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ZLIVANJE CELIC *IN VITRO* Z ELEKTROPERMEABILIZACIJO

DOKTORSKA DISERTACIJA

CELL FUSION *IN VITRO* BY MEANS OF ELECTROPERMEABILIZATION

DOCTORAL DISSERTATION

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On the basis of the Statute of the University of Ljubljana and the decision of the Senate of the Biotechnical Faculty and decision of the University Senate, from the 12th February 2009, the continuation to doctoral postgraduate studies of Biological and Biotechnical Sciences, field Biotechnology was approved. The work was performed in the Laboratory of Cybernetics on Department for Biomedical Engineering at the Faculty of Electro Engineering, University of Ljubljana and in the Department for the Production of Diagnostic Reagents and Research at the Blood Transfusion Centre of Slovenia.

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AB	Cell fusion is a method investigated intensively for its products are used for different purposes in biotechnology and medicine. Electrofusion is a common method for induction of cell fusion, where cells that are destabilized by means of high voltage electric pulses and are at the same time in close contact, fuse. The use of the hybrid cells is defined by the properties of the parental cells; however, it is limited by the yield of the method for cell fusion. In this work some of the many parameters that influence cell fusion were studied for different cells (electric field parameters and medium osmolarity) and results were used in the experiments for hybridoma production. To improve the method new pipette tip chamber was designed for electroporation of cells in suspension with electric pulses in different directions. A contact method (modified adherence method) was developed and used for fusing myeloma fusion partner cells and human lymphoblasts B. In this experiments mouse – human heterohybridoma were produced. Fusion yield was detected with double staining method and comparison between double staining method and clone counting method in production of hybridoma was made.

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AI	Zlivanje celic je metoda, s katero dobimo zlite celice in njihove produkte, ki so uporabni v biotehnologiji in medicini. Elektrofuzija je najpogosteje zastopana metoda za indukcijo zlivanja. Zlivajo se celice z destabilizacijo membrane in sočasnim zagotavljanjem medsebojnega stika dveh celic. Uporabo tako dobljenih hibridnih celic določajo v glavnem lastnosti starševskih celic, v veliki meri pa je odvisna od učinkovitosti zlivanja. V pričujoči nalogi smo za različne vrste celic preučili nekatere od mnogih dejavnikov, ki vplivajo na elektrofuzijo (parametri električnega polja in ozmolarnost medija) ter uporabili dognanja v eksperimentih priprave hibridomov. Z namenom izboljšanja metode smo izdelali novo koničasto komoro za elektroporacijo celic v suspenziji, ki omogoča dovajanje pulzov v različnih smereh. Izpeljali smo tudi metodo za vzpostavljanje stika med celicami (t.i. modificirano metodo pritrjevanja) in jo uporabili za zlivanje mielomskih fuzijskih partnerjev s človeškimi limfociti B. V teh poskusih smo uspeli pripraviti mišje – humane heterohibridome. Učinkovitost zlivanja smo detektirali z metodo dvojnega obravanja s fluorescentnimi barvili in jo primerjali z metodo štetja klonov pri običajnem postopku priprave hibridomov.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

B16-F1 – Mouse melanoma cells

CHO – Chinese hamster ovary cells

CMFDA – CellTracker Green CMFDA (5-chloromethylfluorescein diacetate)

CMRA – Orange-fluorescent CellTracker

CMTMR – CellTracker Orange (Orange-fluorescent tetramethylrhodamine)

EBV – Epstein-Barr virus

EP – electroporation

FY – fusion yield

HAMA – human anti-mouse antibody response

HAT – selection medium for hybridoma selection (hypoxanthine aminopterin thymidine)

HGPRT – Hypoxanthine-Guanine Phosphoribosyl Transferase

HT – medium containing hypoxanthine and thymidine

HuNS1 – human myeloma fusion partner cell line

Ig – immunoglobulin

MoNS1 or NS1 – mouse myeloma fusion partner cell line

OPI – medium containing oxalacetate, pyruvate and insulin

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffer saline

RVD – regulatory volume decrease

INTRODUCTION

1.1 INTRODUCTION IN ENGLISH

This work deals with cell fusion *in vitro*. Cell fusion is necessary in normal physiology of living organisms during its development and later in functioning of specific tissues. Fusion in living organisms is highly specific, governed by specialized proteins. The ability of cells to fuse nonspecifically in *in vitro* conditions can be, however, exploited for production of hybrid cells that can be used in different purposes. Electrofusion is a convenient method and its products can be used for various applications. Electrofusion is efficient if properly used – parameters are optimized for a specific system. Electrofusion is also a "clean" method without use of any harmful chemical or viral agents in the system (cells, reagents, organism); therefore, it can also be used *in vivo*. Electrofusion is influenced by parameters of electric pulses amplitude, duration, number,… and other factors (osmolarity, medium composition, physiological condition of cells,…); however, only some of those influences are underestood. This work deals with seting up a method and optimization of the parameters for electrofusion of cells that are specific for hybridoma technology.

