Mitochondrial inner membrane permeabilization
MMP	Mitochondrial membrane permeabilization
MOMP	Mitochondrial outer membrane permeabilization
MP	Membrane permeabilization
OM	Outer membrane
PTPC	Permeability transition pore complex
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SLO	Streptolysin O
UPR	Unfolded protein response
VDAC	Voltage dependent anion channel

1. INTRODUCTION TO MEMBRANE PERMEABILIZATION

Cell membranes and their properties, such as structure and dynamic function, are at the moment one of the most frequently discussed topics. Progressing research allows to understand their function and to map the structure which contributes to development of new permeabilization techniques or strategies used in order to analyze the intracellular material, moreover, to investigate the molecular base of multiple cellular processes. Another approach using inducible membrane permeabilization is to deliver some extracellular material into certain cells which is normally not allowed to cross the membrane. This allows to deliver special pharmaceutics or other important substances into the cells. Gentle and effective disruption of cell membrane is also the crucial step in a number of laboratory experiments.

Membrane permeabilization is a process of changing the permeability of cell membranes which corresponds to various physiological processes occurring within living cells. Membranes are dynamic tightly packed phospholipid structures maintaining the viability of the cell by their protective, separative or transport maintaining function. They possess unique composition and properties which allows them to be a part of variety of dynamic processes. One of these permeabilizing processes is simple pore formation, maintaining or enhancing the transport of molecules into and out of the cells. Pore forming complexes of proteins can be already present in certain membrane structure or can be created within particular molecular process. Changes of membrane permeability via pore forming proteins or simple osmotic diffusion helps to control the redox balance between intracellular and extracellular environment. Additionally, the permeabilization of mitochondrial and lysosomal membrane is a certain event occurring during the apoptosis, autophagy or necrosis thus plays an important role during the development, life cycle of cells or during certain response to pathogens provided by the immune system.

Disability of cells undergo apoptosis thus staying immortal is the typical property of cancer cells. The regulation of membrane permeabilization, targeting mitochondria or lysosomes, can then serve therapeutic purposes. Possible treatment of cancer by inducing apoptosis from outside or degenerative diseases by the inhibition of cellular death can be achieved. Besides that, the artificially generated membrane permeabilization has been also used in laboratory analysis using chemical or non-chemical methods for disruption of the cell membranes. This bachelor thesis gives an overview of naturally occurring membrane permeabilization, its meaning, importance and use in targeting within the occurrence of certain pathophysiological conditions. From outside induced permanent and transient disruption of cellular or organellar membranes certainly defines the laboratory research, thus methods used to permeabilize membranes are going to be depicted.

2. MEMBRANES OF CELLS

Cell membranes are structures which provide the boundary between living cells and the outside. Furthermore, membrane-bound intracellular compartments, termed organelles, can be distinguished. This chapter provides a short overview of structure and function of various cell membranes.

Almost all biological membranes are structured as lipid bilayers with addition of certain membrane proteins contributing to different functions of membranes. The composition of lipids influences physical properties of the membrane. Phospholipid molecules are composed of two nonpolar hydrophobic tails and a polar hydrophilic head which allows them to form non-covalently bonded structures, membranes. The interaction of hydrophobic tails which seek for being separated from water is achieved and bilayer is formed by enclosure of tails inside of the membrane surrounded by 'water loving' heads of phospholipids. Eukaryotic cell membranes are considered to be dynamic structures behaving according to the proposed fluid mosaic model. This model describes movements of lipid molecules within the membrane. Such movements enable membranes to form specific shapes. Location of hydrophobic fatty acids inside of membrane structure and hydrophilic phospholipid heads outside of the membrane makes sure that the membrane is strongly packed and selectively permeable (Elliot and Elliot, 2009, Lodish, 2013, Yeagle, 2016). In case of prokaryotic cells, especially bacteria, presence of another layers, cell wall, outer membrane or peptidoglycan, has been determined (Islam et al., 2017).

Plasma membrane of eukaryotic cells contains generally a lot of cholesterol, steroid lipid, which increases the stability and rigidity of a membrane. Another lipid molecules tightly associated with cholesterol within membrane structure are sphingolipids. These two kinds of lipid molecules form together tight and strong domains called lipid rafts. Cholesterol is replaced in certain bacterial membranes by molecules known as hopanoids which possess similar abilities to make the membrane more stable (Islam et al., 2017).

The function is attributed to their structure. Membranes play the main role in maintaining the flow of molecules into and out of cells, in giving the cell or organelle proper shape, in providing cell-cell interactions and communication through gap junctions or receptors incorporated in plasma membranes. An important function of membranes which needs to be mentioned is cell compartmentalization. By membrane enclosed compartments termed organelles, such as nucleus, endoplasmic reticulum, Golgi, mitochondria are situated in the cytosolic space. Additionally lysosomes, another membrane surrounded intracellular compartments, appear within the intracellular space. They differ in the structure, function and position within the cells. Their

membranes are also functionally distinct, therefore they differ in their lipid and protein composition. Broad proteomic analysis allows isolation of organelles thus identification of their membrane composition.

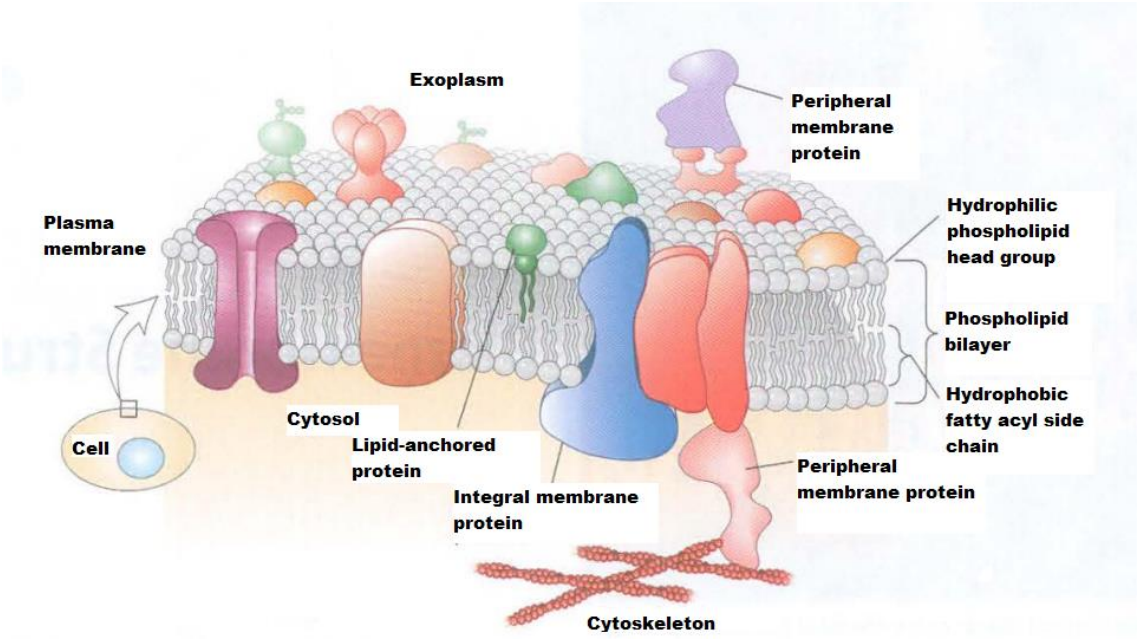

Figure 1: Plasma membrane (Lodish et al., 2013): *Composition of phospholipid bilayer with integral, lipid-anchored and peripheral proteins.*

2.1 The nucleus

The nucleus is enclosed by two membranes, outer and inner, forming the structure called the nuclear envelope. Nuclear membranes are structured as lipid bilayers with the presence of proteins such as integrins and others involved in signal transduction, gene regulation and chromatin positioning. These surface and integrated proteins preserve the unique function of the nucleus. Presence of nucleoporins, channels forming peptides, in the membrane maintains the permeability of nuclear envelope thus allowing the transport of genetic material into and out of nucleus. Besides the capability of component transfer between cytosol and inside of the nucleus, structure of the inside space, chromatin or nucleoli, is preserved by structure of membrane. Perinuclear space is formed between membranes and it is connected to rough endoplasmic reticulum (Ellenberg et al., 1997, Lodish, 2013, Hetzer, 2005). Permeabilization of nuclear membranes has found a place in the genetic analysis.

2.2 The endoplasmic reticulum and Golgi apparatus

Rough and smooth endoplasmic reticulum are parts of another internal membrane system present in eukaryotic cell. Both the endoplasmic reticulum (ER) and Golgi possess specific shape and structure which is maintained by special integral membrane proteins. The ER represents the place where the biosynthesis of variety of components, such as lipids and membrane proteins, occurs. The one single membrane of ER is also structured as phospholipid bilayer, moreover membrane of rough ER is loaded with ribosomes, structures where the translation process of proteosynthesis takes place. ER is within the function connected to another membrane system termed Golgi which is responsible for glycosylation, acetylation or other modification of proteins synthesized in ER. These proteins can retain in Golgi/ ER or can be secreted. Both organelles maintain the proper protein synthesis, modification and folding (Lodish, 2013, Schwarz and Blower, 2016).

2.3 The mitochondrion

Another intracellular compartment is the mitochondrion. Mitochondria serve as a site of biogenesis within eukaryotic cells because of the capability of ATP synthesis via oxidative degradation of variety of compounds. Mitochondria also serve as a main executioner in cell death, both apoptosis and necrosis (Kroemer et al., 2007). Structure and permeability of both mitochondrial membranes are significantly different and dynamic according to their function. Both membranes are formed as lipid bilayers with unique protein composition. The outer membrane is composed of proteins forming pores thus maintaining the transport of small metabolites and molecules. The inner membrane is almost impermeable and higher amounts of proteins, such as enzymes and proteins participating in ATP synthesis can be found within the structure of inner membrane. (Lodish, 2013). The inner barrier is characterized by its membrane maintains the stability of membrane structure and proton transfer. Intermembrane space is determined by outer and inner membrane. This space retains certain proteins involved in further cell signalling such as already mentioned programmed cell death or necrosis. Mitochondrial membrane permeability and its modification has been more closely examined therefore its principle and importance are discussed within subsequent chapters (Kroemer et al., 2007).