1.2 UVOD V SLOVENŠČINI

V tem delu se ukvarjamo z zlivanjem celic v pogojih *in vitro*. Zlivanje celic je v živih organizmih normalno prisoten pojav med razvojem zarodka in tudi kasneje pri delovanju določenih tkiv. Taka »biološka« fuzija je zelo specifičen proces, ki ga nadzorujejo

specializirani proteini. Možnost zlivanja celic izven živega organizma pa predstavlja možnost za pridobivanje hibridnih celic, ki so uporabne v različne namene. Metoda zlivanja celic z visokonapetostnimi električnimi pulzi (elektrofuzija) je primerna za vse možne uporabe produktov zlivanja. V primeru, da optimiziramo parametre za specifičen sistem, v katerem delamo, je elektrofuzija zelo učinkovita. Njena pomembna prednost pred drugimi metodami zlivanja celic je tudi dejstvo, da v sistem (celice, reagente, organizem) ne vnaša nobenih kemičnih ali virusnih agensov, ki bi bili lahko škodljivi. Zato je elektrofuzija primerna tudi za uporabo v *in vivo* pogojih. Na elektrofuzijo vplivajo parametri električnih pulzov (amplituda, trajanje, število,…) in ostali dejavniki (ozmolarnost, sestava medija, fiziološko stanje celic…), ki jih še ne razumemo v celoti. V pričujočem delu proučujemo vzpostavitev metode in optimizacijo parametrov za elektrofuzijo celic, ki jih uporabljamo v hibridomni tehnologiji.

2 SURVEY OF THE LITERATURE

2.1 BIOLOGICAL FUSION

2.1.1 Examples of fusion in living organisms

Membrane fusion is a process whereby two separate lipid bilayers merge to become one. It is essential for communication between membrane-delineated compartments in all eukaryotic cells (Martens and McMahon, 2008). The best-studied process involving membrane fusion is exocytosis, whereby vesicles fuse with the limiting membrane of a cell in order to release their contents (for examples hormones and neurotransmitters) into the intra- or extra-cellular milieu, or to deposit receptors, transporters, channels, or adhesion molecules into the limiting membranes (Vardjan et al., 2007; Ramos and Teissié, 2000). However, large numbers of membrane-fusion events occur between intracellular compartments, such as yeast vacuole, mitochondria and endosomes (Gabrijel et al., 2008). Furthermore, enveloped viruses gain entry into the cytosol by fusing their limiting membranes with host cell membranes (Harrison, 2008).

Cell-cell fusion is a process that involves the conjunction of two separate membranes into a single lipid bilayer surrounding a larger multinuclear cell, in which the constituents of both cells are shared. Cell-cell fusion is a widespread phenomenon in organisms ranging from yeast to humans (Chen and Olson, 2005; Larsson et al., 2008). Cell-cell fusion is essential during fertilization, development and immune response (Martens and McMahon, 2008).

Examples of cell fusion events include the joining of two gametes to generate a zygote (Ogle et al., 2005; Atif et al., 2006), fusion of trophoblast cells in mammalian embryos to form the placenta, fusion of myoblasts during myogenesis (myotube generation), fusion of macrophages, resulting in their differentiation into osteoclasts and giant cell formation (Witze and Rothman, 2002), and fusion between neighboring cells in the eye lens (Shestopalov and Bassnett, 2000). Cell-cell fusion was also extensively studied in *C. elegans*, where almost one third of all somatic cells fuse during epidermis development (Podbielewicz, 2006).

Recent data indicate that additional cell fusions may contribute to tissue repair in the adult (Vassilopoulos et al., 2003a; Wang et al. 2003). Bone-marrow-derived cells have been shown to fuse *in vivo* with hepatic cells, nerve cells and gastrointestinal cells and the theory has been put forward that such fusions may serve to repair damaged or corrupted cells (Alvarez-Dolado et al., 2003; Vassilopoulos and Russell 2003b; Wang et al., 2003; Rizvi et al., 2006).

Although cell-cell fusion is necessary in normal physiology, unregulated membrane fusion may also promote diseases, especially cancer (Paris, 2004). Many tumor cells, for unknown reasons, are particularly fusogenic. Fusion between tumor and normal somatic cells can produce cancer cells with increased metastatic potential (Pawelek and Chakraborty, 2009).

2.1.2 Mechanisms of fusion in living organisms

Lipid bilayers do not spontaneously fuse. Energy must be invested to overcome hydration repulsion between membranes that approach each other and to disrupt the normal bilayer structure of the fusing membranes. This energy is expended on removing water molecules from the cleft between the two membranes, on bending membranes that are to be fused and on creating nonbilayer structures that function as fusion intermediates. The energy to drive biological membrane fusion is provided by highly specialized fusion proteins, called fusogenes.

The structure of the fusogenes, activation mechanisms, and requirements in one or both membranes are very diverse. However, the membrane pathway of fusion through hemifusion appears to be conserved between all classes of viral (Env HIV, HA Influenca …), intracellular SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment receptor), and developmental FF (fusion failure) fusogenes, described first in nematode *C. elegans*.

Most fusion reactions occur via a common stereotypic sequence of events starting with cell-cell recognition, alignment and adhesion. This is followed by an actual fusion event that merges the two cells together by tethering of plasma membranes, formation of aqueous pores between the membranes, and expansion of these pores to clear the membranes from the fusing cells` junction (Sapir et al., 2008).

Theoretical and experimental studies of membrane intermediates in diverse fusion reactions revealed a common membrane remodeling pathway apparently conserved in all membrane-fusion events (Chernomordik and Kozlov, 2003; Chernomordik et al., 1993; Nakatogawa et al., 2007; Xu et al., 2005; Podbilewicz, 2006). Merging of biological membranes is proposed to start with contact between membranes; two opposed biological membranes are separated by 10 to 20 nm. In next step the membranes undergo deformations, causing a local approach. The reaction proceeds via a stereotypic membrane lipid rearrangement, called hemifusion. In this step the outer leaflets, which face each other through intermediate 3 – 5 nm water gap, are the first to fuse, and proceed to mix their lipids. In contrast the inner leaflets remain separated, preventing the mixing of the cytoplasm.

To complete the fusion, the hemifusion intermediate must transform to a fusion pore. The pore might open directly from a fusion stalk or only after an expansion of the hemifusion stalk into the hemifusion diaphragm. The transition from a small flickering pore to a larger expanding pore likely represents the most energy-demanding fusion stage (Chernomordik et al., 2006). The heart of the fusion is the coupling between the proteins and the lipids. Proteins drive membrane rearrangement through a conserved pathway that is defined by the properties of lipid bilayers (Tamm et al., 2003; van den Brink-van der Laan et al., 2004; Atif et al., 2006; Kasson and Pande, 2007).

2.2 APPLICATION OF CELL-CELL FUSION IN BIOTECHNOLOGY AND MEDICINE

Cell fusion in biological systems is highly specific, governed by specialized proteins. Cell membranes are also capable of nonspecific fusion, which can be induced by means of different external mediators, such as polyethylene glycol, viruses, ultrasound and electric field. Induced cell fusion ability is important for biotechnology, medicine and biology research. With induced cell fusion we can produce highly valuable hybrid cells and their products (monoclonal antibodies). Since fusion mechanisms of different membranes (including artificial membranes composed only of lipids) are very similar, induced cell fusion can also be used to study the underlying mechanisms of membrane fusion (Chernomordik et al., 2006).

2.2.1 Production of hybridoma cells for monoclonal antibodies production

Hybridoma technology was developed by Köhler and Milstein already in 1975, and acknowledged by a Nobel Prize in medidine in 1984 (Köhler and Milstein, 1975). The essential part of hybridoma technology is fusion of lymphocytes B (that produce antibodies) with myeloma cells that can be grown *in vitro*. Hybridoma cells, which are produced via fusion and expanded from a single hybrid cell, thus grow *in vitro* and produce antibodies of defined specificity. These, so called monoclonal antibodies, are highly valuable tool, used in many areas (Albitar, 2007)

Since their original description, murine hybridomas have become widely used. Formed by the immortalization of spleen cells from an immunized mouse and subsequent cloning, hybridomas secrete antibodies of a single specificity. The application of murine monoclonal antibodies is widespread in many areas, from biological and medical research to clinical diagnostics. Monoclonal antibodies also have a potential in human therapeutic applications. However their usefulness is limited due to negative side effects including the recipient`s immune response to murine protein (human anti-mouse antibody response; HAMA). There are several ways to reduce or to avoid HAMA, including antibody engineering to remove or replace much of the murine Ig sequence (e.g. chimeric and humanized antibody fragments and antibodies).

The use of hybridoma technology to produce human hybridomas has many advantages. Monoclonal antibodies of human origin elicit fewer problems in a therapeutic setting. One way of preparing human monoclonal antibodies is by using transgenic animals carrying human immunoglobulin (Ig) genes. And last, but not least, some specificities of human antibodies can never be obtained by immunization of mice.

Instead of using a transgenic animal, synthetic repertoires of human antibody fragments using combinatorial phage libraries can be produced. Due to the random association of heavy and light chains, this method has an advantage of allowing the production of monoclonal antibodies with unique specificities. However, this random association will not necessarily represent combinations found *in vivo* and hence will give a distorted view of the immune response.

Another way to produce human monoclonal antibodies is by the transformation of human lymphocytes with Epstein-Barr virus (EBV). However, lymphocytes experience a low rate of infection and successfully transformed cells tend to show unstable growth and secrete low levels of IgM antibody. In addition, the therapeutic use of antibodies derived from EBV-infected cells raises safety concerns (Jessup et al, 2000).

Alternatively, human hybridomas can be prepared by a method based on a traditional fusion approach, substituting murine spleen cells with human lymphocytes. Since first being described (Olsson and Kaplan, 1980) experiments using human myelomas to make human hybridoma have been performed with limited success. Production of human-human hybridomas has been largely hindered by a lack of a good human fusion partners. Human B cell lymphoblastoid cell lines (EBV+) have been used. Their products are characterized by the unstable production of only small amounts of antibody (Ritter and Ladyman, 1995). Human-mouse heteromyeloma and murine myeloma partners were used with efficiency higher than human-human fusions but lower than murine-murine fusions. The existing cell culture banks of murine myeloma fusion partner cell lines are used. Although chromosomal instability is cited as a major problem associated with human-mouse heterohybridomas, extensive subcloning may facilitate the isolation of stably secreting clones.

Aim 4: Obtaining hybridomas from human lymphocytes B and human or mouse fusion partner cell line (human or heterohybridomas) for production of human monoclonal antibodies (human Ab) using different parameters of electric pulses.

2.2.2 Hybrid cells for anticancer immunotherapy

Most human tumors are poorly immunogenic and are capable of evading the host immune system. A compromised immune function is thus a common feature of advanced cancer (Barbuto et al., 2004). A disrupted antigen presentation is the mechanism of immunological escape of tumor cells (Scott-Taylor et al., 2000). Various tumor vaccination strategies have been developed based on dendritic cells, which are the most powerful antigen presenting cells. Fusion of antigen-presenting cells and autologus tumor cells thus represents another promising type of hybrid cells. These hybrid cells could be used as a cell vaccine in cancer immunotherapy (Trevor et al., 2004; Weise et al., 2004; Yu et al., 2005). Use of such hybrid cells as vaccines has been shown to induce potent antitumor immunity (Suzuki et al., 2005; Gottfried et al., 2002).

Dendritic cells are used for fusion with autologous tumor cells, since dendritic cells are the most powerful antigen presenting cells Dendritic cells activate naive T lymphocytes to generate cytotoxic effectors (cytotoxic T lymphocytes). Hybrid dendritic – autologus tumor cells thus express specific tumor antigens and are able to activate T cell mediated responses (Lindner and Schirrmacher, 2002; Schirrmacher et al, 2003).