2.4 The lysosome

Lysosomes are organelles surrounded by a single but tightly packed membrane. The main function of these organelles is degradation of molecules which are transferred into lysosomes within certain degradative biological processes. Certain marked molecules are destroyed in lysosomes and

can later on participate in plasma repair processes or can be restored. Hydrolytic enzymes and low pH values define the intra-lysosomal space. The membranes of lysosomes have the structure of phospholipid bilayer enriched by membrane proteins maintaining the transport and communication between organelle and surrounding cytosol of the cells therefore they partly carry the responsibility for cellular homeostasis. Proteins associated with membrane of lysosomes play important role also in protection of lysosomal membrane from their own degradation by degradative molecules which are localised within the intra-organellar compartment. Cholesterol levels and sphingolipids are tightly connected together within the lysosomal membrane therefore the membrane is thick and rigid. Lysosomal importance has been pointed at and their role within multiple diseases has undergone lot of investigation. Closer attention to lysosomes and their membrane permeabilization is given further in this thesis (Bagshaw et al., 2005, Repnik et al., 2014).

3. MEMBRANE TRANSPORT

Cell membranes are responsible for isolation of intracellular or organellar compartments as well as for maintaining the transport of molecules into and out of the cells. The capability of cell membranes to control what passes through and therefore preservation of composition of internal space is called the selective permeability. Based on the properties of the phospholipid bilayer controlled transport of different molecules through the membrane can be determined. Biological membranes are permeable for small uncharged non-polar molecules which can easily cross the membrane by process termed passive transport. This type of membrane transport requires no energy because it is led by concentration gradient. Concentration gradient is determined by values of the concentration on both sides of the membrane. Membrane simply opens up for certain solutes which then flow from the side of higher concentration to the less concentrated environment. The concentration gradient is also followed by molecules passing through certain channels such as ion channels or water channels. Additional factor for driving ions across the membrane is membrane potential causing different states of polarization. This is well studied in case of neuronal signal transduction. Simple diffusion can be facilitated by certain carrier proteins present within the membrane structure. For certain substances the transport is coupled therefore presence and cooperation of multiple molecules is required.

In contrary, diffusion cannot occur when certain ions and molecules with bigger molecular mass and high polarity want to pass through or the transport against the concentration gradient is supposed to be driven. Transport of these molecules is enhanced by certain transmembrane proteins present within the membranes which are in need of certain amount of energy, thus it is called

the active transport. ATP molecules promote the transport across the cellular or organellar boundaries via hydrolysis of high energetic bonds present in ATP molecules. Because of the complexity and comprehensiveness staying behind this topic, the thesis does not contain detailed description of membrane transport mechanisms (Lodish, 2013).

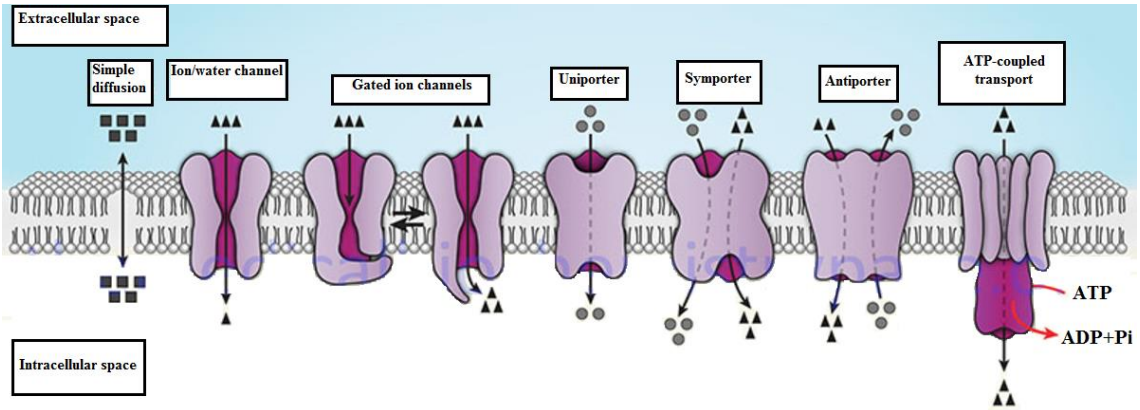

Figure 2: Membrane transport (www.themedicalbiochemistrypage.org)

4. APOPTOSIS

Apoptosis is programmed and closely controlled cell death. Any changes and dysfunctions of these controlled pathways lead to morphological changes in embryonic and postembryonic development or appearance of different diseases. Disability of cells to undergo the apoptosis is one of the features of cancer cells. On the other hand, excessive activation of apoptotic signalling leads to excessive dying of cells (e.g. neurodegenerative disorders or ischemia). The biochemical mechanism of apoptosis has been well studied in case of development the potential targeting strategies and controlling of cell death with cytotoxic or cytoprotective drugs (Kepp et al., 2011).

The process of mostly uncontrolled cell death, necrosis, has been closely linked to apoptosis therefore it is important to distinguish between these two processes. As mentioned above, apoptosis is highly controlled natural cell death while necrosis stands largely for uncontrolled dying of cells within the cellular stress response or response to injuries. Nevertheless some cases of controlled necrosis have emerged but have not been well described yet (Galluzi et al., 2014). Involvement of membrane permeabilization has been proposed in case of both, apoptosis and necrosis. Permanent permeabilization of plasma membrane serves as a characteristic feature of dying cell. Mitochondrial membrane permeabilization appears to be linked to apoptosis and necrosis via release of proteins normally enclosed within the intramembrane space. Consequent disruption of lysosomal membrane has been shown to play major role in process of necrosis and potentially

contributes to mitochondrial membrane permeabilization. All of these mechanisms and their role in cell death is going to be discussed in following chapters (Kepp et al., 2011, Ferri and Kroemer, 2001).

The process of apoptotic signalling is complex and can be accomplished via two major pathways summarized in figure 2. The extrinsic pathway is shown on the left side. Death receptors present on the surface of cells are activated by certain ligands whereby special proteases, caspases, can be activated. The intrinsic pathway depicted on the right side of the figure 2 is activated by several intracellular events, such as DNA damage and within the response to stress stimuli in endoplasmic reticulum. Moreover, also nucleus, lysosomes and Golgi apparatus can be involved in the initiation of apoptosis. The role of mitochondria appeared to be crucial in apoptosis. Especially permeabilization of mitochondrial membrane allows the release of proteins which can activate downstream apoptotic cascade, i.e., effector caspases. (Kroemer et al., 2007, Reed, 2000, Wang, 2016).

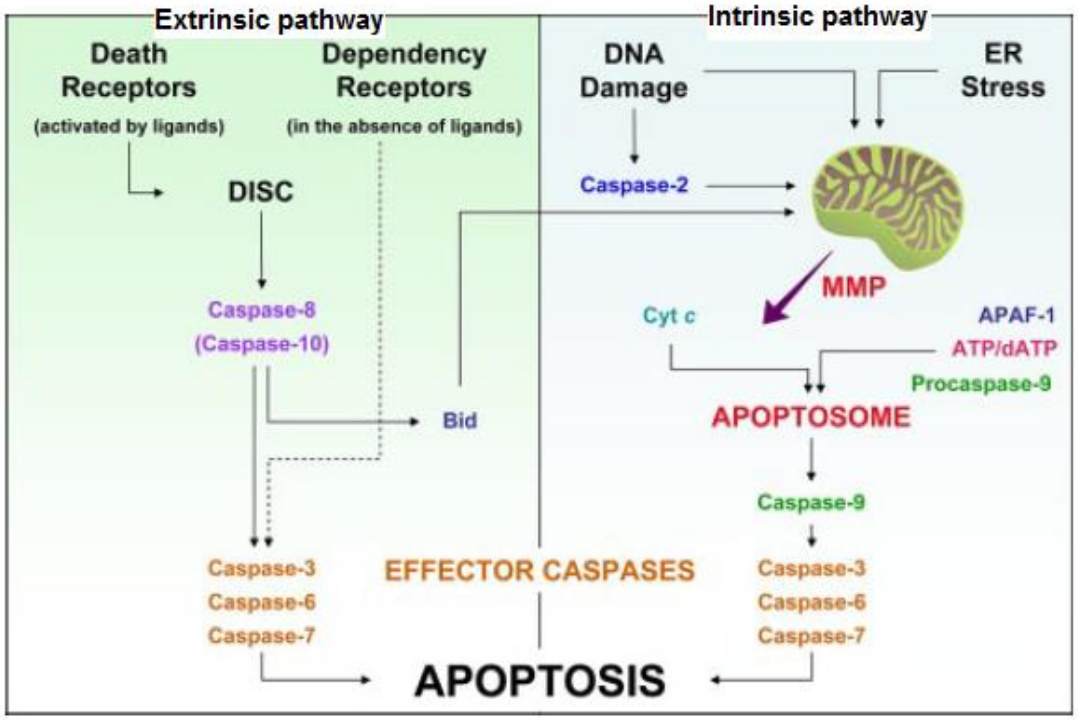

Figure 3: Apoptosis (Kroemer et al., 2007): *Left: Extrinsic pathway based on activation of death receptors or in the absence of ligands using dependency receptors. Forming of death-inducing signalling complex (DISC) which activates caspase-8 or caspase-10. This leads to apoptosis through cleavage of caspase-3, caspase-6 and 7. Another function of caspase-8 or 10 is to activate Bid protein causing mitochondrial membrane permeabilization (MMP). Bid provides the link between extrinsic and intrinsic cascade described on the right side of the figure. Several intracellular signals such as DNA damage or ER stress induce the MMP which leads to release of cytochrome c (Cyt c), proapoptotic factor, which with contribution of apoptosis protease-activating factor 1 (APAF-1) and ATP/dATP assemble the large protein structure, apoptosome. Apoptosome is capable of activation of caspase 9 followed by activation of the effector caspases 3, 6 and 7 which promote apoptosis. DNA damage can favour the MMP also through caspase 2.*

5. MITOCHONDRIAL MEMBRANE PERMEABILIZATION

Mitochondria are known as organelles serving as a metabolic centre of cells. They are responsible for the production of energy in the form of ATP by oxidative phosphorylation located in the inside of mitochondria. In addition, they possess the ability to control and participate in a variety of other processes, including synthesis of biomolecules, production of reactive oxygen species, cell death etc. (Kroemer et al., 2007). Presence of two membranes surrounding the mitochondria has been shown. The inner membrane is barely permeable to ions and water whereas the outer membrane has been shown to be loaded by certain proteins allowing the small metabolites and solutes up to 5 kDa to pass through. The structure named voltage dependent anion channel is the key protein complex (Kroemer et al., 2007). The inner membrane is determined by its inner mitochondrial transmembrane potential ($\Delta\Psi m$) created by proton gradient required for oxidative phosphorylation and the following ATP synthesis (Kroemer et al., 2007).

Mitochondrial membrane permeabilization (MMP) has been identified as a hallmark of early apoptosis. Occurrence of mitochondrial outer membrane permeabilization (MOMP) can lead to release of certain proteins localized within the intermembrane space which are normally not allowed to cross the outer membrane. These proteins released from mitochondria to the cytosol trigger the apoptotic pathways leading the cell to immediate death (Henry-Mowatt et al., 2004).

Two membranes, outer membrane (OM) and inner membrane (IM), together build the mitochondria. Both of them has a different composition and properties, such as permeability described above. Different mechanisms of membrane permeabilization which can occur under the physiological and patophysiological conditions can be distinguished (Kroemer et al., 2007, Jacotot et al., 1999, Breckenridge and Xue, 2004).

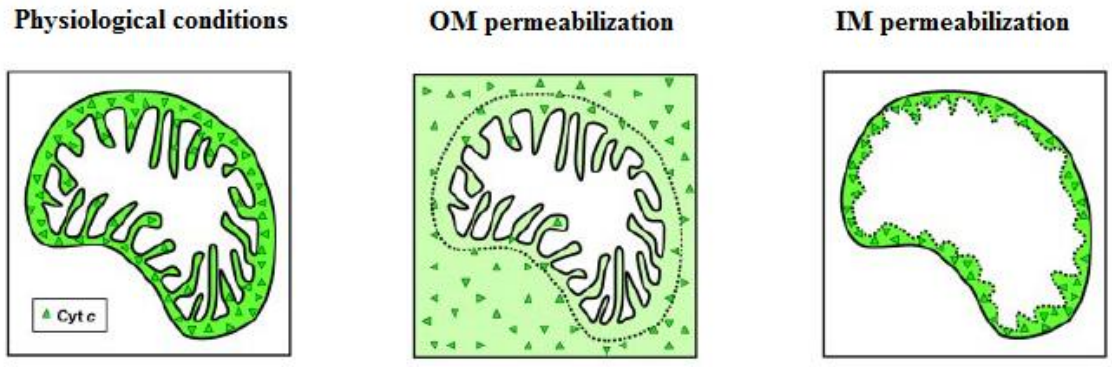

Figure 4: Mitochondrial membrane permeabilization (Kroemer et al., 2007): *Illustration of mitochondrial membrane integrity under physiological conditions, in case of outer membrane (OM) permeabilization and inner membrane (IM) permeabilization. Visualization is based on monitoring the retention or release of cytochrome c (Cyt c.) from intermembrane space.*

5.1 The outer membrane permeabilization

Outer membrane is the direct barrier between cytosol and intermembrane space (IMS) of mitochondria. Proteins of IMS, such as cytochrome c and other pro-apoptotic factors, factors favouring the apoptosis, can be released from the inside of these organelles into cytosol as a result of their permeabilization. Therefore, the mediation of mitochondrial outer membrane permeabilization (MOMP), appeared to be tightly regulated process. Understanding the mechanisms, under which conditions MOMP occurs, which compounds take part in the process or which substances can mediate the disruption of outer mitochondrial membrane helps to develop the selective targeting therapy used in treatment of multiple diseases.

Bcl-2 family of proteins can be named as the main family of interest because of its crucial effect on the mitochondrial outer membrane. These proteins can be sorted into two groups according to their abilities to initiate or inhibit the further apoptosis of cells mediated via MOMP. Bcl-2 and Bcl-XL are anti-apoptotic proteins which have protective effect on mitochondria, thus MMP does not occur under their protection. The basic mechanism of their operation can be summarized as neutralization of pro-apoptotic proteins which are objects of following discussion. These proteins are able to act on a mitochondrial outer membrane and causing its permeabilization which favours apoptosis. Bax, Bak, Bid and Bad belong to this group of proteins (Chipuk and Green, 2008, Green and Kroemer, 2004).

Bax can be found within the cytosol and has direct effect on OM. This protein contains three BH domains allowing Bax to be inserted into OM of mitochondria. Bax undergoes some conformational changes and forms pores which disrupt the structure of the membrane. Bak, in contrary to Bax, is a protein already present in mitochondrial membrane, nevertheless the mechanism of pore formation appears to be similar. Two other mentioned proteins favouring apoptosis, Bid and Bad, are proteins facilitating the action of Bax or Bak. They provide stronger interaction with anti-apoptotic proteins, therefore allows inhibited Bax or Bak to be released and to act on the outer mitochondrial membrane via pore formation (Tait and Green, 2010).

Voltage-dependent anion channel (VDAC) is considered major participant in outer membrane permeabilization process. This transporting membrane protein is responsible for the certain permeability of OM. Metabolites <5000 kDa are allowed to pass through the membrane after VDAC opening. It has been suggested that VDAC could be a site of Bax, Bak, Bcl-XL and Bcl-2 action. These proteins can directly cause the opening or closure of VDAC. Physiologically The VDAC is in charge of what passes through the outer membrane. Nevertheless the irreversible

openings leading to programmed cell death via release of apoptotic factors can be created under certain conditions (Kroemer et al., 2007, Tsujimoto and Shimizu, 2000).

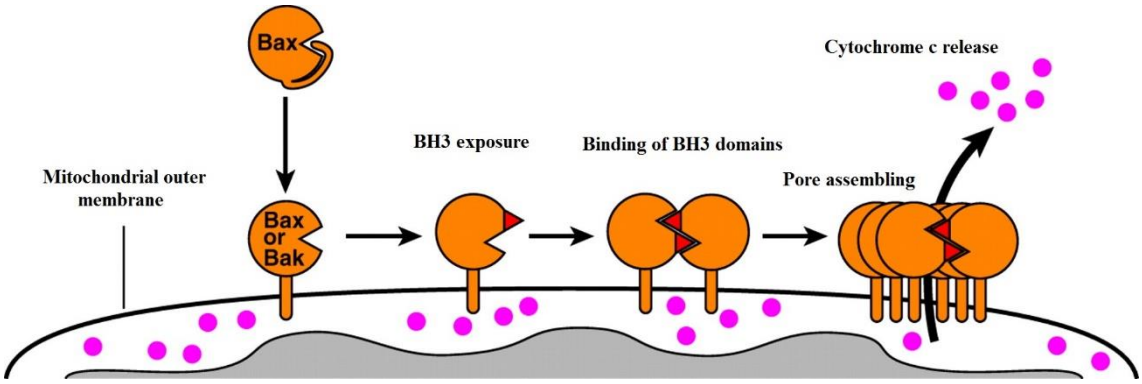

***Figure 5: Action of Bax or Bak proteins (Dewson and Kluck, 2009)**: Pore formation as a result of the oligomerization of pro-apoptotic Bax and Bak..*

5.2 The inner membrane permeabilization

The major impact on apoptosis induction via MMP has the permeabilization of outer mitochondrial membrane. What is the role of the inner membrane in apoptotic signalling is still a matter of current research. However, some observations have been made. The inner boundary is permeable for small solutes, including water and ions. This allows the membrane potential to be established across the membrane. Collapse of this potential can lead to the destabilization of phospholipid structure and inner membrane permeabilization. This change of permeability of inner membrane happens via formation of permeability transition pore (PTP) which is a channel formed in the inner membrane of mitochondria. Major component with pore-forming abilities is adenine nucleotide translocator (ANT). This protein is under physiological conditions responsible for ADP/ATP exchange through the inner membrane, nevertheless in case of apoptotic signalling, can form larger and permanent pores in the inner mitochondrial membrane. ANT, part of assembled permeability transition pore complex, has been shown to interact with multiple proteins playing roles in maintaining or enhancing the MMP. One of these substances is cyclophilin D (CyPD) which is present in mitochondrial matrix of mammalian cells. CyPD binds the ANT and thus partly regulates the function of the whole complex. Its participation in certain pathophysiological processes has been proposed. CypD can nevertheless serve as potential target in regulation of MMP from outside (Giorgio et al., 2010, Lemasters et al., 2002). To allow solutes to be transferred across both mitochondrial membranes, the cooperation of both OM and IM components is required. Formation of dynamic structure, yet not fully understood, named permeability transition pore complex occurs.

5.3 The permeability transition pore complex

Permeability transition pore complex (PTPC) is a mitochondrial membrane channel which is composed of several proteins and connects outer membrane (OM) with the inner membrane (IM) of mitochondria. Full protein composition was not discovered yet nevertheless components such as voltage-dependent anion channel (VDAC) and adenine nucleotide translocator (ANT) have been proposed to play major roles in forming the PTPC by providing the contact site between both mitochondrial membranes.