2.2.3 Insertion of cell receptors into the target tissue

Cell-tissue fusion technology may be employed to establish unique and novel animal models for investigating receptor-mediated processes *in vivo*. Bioengineered animals that

express host- or tissue-specific microbial attachment receptors should be susceptible to infection by those etiological agents to which unaltered animals are naturally resistant (Grasso et al., 1989; Heller and Grasso, 1990).

2.2.4 Study of electrophysiological properties of cells

Fused cells of large diameters, so called giant cells (30 to 65 μm) were produced by cell fusion. The large size of these cells allows studying their electrophysiological properties. Studies showed that values for the area-specific membrane capacitance and the area-specific conductivity of the giant cells are in the range of that of the parental cells. More importantly, the substantial increase in the number of the membrane proteins due to the large membrane areas paves the way for the characterization of ion transporters that are not accessible on a single cell level because of a low turnover-number and, in turn, of low current amplitudes (Zimmermann et al., 2006).

2.2.5 Targeted tissue regeneration

Bone marrow-derived cells can contribute to the regeneration of diverse adult tissues, including brain, liver and heart, following bone marrow transplantation (Rodić et al., 2004). These unexpected events were initially considered as a result of transdifferentiation of bone marrow-derived cells, supporting the emerging idea of extended plasticity of adult stem cells. However, the consequent studies have clearly demonstrated that spontaneous cell fusion, rather than transdifferentiation, was the primary cause for unexpected cell fate-

switches of bone marrow-derived cells into hepatocytes, Purkinje cells and cardiac myocytes *in vivo* (Vassilopoulus et al., 2003, Alvarez-Dolado et al., 2003; Wang et al., 2003).

Taken into account that cell fusion may account for many examples of tissue repair after transplantation, ultimately it could be used clinically, and thus the safety and efficacy of approaches based upon it (induced fusion) needs to be considered (Rodič et al., 2004, Duelli and Lazebnik, 2003). It was suggested that fusion enhances the metastatic potential of tumors; however, the available data do not demonstrate that fusion between non-transformed, normal cells promotes genetic instability or leads to cancer without additional or pre-existing genetic changes. In addition, since *in vivo* cell fusion is presumably an ongoing process that occurs normally, therapeutic interventions may not increase whatever risks are already present (Vassilopoulos and Russel, 2003).

Delivering a complete normal chromosome by cell fusion also avoids the risks of integrating viral (or non-viral) vectors, which can disrupt and activate chromosomal genes and have led to the formation of leukemias in children who received retroviral vectors to treat severe combined immunodeficiency (Hacein-Bey-Abina et al., 2003).

2.3 ELECTROFUSION

Spontaneous fusion of somatic cells *in vitro* was originally demonstrated already in 1961. Cells with mixed karyotypes were identified after co-culturing of cells of two different cell lines together (Barski et al., 1961). In early seventies, methods were invented that adopted viruses and polyethylene glycol as means for fusion of protoplasts and animal cells. In late seventies first reports appeared of fusion, caused by external electric field, e.g. electrofusion (Senda et al., 1979). It was shown that electric field can cause fusion of different types of membranes (lipid bilayers, liposomes and different types of cells…). Therefore, the conclusion was made that fusogenicity (an ability to fuse) is a common property of all membranes.

Electrofusion turned out to be an effective, reproducible, and medically safe system. Researchers obtained at least comparable to substantially higher efficiencies with electrofusion in comparison to polyethylene glycol fusion (Hui and Stenger, 1993; Lindner and Schirrmacher, 2002; Gottfried et al., 2002; Skelley et al., 2009). The most important feature of electrofusion in this concern is that it can be (and should be) optimized for different systems (for different combination of cells, cell-tissue systems, etc.). Therefore, even for the systems, where fusion yields are still low, in principle there is a possibility to improve it by optimizing protocols.

The important advantage of electrofusion is also that it does not include the use of any potentially harmful agents like chemicals or viruses. It can be; therefore, used *in vitro*, for

preparation of products intended for therapeutic purposes and also applied *in vivo* (for tissue regeneration or eliciting immune response). Because of the great value of fused cells and their products in biology and medicine, many researchers work on the optimization of the process of cell electrofusion.

Fusion is a two step process. Membranes are normally stable structures that resist to external influences. The first necessary step of the process is, therefore, a destabilization of membranes that enables fusion by causing structural rearrangements within the membrane. The second necessary step is a close contact between membranes that enables mixing of the two lipid bilayers.

2.3.1 Membrane destabilization

Membrane destabilization is achieved by electroporation. This means that short, high voltage electric pulses are used to create hydrophilic pores in the membranes. We also say that membrane is permeabilized (more permeabile for otherwise unpermeant molecules). These pores can be reversible and reseal after some time. In the case of overcritical membrane voltage, irreversible breakdown occurs, when cell dies (Kotnik et al., 1997; Kotnik et al., 2005). In electrofusion, membrane destabilization is achieved by means of reversible electroporation.

One can say that electroporated membrane (destabilized with electroporation) is also a fusogenic membrane, i.e. capable of fusion. The magnitude of the induced transmembrane voltage needed for fusion is approximately the same as that for electroporation. In addition to the absolute value of the induced transmembrane voltage, good correlation was found between electroporation and electrofusion process also in terms of other electrical parameters, such as number of the pulses, their duration and the direction of electric field (Zhelev et al., 1988; Teissié and Rols, 1993; Trontelj et al., 2008).