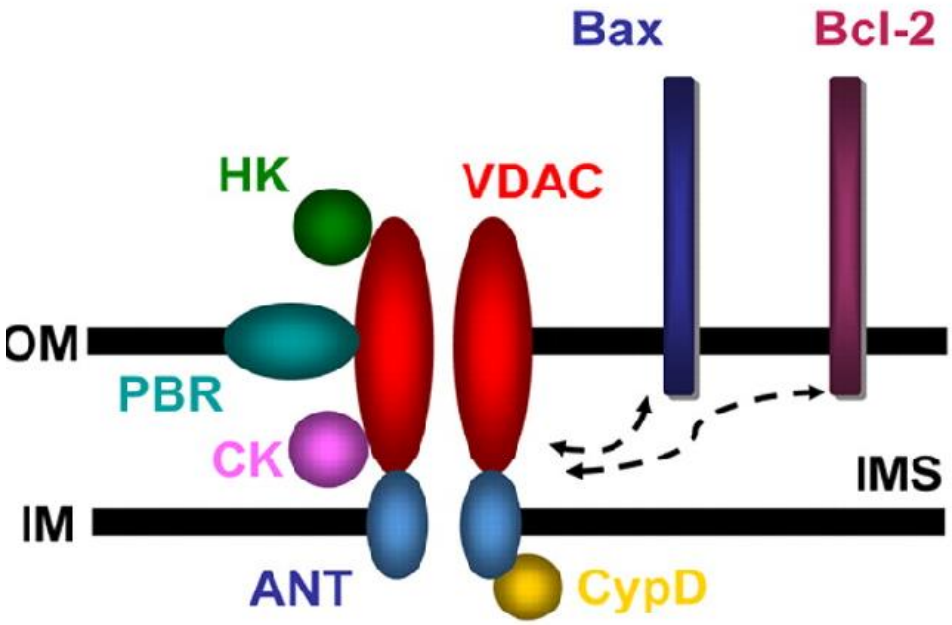

Figure 6: Permeability transition pore complex (PTPC) (Kroemer et al., 2007): *PTPC is composed of two major components, such as voltage-dependent anion channel (VDAC) and adenine nucleotide translocator (ANT). There are present as well some additional components, such as hexokinase (HK) interacting with VDAC from cytosol, creatine kinase (CK) coming from intermembrane space (IMS), peripheral-type benzodiazepine receptor (PBR) inserted in outer membrane (OM) and cyclophylin D (CypD) present in mitochondrial matrix and binding to ANT. Bax and Bcl-2 proteins, modulating the pore activity, are also shown in the picture.*

PTPC complex is located on contact site of mitochondrial inner membrane and outer membrane. Formation of the PTPC and its opening under certain circumstances, which are still a part of broad discussion, can lead to mitochondrial outer and inner membrane permeabilization. When the channel undergoes conformational changes leading to its opening, inner mitochondrial membrane potential changes thus its permeability changes too. Inner mitochondrial space starts swelling which subsequently breaks the membrane and allows the release of pro-apoptotic factors into intermembrane space. Consequential rupture of outer membrane, i.e. outer membrane permeabilization, causes the release of these factors out of the intermembrane space and the apoptotic cascade can be triggered. As already mentioned, understanding the mechanism

of permeabilization of both membranes and their potential cooperation can help to develop and/or to improve the treatment of variety of diseases. CypD can be mentioned as an example of pharmacological target of cyclosporin A in mitochondria. This drug is capable of selective inhibition of CypD expression therefore mitochondrial membrane can be protected from permeabilization and programed cell death does not occur. Nevertheless, it has to be taken into account that there are other factors playing important roles in the mechanism of membrane permeabilization thus this is just a one way of potential targeting (Vieira et al., 2000).

6. INFLUENCE OF OTHER ORGANELLES

As previously described, mitochondria play a crucial role in cellular apoptosis. Moreover they are considered the central organelles in programmed cell death. Apoptosis is tightly regulated process and except mitochondria, contribution of other cellular compartments and biochemical processes has been proposed. Organelles such as ER, Golgi or lysosomes do not stay intact during the apoptotic process. They undergo certain biochemical changes or even destruction, therefore, they have been suspected from being involved in the performance of programmed cell death. They can act as direct activators of caspases or they can maintain the inter-organellar cross-talk between those contributors and mitochondria. As a result of their action the apoptotic pathway including MMP can be stimulated. It needs to be mentioned that these processes, caspases activation and MMP are interconnected. Tight cooperation has been proposed and shown in the following figure (Ferri and Kroemer, 2001). Closer look at contribution of the nucleus, ER, Golgi and lysosomes in cell death direct and especially apoptosis mediated via mitochondrial membrane permeabilization is given within this chapter.

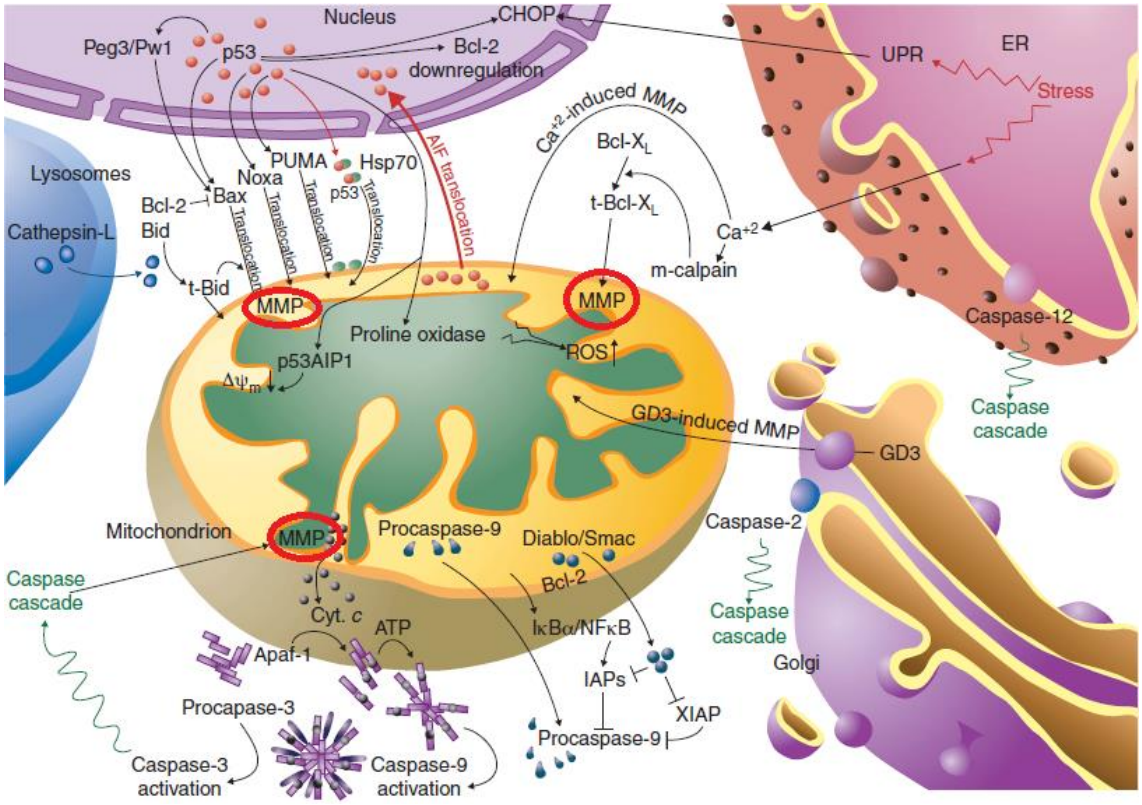

Figure 7: Regulation of mitochondrial membrane permeabilization (Ferri and Kroemer, 2001): *Contribution of Endoplasmic reticulum (ER), nucleus, Golgi apparatus and lysosomes to mitochondrial membrane permeabilization (MMP).*

6.1 Endoplasmic reticulum

Endoplasmic reticulum (ER), is involved in sensing the local cellular stress. It possesses chaperones, calcium channels and calcium binding proteins which can trigger the mitochondrial membrane permeabilization. Moreover some Bcl-2 proteins are placed within the ER. Two major processes involved in the initiation of apoptosis can be mentioned based on these components of ER - calcium signalling and unfolded protein response (UPR).

Unfolded protein response is a complex process involving a number of enzymes and transcription factors. It occurs in the ER in case of incorrect protein folding within the response to ER stress. In this case the defect in protein folding can be repaired or the cell death cascade can be initiated (Cao and Kaufman, 2012). Because of the amount of molecules involved in mechanism of UPR initiating the apoptosis and its complexity the process is not discussed in details. Nevertheless the final step in UPR regulation has shown the downregulation of familiar Bcl-2 anti-apoptotic protein expression thus induction of apoptosis is favoured (Ferri and Kroemer, 2001).

The second mentioned process by which ER contributes to initiation of apoptosis and MMP includes calcium ions stored within the intracellular space. Procaspase-12, similar to caspase 4,

is connected to the ER membrane from the outside. Mobilization of calcium ions during the ER stress response leads to the activation of this caspase by calcium dependent protease named m-calpain. This results in following induction of direct caspase mediated apoptotic signalling. Moreover, the m-calpain molecule has been shown to cleave Bcl-XL protein which has the role of a pro-apoptotic factor maintaining the permeabilization of mitochondrial membrane (Ferri and Kroemer, 2001). It has been proposed that calcium ions are involved in more complex regulation of MMP which are not fully understood yet. Two main mechanisms related to ER regulation of MMP, UPR and calcium influence, has been pointed at in this chapter.

6.2 Golgi apparatus

Mediation of apoptosis can be enhanced by contribution of Golgi apparatus (GA). Proteins, especially caspase-2, which is involved in triggering MMP, is located in the membrane of GA (Ferri and Kroemer, 2001). Another important molecule appeared to be related to this topic. The ganglioside GD3, as a pro-apoptotic mediator, can be generated by GD3 synthase within the Golgi. Translocation of GD3 from GA to mitochondrial membrane is involved in MMP mediated apoptosis. This molecule directly affect the transmembrane potential of mitochondrial membrane therefore the permeabilization is caused and cytochrome c, AIF or caspase 9 are released from the intramembrane space. These released molecules then participate in further downstream apoptotic signalling (Rippo et al., 2000).

Golgi apparatus has been also linked to cancer because of the protein glycosylation which occurs within this organelle. Glycosylation is a process of posttranslational modification when additional sugar residues are added to glycoproteins or glycolipids made in ER thereby glycan molecules are synthesised. This process is important for further molecular trafficking, cell growth or cell survival (Stowell et al. 2015). Inhibition of this process can possibly induce the apoptosis. The treatment using the cancer selective inhibitors has been studied by the number of researchers (Ferri and Kroemer, 2001, Goss et al., 1995).

6.3 Nucleus

Another important process involved in initiation of apoptotic signalling is DNA damage which is localised in nucleus. The p53, an important tumor suppressor and transcription factor, may be the key molecule in the apoptotic initiation pathway due to its capability of regulation of the expression of certain Bcl-2 family proteins. Genes coding for nti-apoptotic members of Bcl-2 protein family can be downregulated by p53 in the nucleus. In addition to downregulation, upregulation of genes representing pro-apoptotic proteins such as Bax, PUMA or Noxa can be

induced by p53 molecule. These proteins are then transferred to cytosol where they can target the mitochondrial membrane thus induce MMP leading to cellular death (Oda et al., 2000). Not only this mechanism linked to the nuclear contribution, has been proposed to favour the apoptosis. The p53 molecules can induce MMP via regulation of expression of several other proteins which can be involved in operation of reactive oxygen species or act via direct loss of membrane potential. Nevertheless, these mechanisms have not been completely understood yet and p53 regulation is still the major part of many current research projects (Ferri and Kroemer, 2001).

6.4 Lysosomes

Lysosomes are organelles involved in autophagy and necrosis. Besides these two death mechanisms, apoptotic process might involve lysosomes as well. It has been proposed that lysosomes can cooperate with mitochondria in initiation of apoptosis via contribution to MMP (Ferri and Kroemer, 2001). The lysosomes and mitochondria remain closely connected within the process of cell death. Key molecules implicated in this complex relationship are cathepsins. Cathepsins are major proteases involved in number of biological processes such as antigen presentation, cell signalling, degradation or processing of different molecules (Turk et al., 2012). In case of lysosomal membrane destabilization cathepsins can be released from the intra-lysosomal space, thus serving the purpose of cellular death. Translocation of cathepsins to mitochondria leads to destabilization of mitochondrial outer membrane and release of cytochrome c, one of the main mediators of programmed cell death. The induction of MMP enhanced by cathepsins has shown that these proteases can transform Bid molecule into t-Bid. Bax and Bak pro-apoptotic proteins are regulated by t-Bid, therefore, they can undergo oligomerization on the outer mitochondrial membrane. As a result of this event, pores in the membrane are created (Repnik et al., 2014, Nylandsted et al., 2004).

This chapter has clarified the contribution of lysosomes to MMP induction nevertheless the independent activation of caspase cascade has been proposed to be triggered by lysosomal membrane permeabilization. The mechanism has not been clearly described yet but the existence of LMP, its induction connected to the cell death and, on the other hand, the inhibition of LMP leading to the cell survival has found the place in the treatment of multiple diseases.

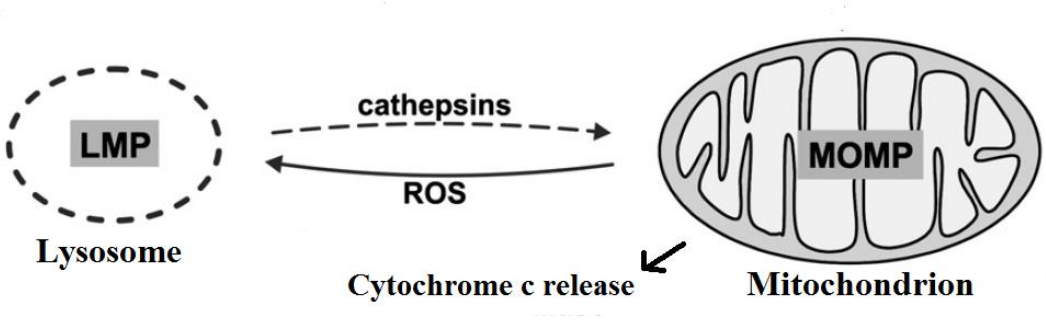

Figure 8: Cathepsins (Repnik et al., 2014): *Release of cathepsins from lysosomes within the response to oxidative stress and cathepsins translocation to mitochondria is depictured. Translocation is followed by destabilization of mitochondrial membrane and cytochrome c release. Lysosomal permeabilization (LMP) is proposed to be connected to mitochondrial outer membrane permeabilization (MOMP).*

7. LYSOSOMAL MEMBRANE PERMEABILIZATION

This chapter is going to provide some information about lysosomal membrane permeabilization (LMP) which has been largely studied in case of cancer treatment. Lysosomes are membrane-bound organelles which contain certain hydrolytic enzymes responsible for degradation of cellular material when needed. Because of the possible rupture of the lysosomal membrane these enzymes can be released from intracellular compartment and trigger the programmed cell death. The induction of cell death appeared to be possible via independent activation of caspases cascade due to direct LMP and also via tight cooperation with mitochondria, closely via the influence on destabilization of mitochondrial membrane. The exact mechanism of lysosomal action in apoptosis and the cooperation with mitochondria has not been fully described yet nevertheless the importance of LMP and its direct targeting is certain (Repnik et al., 2014, Nylandsted et al., 2004, Ferri and Kroemer, 2001).

The content of lysosomal membrane certainly plays a role in lysosomal membrane permeabilization. Not only levels of cholesterol and sphingolipids matter but also the composition of membrane associated proteins, which can serve as targets for induced selective permeabilization. Pore formation and followed disintegrstion of lysosomal membrane also allows the transfer of certain compounds into the lysosomes therefore the delivery of drugs and other therapeutic agents into cytosol of cells can be achieved (Repnik et al. 2014).

Two major mechanisms have been studied in case of lysosomal membrane permeabilization. The first one includes the lysosomal membrane disruption caused by increase of osmotic pressure within the lysosomal organelle. Increased pressure inside of lysosomes leads to their lysis. The integrity of lysosomal membrane can be also disrupted by certain compounds acting as surfactants. These are agents or compounds generally changing the surface tension of different molecules thus the direct membrane destabilization can be induced. It is necessary to mention that

lysosomes are part of dynamic endocytic pathway, therefore, later stages of this organelle already underwent certain changes, such as accumulation of different components or changes in the membrane composition, which might favour LMP (Repnik et al., 2014).

Compounds used for effective lysosomal membrane permeabilization are known as lysosomotropic agents which can accumulate within the lysosomes and cause their lysis from the inside of the organelle. These compounds are selective for lysosomes, nevertheless they act similarly as detergents which are going to be described on one of the following chapters.

8. TARGETING THERAPY

Theoretically speaking, all compounds involved in mechanisms which imply certain permeabilization of different membranes described above can serve as potential targets. These targets would allow potential regulation of membrane permeabilization (MP) therefore the regulation of apoptosis which has been identified as a source of multiple diseases affecting for instance the central nervous system, cardiovascular system or other organ systems in human body. Moreover, the inability of cells undergoing the apoptosis, immortality, is a major feature of cancer cells. It has been proposed that certain targeting might favour the cancer treatment. Lately discovered importance of mitochondria and their progressive targeting allowing the selective induction of mitochondrial membrane permeabilization followed by release of pro-apoptotic factors gained attention of scientific world (Gogvadze et al., 2008, Galluzzi et al., 2006).

The cell death can be controlled in both ways, enhanced or inhibited, via MP in context of various pathological process responsible for particular diseases, such as neurodegenerative diseases or cancer. Therapeutic control of MP, especially mitochondrial MP, is considered to be one of the most promising strategies applied in the treatment of diseases based on uncontrolled apoptosis. Either pharmacological or genetic approach can be chosen (Reed, 2002). Determination of protein structure allowed by nuclear magnetic resonance analysis might lead to discoveries of antagonists or inhibitors of Bcl-2 family of proteins which, as already mentioned, play an important role in MP regulation. These synthetic peptides have become part of broad field of research especially in the field of developing cancer therapies (Costantini et al., 2000).

9. EXTERNALLY INDUCED PERMEABILIZATION

Membranes create boundaries which isolate the intracellular compartment. Together with determined membrane permeability and other properties viability of each cell is maintained. The induced permeabilization of cell membranes and isolated organelles allows the access to the intracellular matrix and compartments of cell or organelle. Disruption of membranes is mostly performed as a first step in multiple analysis, such as protein extraction, study of intracellular processes or genetic manipulation. Efficient membrane permeabilization also allows therapeutic agents or antibodies to be delivered into cells and organelles when needed. Permanent membrane permeabilization stands for cell lysis which can be performed when simple isolation of intracellular or organellar components is desired. Moreover, permeabilization techniques used for transient poration or partial disruption of membrane structures appeared to be important for drug delivery and other experiments.