The most important and known electrical parameters governing membrane electroporation are the pulse amplitude (enlarges electroporated membrane fraction) and pulse duration and number of pulses (enlarge the density of membrane defects) (Ramos and Teissié, 2000a).

Another way of enlarging the electroporated membrane area without reducing the survival of the cells that has not been studied until lately is changing electric pulse direction (Reberšek et al., 2007); i.e. delivering electrical pulses in different directions to the cells. From the theory of electroporation (Valič et al., 2003; Kotnik et al., 2001) and already performed experiments (Miklavčič, 2001; Faurie et al., 2004; Trontelj et al., 2008) it follows that applying pulses to cells in different directions causes electroporation of different areas of the cell membrane. Application of pulses in different directions thus increases the total electroporated fraction of the membrane.

2.3.2 Contact between membranes

Fusion is achieved only when cells with destabilized membranes are in close contact. Cells normally do not approach each other to the distance that is small enough to allow fusion (≈1 nm) because of repulsion caused by negative charge on their surface. This repulsion force has to be overcome to enable mixing of lipid molecules from two different membranes. As said earlier, electrofusion is two-step process: (a) cell membrane has to be brought into fusogenic state and (b) close physical contact between two fusogenic membranes has to be established. The process of electrofusion has been successfully performed in both possible sequences; that is in pulse first (PF) and contact first (CF) protocols. Methods for establishing of the cell contact; however, depend on which protocol is used. Below, methods for contact first protocols are described.

A wide variety of approaches has been used to induce cell contact; including rolling cells in plastic tubes (Neumann et al., 1980), using a custom designed chamber (Jaroszeski et al., 1994a), fusion of cells in a pellet (Abidor et al., 1994) and using filters and depression (Ramos et al., 2002). In addition to these mechanical approaches a specific avidin-biotin bridging between cells was used. This method is, however, expensive and very specific (Lo et al., 1984).

The most widely used method for contact achievement is dielectrophoresis, where cells are aligned in a pearl-chain formation due to a non-homogeneous alternating electric field of low amplitudes (Vienken and Zimmermann, 1985; Neil and Zimmermann, 1993; Dimitrov,

1995). Today all commercially available electrofusion systems use this method for achieving cell-cell contact. However, in order to use dielectrophoresis, electroporation media of low ionic conductivity should be used that reduce cell heating, which is not beneficial for cells (Jaroszeski, 1994b). On the other hand, it enables a development of microchips on which process can be automated and accelerated (Fox et al., 2006; Cao et al., 2008).

Another method was described, where cell contacts were spontaneously established in the confluent cell culture (Teissie et al., 1982; Finaz et al., 1984; Blangero et al., 1989). In the confluent cell culture; however, cells with many nuclei often form. These cells are unlikely to survive and proliferate. Later, a modified adherence method was developed, where cells are plated in appropriate concentration for a short time (20 min) to form cell-cell contact (Ušaj et al., 2009a).

During this short incubation period cells slightly adhere to the surface and create contacts between themselves. Since cells are adhered to the surface of the plate, the media can be exchanged and electroporation performed, yet cells still preserve their spherical shape. With this method (providing that proper cell concentrations are used) fused cells are obtained that have only small number of nuclei and are, therefore, more likely to survive and proliferate.

In all methods described above, contacts between cells are established before the electroporation (contact first protocol). A different sequence of the two crucial events

(pulse first protocol) would theoretically enable us to electroporate different fusion partner cells with different electric pulses and establish contact afterwards. Treatment of cells with different pulses is important in cases, where cells to be fused are of different sizes; therefore, different parameters of electric pulses are needed for optimal electroporation of both types of cells. Such method was reported and consists of cell centrifugation after electroporation (Sowers et al., 1986; Teissié and Rols, 1986).

For pulse first methods it is important to establish cell contact in a very short time after electroporation when cell membranes are still fusogenic (Teissié and Ramos, 1998). This time is within seconds or minutes and presumably depends on intensity of electric pulses and temperature of the electroporation medium (Dimitrov and Sowers, 1990).

Aim 5: Development of an electrode chamber and system for PF electrofusion with separate treatment of two fusion partner cells.

Optimal cell membrane electroporation parameters are different for cells that are different in size (Kotnik et al., 1997) but also for different types of cells (Neil and Zimmermann, 1993; Čemazar et al., 1998; Čegovnik and Novakovič, 2004; Yu et al., 2008). Each type of cells that is to be fused has to be, therefore, analyzed for optimal electroporation parameters.

Aim 1: Analysis of different cell types before electrofusion: to determine efficiency of electroporation for different cells at different values of electrical parameters.

2.3.3 Other parameters that influence electrofusion

Even though electrofusion of biological cells is potentially a useful method, achieving sufficiently high efficiency still requires extensive trial-and-error experiments. One of the earliest approaches to improve electrofusion yield was the use of hypoosmolar electrofusion medium that resulted in a considerable increase in fusion yield (Ahkong and Lucy, 1986; Schmitt and Zimmermann, 1989; Zimmermann et al., 1990; Foung et al., 1990).

To ensure the improvement of fusion efficiency in hypoosmolar medium the duration and the osmolarity of the hypoosmolar treatment has to be carefully selected (Ušaj et al., 2009b). Rapid cell swelling in the hypoosmolar environment due to influx of water namely triggers regulatory volume decrease. If it is triggered before the induction of cell fusion, it can inhibit the positive effect of the hypoosmolar treatment on electrofusion by reducing cell size, restoring microvilli and excessive leaking of cytosolic electrolytes (Shukorukov et al., 2005; Kiesel et al., 2006). The prolonged treatment thus leads to poor fusion efficiency and also decreases cell viability (Foung et al., 1990; Rehman et al., 1992).

Aim 2: Analysis of different cell types before electrofusion: to determine behaviour of cells in hypoosmolar medium.

Electrofusion is further affected by electrical conductivity of the electroporation medium and the medium composition. The presence of bivalentcations caused the increase in fusion

yield (Matibiri and Mantell, 1995; Rols and Teissie, 1989; Stenger et al., 1991; Stenger and Hui, 1986).

2.4 FUSION YIELD

Even though electrofusion of biological cells is an efficient and potentially useful method, the achievement of sufficiently high efficiency still requires extensive trial-and-error experiments. A low number of obtained hybridoma cells is still the main problem for human monoclonal antibodies production and cancer immunotherapy and their instability (Yu et al., 2008).

2.4.1 Determination of fusion yield

Fusion yield has been determined by different methods. In hybridoma technology an adequate method exists, where hybridoma cells are first selected with selection media and their clones are counted later using phase contrast microscopy. This method is accurate and detects only hybridomas (cells that were produced from at least one lymphocyte and at least one fusion partner, i.e. myeloma cell) that survived the procedure and proliferate.

From the beginning the methods used in research was counting cells that contain more than one nucleus using phase contrast microscopy. This method is not very appropriate because it is difficult to determine visually the number of nuclei and because of the nuclei fusion. This method was later substituted with different staining methods. Double staining method,

e.g. with membrane dyes DiI and DiO was not accurate due to the transfer of dyes molecules between two membranes in close contact, without fusion event. The most often used method in research of electrofusion is the double staining method using two fluorescent cytosolic dyes. Cell tracker dyes used for this purpose are green CMFDA and red CMTMR; the later can be replaced with newer orange CMRA.

Hybrid cells are thus detected microscopically by counting cells that are labeled with both dyes or by flow cytometer. In flow cytometer, the fusion yields are slightly higher because cell clumps are counted as fused cells (Jaroszeski et al., 1994; Gabrijel et al., 2004). Flow cytometer, though, enables evaluation of large samples of cells, which is necessary when fusion yields are low.

The most exact method for fusion yield determination is colony counting method in hybridoma technology. This is the only method where fused cell survival and proliferation is taken into account. The average results obtained with fluorescent dyes are usually much higher than results obtained with colony counting method in hybridoma technology (which are in the range of 0,001 % to 1 %), although an exact comparison have not been made.

Aim 3: Testing of double staining method using CMFDA and CMRA and comparing this method with other methods (hybridomas colony counting and new method, based on cell size measurements).

2.5 RESEARCH HYPOTHESIS

- Cell electroporation and fusogenicity are controlled by the parameters of the electric pulses used (amplitude, number, length, repetition frequency). While preserving cell survival level we can improve fusion yield by applying pulses in different directions, the opposite to only one direction. In this way, different parts of the cell membrane are electroporated. Because of the mainly experimental nature of determination of the optimal electrical parameters of cell electroporation, it is necessary to determine optimal conditions for each (fusion partner) cells separately.

- The most important non-electrical parameter influencing cell fusion is the osmolarity of the fusion medium. Namely, the cell volume changes when the osmolarity is changed. Cells enlarge in hypoosmolar medium and shrink in hyperosmolar medium. The changing of the cell volume influences key phases of the cell fusion process, predominantly when achieving cell contact. Cell enlarging includes smoothing of the surface thus offering larger part of the surface for contact with the surface of another cell.

- More parameters described in the survey, known and yet unknown, influence cell electrofusion. Temperature is an important factor, influencing the kinetics of the cell resealing. At lower temperatures cells divide slower, which makes the temperature a crucial factor for "pulse first" methods. Another important parameter is also the presence of bivalent cations like Ca^{2+} in Mg^{2+}.

- Detection of cell fusion is problematic for many reasons. The use of membrane and nucleus dyes is only appropriate when observing separate cells and not for the statistical study of larger samples. The double staining method is potentially useful; however, it requires additional time for the pre-staining of the cells. Moreover, the dyes could change the properties of the cell surface. Based on the cell size difference between the fused and the non-fused cells it might be possible to determine fusion yield by means of measuring the cell volumes. In addition to using cell size as a detection method, it might also be used for selection of fused cells.

- Cell size is one of the crucial parameters that determines the cells' sensitivity to electric pulses. Larger cells are electroporated at lower pulse amplitudes, while smaller cells stay intact, which represents a problem when fusing cells of different sizes. This problem could be overcome by using a cell fusion method that uses different electrical parameters to optimally electroporate different cells and brings them into close contact after that. In such situation, only "pulse first" methods can be used.

2.6 EXPECTED RESULTS AND ORIGINAL CONTRIBUTIONS TO THE RESEARCH AREA

Aim 1: We will investigate the influence of different parameters of the electric pulses (the field strength or pulse amplitude, number of pulses, their length and repetition frequency) on electroporation efficiency for each (fusion partner) cells. We will focus on the investigation of electrical pulse protocols with pulses in different directions.

Aim 2: We will study the influence of medium osmolarity on cell fusion. We will determine the swelling dynamics of the cells in hypoosmolar medium. The data obtained will be used to improve the effectiveness of cell fusion.

Aim 3: We will test the double staining method and compare it to the new method for detecting cell fusion (cell size measurements). We will study the usefulness of this new method especially for fusing cells differing in size.

Aim 4: Electrical parameters influencing cell fusion will be studied for the cell line and the untransformed cells we will be using as fusion partners in hybridoma technology for production of hybridomas.