Different origin and composition of membranes, or eventually cell walls in case of plants or bacteria, have been previously pointed at and so have to be considered for choosing the right technique in each analysis. Also the nature of further downstream analysis has to be taken into consideration in order to choose the right agents or technique to efficiently disrupt the membrane of cells. Breaking down the membrane, temporary or permanently, and such enter the intracellular space can be performed via multiple ways. Mechanical, acoustic, optical, electrical or chemical approaches can be used for efficient cell lysis and permeabilization (Brown and Audet, 2008, Islam et al., 2017). This chapter provides an overview in commonly used techniques enabling transient and permanent membrane permeabilization. For the lucidity of the context the methods have been sorted into two groups based on the nature of certain methods. Methods applying chemical treatment of cells and methods with no use of special chemicals have been listed.

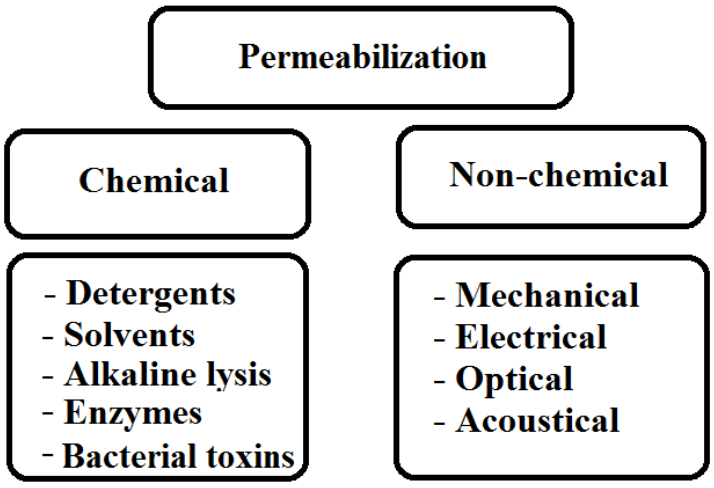

Figure 9: Methods used to membrane permeabilization: *Two sections of methods have been created based on the character of certain method.*

9.1 Non-chemical methods

Methods listed as non-chemical methods review approaches which do not include methods using certain chemicals such as enzymes, detergents or other agents to disintegrate the membrane structure in order to perform, for instance, certain downstream analysis of isolated components or to maintain drug delivery. These methods use mechanical, electrical or optical approaches. The special equipment, tools enabling membrane disruption and further analysis of intracellular space, organelles or integrated membrane compounds has to be available in case of using non-chemical methods.

9.1.1 Mechanical methods

Mechanical cell lysis uses direct mechanical disruption of cell membranes by application of particles with sharp surface for induction of cell lysis. Within application of these methods cell membrane and even cell wall is mostly broken therefore the use on organisms which possess thick cell walls is possible. Moreover, bacteria can evolve certain resistance to chemical treatment therefore the mechanical lysis can be used. Using mechanical approaches is considered to be suitable for analysis of intracellular compartment and parts of membrane structure. Mixers and blenders could be used for disruption of plant or animal tissues. Within using this technique the whole tissue is processed. Cells are grinded, homogenised and intracellular compartment is accessible. This method involves using of reducing agents and inhibitors which prevent degradation of extracted enzymes and proteins (Roe, 2001, Vandeventer et al., 2011).

Yeast, algae, fungi and microorganisms are organisms used in multiple analysis therefore suitable method for their disruption is needed. Bead mill could serve as a suitable disruptive technique for release of intracellular components. Flexible bead size, volume and speed allows to increase the efficiency within certain tissues. Shearing forces mechanically with accurate pressure disrupt the membrane of cells which are grinded between the beads. Beads parameters and time of performed technique appeared to be crucial for potent application. Advantage of using these techniques is that no closer attention to the buffer is needed. Lysis can be performed in physiological buffer because the disruption is only mechanical. The fact that intracellular components do not leave the physiological environment allows their release in their native forms which favours further downstream analysis (Roe, 2001).

High pressure homogenizers are considered another method used especially for large scale disruption of microbial cells. Cell suspension is forced through orifice at high pressure while being cooling down. Increased temperature could have an effect on functions and structure of proteins

or enzymes of interest. By repeated pumping of cell suspension through the orifice under the suitable pressure conditions, cell membrane disruption is achieved (Roe, 2001).

Another method which involves significant, but this time desired, heat is a simple heat shock application. Cells are incubated at higher temperature. Then the temperature is decreased in a short time period. Temperature and number of cycles depend on the kind of cell type and experiment. As mentioned before heating does not allow proteins to be further analysed because of their denaturation and loss of activity. Nevertheless this simple heat shock method allows us easily enter the cellular compartment, especially microbial cells. Shock using procedures applied on cells can also use the osmotic pressure. Incubation of cells in solution of high osmotic pressure and immediate transfer to environment with lower pressure changes the flow of water across the membrane thus the membrane can be disrupted (Roe, 2001).

Controlled freezing and thawing is applicable on animal cells because they do not possess the cell wall. This method has been shown to be selective for plasma membranes. (Mardones and González, 2001). However protein denaturation can occur due to need of repetition of cell cycles thus repeated changing of temperature levels.

9.1.2 Electrical methods

Application of short electric pulses on membrane is called electroporation. This technique, also reviewed as electropermeabilization, became popular in genetic analysis and molecular transfer especially plasmids DNA, RNA, proteins or drugs (Faurie et al., 2005). Membrane structures are exposed to electric field created by positive and negative electrodes. External electric field generates transmembrane potential which, above the certain threshold, is capable of membrane destabilization. After electric pulses reach the membrane and cross the threshold, water may enter the cell. Because of hydrophobic and hydrophilic composition of phospholipid bilayer, transient hydrophilic pores are proposed to be formed. This threshold, thus size and exposure time of applied electric field, differs with type of cell and organelle. Both, the method itself and used equipment, did undergo multiple experimental optimizations and set-ups in order to achieve efficient permeabilization of different membranes as possible (Gehl et al., 2003.). Moreover, both irreversible and transient permeabilization can be achieved. Transient pore formation induced by electric field enhance delivery of chemotherapeutics in tumor cells. Application of ultra-short pulses in a nanosecond scale allows permeabilization of organelles inside of a cell without breaking the integrity of plasma membrane thus allows potential delivery of cytosolic compounds directly into organelles. Direct permeabilization for instance of mitochondria via electroporation brings possibility to study downstream pathways of apoptosis linked to MMP (Schoenbach et al., 2001).

Irreversible electroporation may find use in certain cancer treatment via re-distribution of membrane-linked receptors important for further immune-cell signalling. These certain changes could induce immune cell response to tumor cells (Goswami et al., 2017).

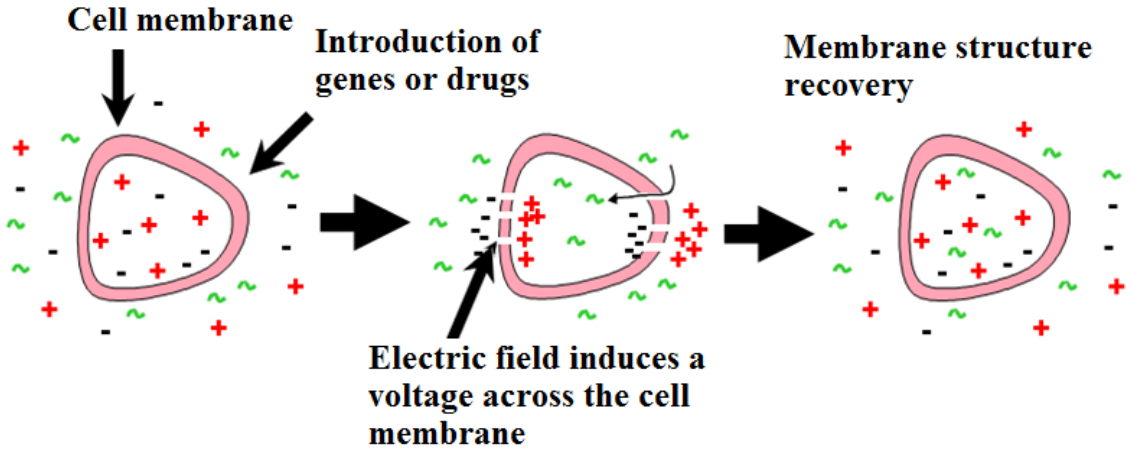

Figure 10: Electroporation (Xiong et al. 2014): *Gene or drugs are introduced to the cell. Membrane pores are created within the application of electric field therefore extracellular molecules can enter the cell. After cell stopped being exposed to electric pulses the membrane structure is reformed.*

9.1.3 Acoustical methods

Certain laboratory uses favour performing membrane permeabilization by ultrasonication. This is another relatively simple technique using acoustic approach to break down the cell membrane permanently thus cause the cell lysis. Besides the lytic effect the transient permeabilization for gene delivery experiments can be invoked by using acoustic approaches. This approach causing transient permeabilization of cell membranes is reviewed as sonoporation and found assertion in development of new gene therapies and cell transfection mechanisms (Miller et al., 2002). Ultrasonic waves generated in ultrasonicator create high pressure but small size areas within the cell suspension. Occurrence of cavitation bubbles follows and later on the cell membrane is permanently or temporally disrupted in these certain areas with small pore which has been created after application of ultrasonic sounds. This technique, in case of cell lysis, nevertheless involves significant heating which often leads to denaturation of some proteins therefore further analysis of proteins may not be possible. (Roe, 2001, Miller et al., 2002). Acoustic approaches are shown to be possibly used for enhancing treatment of tumor cells by direct membrane targeting or by non-invasive approach for sufficient drug delivery into tumor cells (Prentice et al., 2005).