Aim 5: We will design new electrodes or electrode chamber that will enable us to reduce the number of cell manipulations needed and to shorten the period from pulsing to achieving cell contact. These electrodes will be designed in a way which will allow further upgrading and the use of separate treatment of both fusion partner cells at the same time.

3 MATERIALS AND METHODS

Our work was performed in two different laboratories. Lymphocytes B isolation, hybridomas production and evaluation experiments were performed in the Centre for the Production of Diagnostic Reagents and Research (CPDRR) at the Blood Transfusion Centre of Slovenia and all other experiments were performed in Laboratory of Biocybernetics at the Faculty of Electrical Engineering.

3.1 CELLS, CULTURE MEDIA AND CHEMICALS

3.1.1 Culture media and chemicals

Eagle's minimal essential medium (EMEM), DMEM (Dulbecco's modified Eagle medium), Ham's Nutrient Mixtures (F-12 HAM), RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, 2-amino-6-methylmercaptopurine, glucose, sucrose, sodium pyruvate, HEPES, Ficol – Histopaque – 1077, dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), magnesium chloride, $MgCl_2$, trypsin, EDTA, HAT, HT, OPI, nigrasin, sodium bicarbonate, PBS (Mg- Ca-) cell grade, BSA were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Germany). Antibiotics (crystacillin and gentamicin) were purchased from Lek (Ljubljana, Slovenia). CD19 beads for isolation of lymphocytes B from fresh PBMC (peripheral blood mononuclear cells), LS columns for positive selection of cells with the MidiMax separation unit were purchased from Miltenyi Biotec (Miltenyi Biotec GmbH, Germany). Fluorescent dyes propidium iodide, CMFDA

and CMRA were purchased from Molecular probes (Invitrogen, USA). Human AB serum was obtained from CPDRR production, Ecoclone.

3.1.2 Isoosmolar and hypoosmolar buffers

Iso- and hypo-osmolar buffers (phosphate buffer saline – PBS) of osmolarities 260, 200 and 93 mOsm (mOsmol/kg) and conductivity 1.62 mS/cm were used (Table 1). The osmolarity of solutions was determined with Knauer vapor pressure osmometer K-7000 (Knauer, Wissenschaftliche Gerätebau, Germany). Buffers pH was adjusted to 7.2.

Table 1: Composition of hypo- and isoosmolar media used in the experiments.

Preglednica1: Sestava medijev (hipo- in izoosmolarni), uporabljenih v poskusih.

ingredients	isoosmolar	hypoosmolar
K_2HPO_4/KH_2PO_4	10 mM	10 mM
$MgCl_2$	1 mM	1 mM
sucrose	250 mM	75 mM
pH	7,2	7,2
conductivity	1,62 mS/cm	1,62 mS/cm
osmolarity	285 mOsm	93 mOsm

3.1.3 Cells

All cell lines were cultured in CO_2 incubator (Kambič, Slovenia and Hereaus, UK) in humidified atmosphere at +37 °C and 5 % CO_2 in appropriate culture media. Mouse melanoma cell line (**B16-F1**) were cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS), and L-glutamine. Chinese hamster ovary cells (**CHO**) were cultured in F-12 HAM medium supplemented with 10% FBS, gentamicin, crystacillin and L-glutamine.

Mouse myeloma cells (**NS1**) were cultured in DMEM medium (4,5 g/L glucose, 10 mM HEPES, 1,5 g/L sodium bicarbonate) supplemented with 13% fetal calf serum (FCS), penicillin (Pliva, Croatia), streptomycin sulfat (Sigma, USA) and L-glutamine. Human myeloma (**HuNS1**) and hybridoma cells were cultured in RPMI-1640 medium supplemented with 4,5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1,5 g/L sodium bicarbonate, 0,02 mM 2-amino-6-methylmercaptopurine, 15% AB serum (ABS), gentamicin, crystacillin and 2 mM L-glutamine. L-glutamine is an essential amino acid that can be used as an alternative source of carbon and as a precursor for nucleotides. It was added in the media for culturing of myeloma and hybridoma cells every 14 days, since it decomposes in short time at higher temperatures.

Lymphocytes B were isolated from peripheral blood obtained from healthy volunteers (permission of ethical commission No. 193/02/09 dated 17.4.2009) and kept in phosphate

buffer saline (PBS) until electroporation or electrofusion experiments were performed. Cell lines were grown in 25 cm^2 culture flasks (TPP, Switzerland). Adherent cells were exposed to 0.25% trypsin / EDTA solution for 1 minute. Trypsin solution was then removed and 5 ml of culture media was added. Cells were gently rinsed from the bottom with plastic pipette and homogenous cell suspension was prepared.

3.1.4 Isolation of lymphocytes B from Buffy coat

10 ml of Histopaque - 1077 was added into a 50 ml conical centrifuge tube and brought to the room temperature. 20 ml of diluted blood (1:1 with PBS) was carefully layered onto the Histopaque. The tube was centrifuged at 700 x g for 30 minutes at room temperature. After centrifugation, the 0,5 cm of the opaque interface containing mononuclear cells was carefully aspirated with a Pasteur pipette and transferred carefully into a clean conical centrifuge tube. The tube was filled up with PBS and mixed by gentle aspiration and centrifuged at 400 x g for 15 minutes at room temperature. The supernatant was aspirated and discarded. Cell pellet (peripheral blood mononuclear cells) was then resuspended with PBS, mixed by gentle aspiration and centrifuged at 300 x g for 10 minutes at room temperature. The last step was repeated two or three times to wash out the remaining Histopaque solution.