9.1.4 Optical methods

Optical lysis is a high speed technique relatively recently used in single cell lysis. Speed of this technique allows analysis of multiple highly dynamic and time sensitive processes in certain

cells. This technique enables fast creation of various gaps in membrane on cellular and subcellular levels without any damages of macromolecules and components within inside of cells. Principle of this technique is in pulsed laser radiation via laser microbeams. Focused laser light beams causes propagation of a shock wave therefore a dynamic cavitation bubbles can be created. Formation of bubbles disintegrates the membrane structure. Light responsible for induction of membrane permeabilization can be modulated thus cause the permeabilization on both cellular and subcellular level (Rau et al., 2006, Brown and Audet, 2008). New approach has been tested to improve selectivity and efficiency of laser induced cell lysis. This approach involves use of nanoparticles bound to membranes which can respond to laser light thus enhance the membrane permeabilization. Two parameters, light and parameters of used particles such as binding selectivity and size, can be controlled and manipulated to achieve desired selective membrane permeabilization. Optimization allows another technique to sufficient gene therapy and gene transfection to be created (Yao et al., 2005).

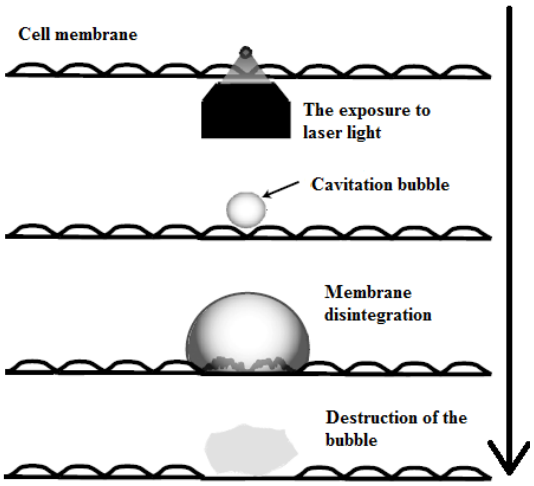

Figure 11: Laser lysis (Broun and Audet, 2008): *Scheme of laser lysis showing laser focusing followed by formation of cavitation bubble which disintegrate the membrane in zone of lysis. Bubble is then destructed and membrane structure is disrupted.*

9.2 Chemical methods

One of the many possibilities of cell treatment is to use certain chemicals. The release of intracellular or membrane bound compounds while not fully breaking the membrane structure is allowed by using special agents such as detergents, chaotropic agents, solvents, chelating agents or enzymes. Solubilisation of membrane compounds such as lipids and proteins is possible by using agents as detergents. Simple separation of supernatant follows. Chemical treatment of cells has to be chosen wisely according to further downstream analysis nevertheless treating cells with chemicals is mild and sensitive to released components. Moreover using chemicals can be performed within

a process called pre-treatment which precedes the non-chemical lysis. Cooperation of methods then differs from research to research. This chapter is focused on application of detergents, other membranolytic agents, enzymes and bacterial toxins.

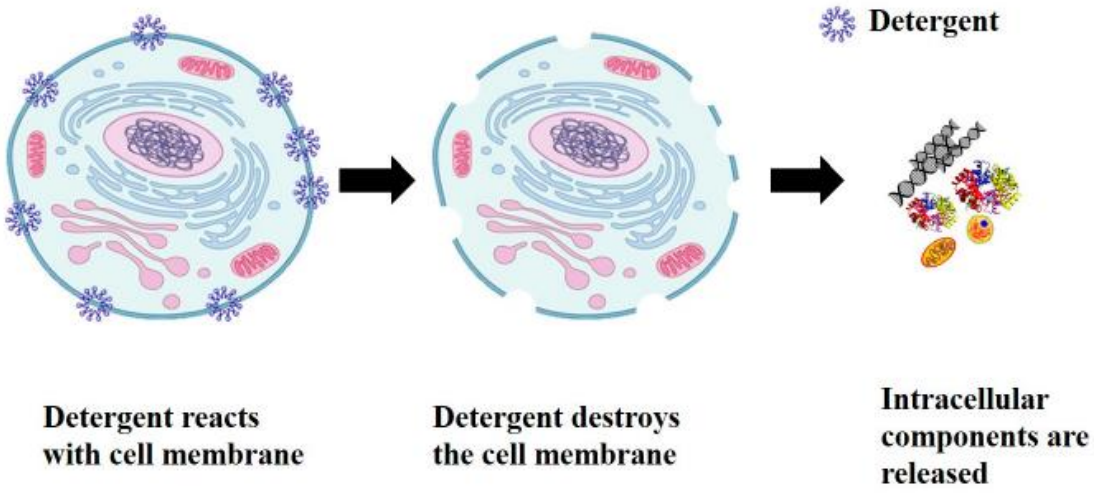

Figure 12: Chemical lysis (Islam et al., 2017): *Detergents target the membrane and allow release of intracellular components.*

9.2.1 Detergents and other agents

Detergents are, for their special properties, widely used molecules for membrane permeabilization. They show amphipathic character which means that they possess polar group along with the hydrophobic part of their molecule. Detergents are soluble in water and they are characterized with creation of micelles in the water. Micelles are structures created by molecules of certain detergent which enclose the hydrophobic carbon chains by polar heads forming the mostly rounded micellar structure. Membranes are structures which possess the same capability of protection the hydrophobic tails therefore the stable lipid bilayer can be formed. Detergents act on membrane by copying the structure of biological bilayers. They incorporate themselves into the membrane. With high concentration of used detergents the membrane structure can be broken and micelles of both detergent and membrane proteins can be constructed. This allows the further isolation of certain micelles, washing out the detergent molecules and proteins, which were successfully dissolved from the membrane, analysis can follow. Low concentration on the other hand does not have to create desired pores due to not strong enough bound breaking between lipids, proteins bound to lipids or proteins bound together. Mammalian cells are surrounded just by plasma membrane thus the use of detergents is efficient. In case of presence of cell wall detergents have to be combined with another more suitable method. When the detergent reaches the membrane, the strength and properties of certain detergents play a role in speed of cell lysis and efficiency the proteins are extracted with (Brown and Audet, 2008, Bhairi and Mohan, 2001).

Detergents are already well characterized group of permeant agents used in cell treatment. Therefore they can be sorted into three groups by their character. The first group contains ionic detergents. Head of these detergent molecules can be positively or negatively charged. Negatively charged sodium dodecyl sulfate, abbreviated as SDS, can be listed within this group. SDS is considered to be strong detergent with high affinity to membrane proteins providing fast membrane solubilisation. Detergents which belong to this group of molecule has the certain tendency to break protein bonds therefore isolated proteins cannot be analysed after their use in their native form. Therefore the method is preferable in need of simple and fast entering the intracellular space (Islam et al., 2017).

Non-ionic detergents on the other hand do not contain charged head. A broad use of non-ionic detergent molecules has been shown in protein analysis when the untouched nature, structure and activity, is desired. These molecules, such as agents called Triton X-100, Tween and others, happened to show no denaturation effect on protein molecules. Nevertheless the permeabilization of the membrane structures appeared to be slower than in case of using SDS. Moreover the SDS exhibits the certain affinity to proteins and causes the fast disruption of lipid-protein bond. This specificity has not been observed in case of Triton or Tween thus all bonds present within the membrane can be disrupted by using non-ionic detergents (Jamur and Oliver, 2009).

The last group consists of zwitterionic detergents which possess properties of both ionic and non-ionic detergents. They do not provide a charge on the surface of head of detergent molecule but presence of denaturation has been shown.

Choosing the right detergent with sufficient properties and setting up the correct conditions under which the permeabilization is going to be carried on is crucial for efficient and correct performance of the experiment. What needs to be kept in mind is the concentration of detergent and its nature such as presence of charge, solubility, possible toxicity or purity. Character of following downstream analysis with properties of proteins undergoing certain analysis also determine what disruptive method should be choose. Using detergents can bring certain interferences to experiment based on properties of certain detergent molecules. Aromatic character of certain detergents can show the undesired interference in further spectral analysis. Charge of ionic detergents can influence the product separation based on charge of proteins of interest. Experiment needs to be carried out under the proper pH and temperature which influences the efficient concentration for micelle formation thus the membrane permeabilization (Bhairi and Mohan, 2001).

Detergents allow proteins from intracellular space, plasma membrane or organellar membrane to be more closely analysed. This found a place importantly in identification of components by using certain antibodies or generally immunohistochemical approaches.

To localize the cellular components cell fixation is needed. Fixation is a process of using certain agents, coagulants or crosslinking agents, to preserve the cellular structure. Fixation can precede the process of membrane permeabilization therefore fixatives and for example detergents are used. Nevertheless certain agents has been shown to serve both as permeant and fixatives. Efficient fixation and permeabilization can be achieved by organic solvents (Bhairi and Mohan, 2001, Javois, 1999).

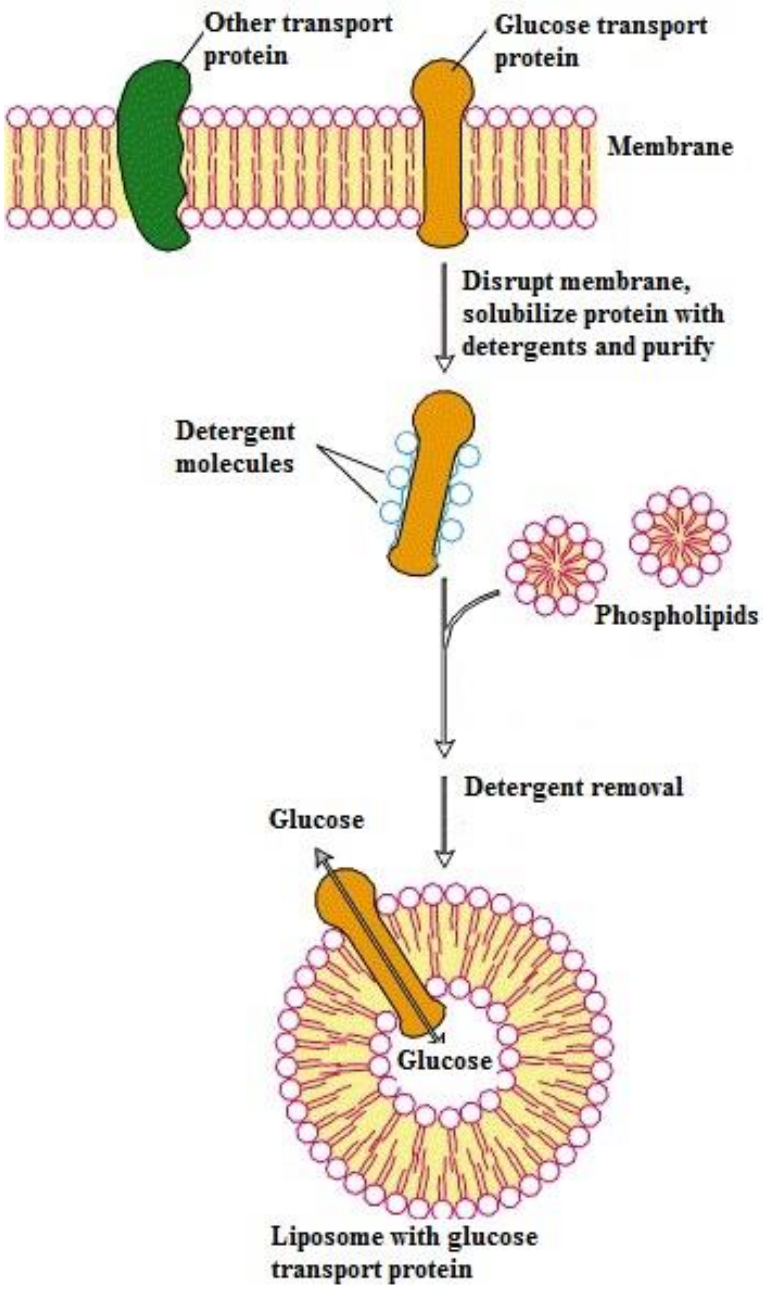

Figure 13: Application of detergents (Lodish et al., 2000): *Certain membrane proteins can be isolated by using detergents to disrupt the membrane structure. Detergent molecules induce micelle formation and protein of interest can be isolated.*

Organic solvents act on membrane by dissolving the lipids therefore the openings are created and molecules of interest, for example antibodies, can pass through the membrane into the cytosol. This simple technique enables the release of intracellular components of yeast or bacteria without any further damage of released components. One major advantage of using organic solvents is that they can also serve as fixative agents therefore the cellular structure can stay fixed and at the same time permeabilization is carried on. Alcohol, acetone or ethers can be named within the group of widely used solvents. Cell lysis can be also achieved by using chaotropic agents. Urea, EDTA, ethanol, guanidine or certain salts belong to the most known ones. These agents are capable of making hydrogen bonds falling apart therefore capability of reducing the hydrophobic properties of membrane structures is provided (Bhairi and Mohan, 2001, Javois, 1999).

Another famous approach of cell treatment has been proposed. Special treatment of cells is achieved by using certain plant derivatives known as saponins. These are surface active glycosides or steroids taking part in certain physiological processes of plants and animals such as stimulation of immune system, growth or feed intake and digestion. Closer attention has been paid to their effect on cellular membranes therefore their use in induced membrane permeabilization has been found. Saponins act via permanent but also a transient breakage of bonds connecting phospholipids and cholesterol molecules within the lipid bilayer. Transient openings creation made them important especially throughout the antibody manipulation (Francis et al., 2002, Javois, 1999).

9.2.2 Alkaline lysis

Certain technique using combination of chemical and electrical approach is now additionally mentioned. Alkaline lysis is a technique using at electrodes generated hydroxide ions. At certain concentration those OH- ions are capable of inducing the membrane permeabilization (Brown R. B. and Audet J., 2008). Ions come from the lysis buffer which is composed of sodium hydroxide (NaOH). Hydroxide ions possess the capability of breaking the bond between fatty acids and glycerol esters in the membrane therefore the membrane structure is destabilized. In addition to NaOH the lysis buffer can contain detergent such as SDS which helps membrane and proteins to be solubilized (Islam et al., 2017).

9.2.3 Enzymes

Enzymatic lysis is an approach using certain enzymes to break the membrane. Certain cell types require certain specific lytic enzymes for the performance of efficient cell lysis. Enzymes such as pectinases could serve as lytic compounds applicable on plant cells, yeast cells on the other hand

could be treated with chitinase. Lysozyme has been shown to be sufficient and suitable for lysis of microbial cells. These enzymes are capable of breaking the glycosidic linkage within the membrane thus enhance the membrane structure disruption. Peptidoglycan inherent in bacterial membranes can be easily targeted by certain enzymes such as lysozymes nevertheless presence of an extra layer surrounding cells in case of gram-negative bacteria requires cooperation of enzymes with detergents. Treatment of bacterial cell has undergone multiple alternations and use of lytic enzymes differs research from research. This highly biologically selective lytic method has been found desired in the field of genetics, especially in recombinant proteins isolation from bacterial hosts (Islam et al., 2017, Asenjo et al., 1988).

9.2.4 Bacterial toxins

Direct effect on biological membranes has been detected within actuation of bacterial toxins. These proteins, either small or big, generally target certain components, proteins or lipids, of cellular plasma membrane and mostly induce pore formation thus enter of pathogens into cells is allowed. The elucidation of the process via which bacteria damage the plasma membrane structure of mammalian cells in case of bacterial infection has given the toxins the laboratory use. Delivery of certain macromolecules to cells could be done by introducing toxins to membranes. Moreover the permeabilization induced by bacterial toxins can be temporal therefore the viability of cell can be preserved. Suddenly the attention has been drawn to bacterial toxins (Rappuoli and Montecucco, 1997).

Use of some bacterial toxins has been discovered as method satisfyingly allowing access to the inside of the cell via creation of pores in bio-membranes. As an example α-toxin of *Staphylococcus aureus* can be mentioned, pneumolysin of *Streptococcus pneumoniae* or streptolysin O (SLO) produced by *Streptococcus pyogenes*. Aggregation of mentioned toxin molecules enhances creation of nicely formed pores. This allows exchange of different sized molecules. The width of pores is dependent on used toxin and variation in their concentrations or doses. Use of bacterial toxins requires the knowledge of sensitivity of particular cell type to certain toxin. With this knowledge pores of certain size can be created and certain analysis or delivery of macromolecules can be performed.

Streptolysin O is a proteinaceous exotoxin delivered from *Streptococcus pyogenes*. Its use favours the creation of large pores of the shape of rings in the membrane bilayer. When pores are created, antibodies or other macromolecules can be delivered into the cell. Streptolysins belong to the group of cholesterol-binding toxins therefore the use is focused on membranes which contain cholesterol molecules. Presence of cholesterol allows toxins to bind the membrane. After binding

their target the oligomerization follows and certain structural changes occur within the membrane. These oligomers, formed on or in the membrane structure, are crucial for further pore formation. (Rappuoli and Montecucco, 1997, Walev et al., 2001).

Another bacterial toxins can serve as a suitable alternative for pore assembly in the cell membrane. Action of α-toxin of *Staphylococcus aureus* has been shown to sufficiently cause the permeabilization of plasma membrane. The α-toxin is capable of binding to the lipid bilayer and induce the cell lysis. This has been implicated especially in case of erythrocytes where the action of α-toxin is followed by haemolysis and haemoglobin release. The mechanism of its action is presumably based on formation of its oligomers on the membrane which leads to the ring channel assembly, thus ion leakage which results in cell lysis. Pores created by α-toxin appeared to be smaller than in case of SLO therefore the use in cell biology has been restricted to delivery of substances of lower molecular weight (Belmonte et al., 1986, Tartakoff and Wilson, 1989).

Two commonly used and known bacterial toxins has been used to depict the action of toxins on membrane. Variability in size of pores has been pointed at. Moreover the use in cell biology has been proposed. Pores serve as an entry for certain molecules whose molecular action can be then studied in living cells. Besides the delivery of biologically important compounds the treatment of cancer cells using bacteria producing certain toxins has been proposed. Bacteria which underwent some gene modifications can serve as selective drug delivery agents targeting certain tumor cells. Toxins produced by those organism effectively form pores in tumor cells thus therapeutic agents can possibly enter the tumor cells. Moreover those therapeutics can be based on structure of bacterial toxins and possess their cytotoxic activity (Jain, 2001). A variety of other bacterial toxins could be applicable in specific membrane targeting. Because of the certain potential of the selective membrane permeabilization their application still undergoes many modifications and improvements.

10. CONCLUSION

The phenomena known as membrane permeabilization has been looked at in this thesis. Permeabilization, certain changes of membrane permeability for macromolecules and normally non-permeable solutes, occurs within physiological and pathophysiological processes. Nevertheless, disruption of membrane structure by application of different approaches can be achieved in both temporal and permanent manner from outside. One of the many signs of dying cells are ruptures in cellular membrane therefore the process of membrane permeabilization has been linked to cellular death. Moreover certain organelles such as mitochondria have been shown to participate in apoptotic onset. Lysosomes are mostly involved in necrotic death. Nevertheless it has been proposed that they may have a particular effect on induction of mitochondrial membrane permeabilization. Nucleus, endoplasmic reticulum and Golgi apparatus also contribute to the induction of mitochondrial membrane disintegration. To be even capable of analysing certain dynamic cellular processes, methods damaging the membrane structure had to be created. The principle and use of methods capable of permanent or transient membrane permeabilization using chemical and non-chemical approaches have been described in this thesis. Certain attention has been paid to targeting therapy which in one way favours cancer treatment by induction of apoptosis. Inhibition of apoptosis thus preservation of cellular viability has found the place in treatment of neurodegenerative diseases or neuronal injuries. Life and death of cells are very complex processes and understanding the mechanism can improve the treatment of multiple diseases. As mentioned before methods used to make cells more permeable for certain compounds such as antibodies or therapeutics, cellular direct lysis and the induction of apoptosis still undergo wide optimisation and every year certain improvement might be made.

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