Freshly isolated PBMC (peripheral blood mononuclear cells) were then resuspended in 20 ml Mac buffer (PBS with 0,5 % FCS and 2 mM EDTA) and centrifuged at 300 x g for 10

minutes at room temperature. Pellet was resuspended in 1 ml Mac buffer and 50 µl CD19 microbeads were added. Cells were then incubated on ice for 25 minutes, shaken from time to time. 20 ml ice cold Mac buffer was then added and centrifuged at 300 x g for 10 minutes at +4 °C. CD19 microbeads were developed for direct magnetic isolation of CD19+ B cells. As a B cell lineage marker, the CD19 antigen is expressed from the early pro-B cell stage to the B cell lymphoblast stage, but the expression is down-regulated upon B cell maturation to plasma cells. The CD19 antigen is further expressed on most malignant B cells and on a subset of follicular dendritic cells.

The device for cell isolation was assembled in laminar flow (column was placed in magnet holder; filter was put on the top of it). 3 ml Mac buffer (ice cold) was run through to wet the column. Cells were resuspended in 3 ml Mac buffer (ice cold) and loaded on top of the filter. After the last drop came out of the column, it was washed two to three times with 3 ml of Mac buffer (ice cold). The column was then placed on empty 15 ml tube and positive cells were pressed out of the column and collected in a tube. The tube was filled with PBS and centrifuged for 10 minutes at 300 g. Purified CD19 positive cells were then kept on +4 °C until experiments were performed.

3.1.5 Preparation of cells for feeder layer

The remaining mononuclear cells (after CD19 positive cell isolation) were irradiated to make them unable to proliferate thus preventing them from rapidly expanding throughout

the cultures at the expense of the hybrid cells. These cells were used as the feeder layer cells in post – electrofusion processing of the cells in the hybridomas production experiments. Mononuclear cells may be superior to other cell types for that purpose because they contain phagocytic cells capable of ingesting contaminating microorganisms and cellular debris, thus eliminating some of the toxic substances released into the medium by the dying cells. Feeder cells are thought to work by providing essential cellular contact to the developing cell clones as well as by secreting vital growth factors (Ritter and Ladyman, 1995).

3.2 THE EFFICIENCY OF CELL ELECTROPORATION

In order to expose cells to electric field, electroporation cuvettes with 2 mm gap (Eppendorf, Germany) or plate electrodes with 2 mm gap (made in our laboratory) were used. Cell suspension prepared in cell culture media was counted and aliquots of 8 x 10^5 NS1 and huNS1 cells or 5 x 10^5 lymphocytes B were prepared, centrifuged (300 x g, 5 min, +4 °C) and kept at +4 °C. For each electric field amplitude (and control) one vial was used. Supernatant was carefully removed and NS1 or huNS1 cells were resuspended in 360 µl of 200 mOsm hypoosmolar buffer.

Electroporation was performed two minutes after the start of hypoosmolar treatment. Fifteen seconds before pulse application, 40 µl of 100 mM propidium iodide was added and 400 µl of cell suspension (8×10^5 cells/ml for NS1 and huNS1) was transferred to

electroporation cuvette. For lymphocytes B, cells were resuspended in 45 µl of 200 mOsm hypoosmolar buffer. Fifteen seconds before pulse application, 50 µl of 100 mM propidium iodide was added and 50 µl of cell suspension (5×10^5 cells/ml) was transferred between the plate electrodes.

Cells were permeabilized by 8 rectangular pulses (pulse duration 100 µs, repetition frequency 1 Hz) with different pulse amplitudes. For negative control we used 0 V/cm and for positive control 2400 V/cm (3600 V/cm for lymphocytes B) were delivered. In negative control samples no pulses were delivered. Electroporation efficiency was determined spectrofluorimetricaly by the intensity of fluorescence of propidium iodide that enters only electroporated cells. Cell suspension was plated in four microplate wells (384 – black well microplates, Greiner Bio-One, Germany). Fluorescence was measured with microplate reader Infinite M200 (Tecan, Switzerland) at 535 nm excitation and 617 nm emission wavelength controlled with PC software i-Control (Tecan, Switzerland) 3 minutes after pulse application. Electroporation efficiency EP [%] of treated cells was defined as

$$EP = \frac{(A_{TC} - A_{NCC})}{(A_{PCC} - A_{NCC})} \times 100\,, \qquad (1)$$

where A_{TC} is the fluorescence value of treated cells [relative fluorescent units R.F.U], A_{PCC} is the fluorescence value of positive control cells that represents 100 % electroporation efficiency [R.F.U] and A_{NCC} is the fluorescence value of negative control cells that

represents 0 % electroporation efficiency [R.F.U.]. The mean EP values (± STD) for given amplitude were calculated from at least three independent experiments.

3.3 BEHAVIOR OF CELLS IN STRONGLY HYPOOSMOLAR (100 MOSM) MEDIUM

3.3.1 Cell size measurement and analysis

We measured cell volume changes using a protocol which allowed rapid exchange of media. For that purpose we used 9,2 cm^2 tissue culture Petri dishes (TPP, Switzerland). Before microscopic measurement, cells in suspension were counted by hemacitometer and 5 x 10^5 cells were placed on Petri dish. B16-F1 and CHO cells were incubated at +37 °C for 20 - 40 minutes in culture medium to allow cells to slightly adhere. Cells adhered enough to stay on place while medium was exchanged and their round shape was preserved.

NS1 is a suspension cell line that always retains round shape; therefore, these cells were cultured for one day, during which time they adhered to the surface enough for performing such experiments. Culture medium was removed and cells were washed with 1 ml isoosmolar buffer leaving 300 µl to avoid drying of the sample during acquisition of the first image (represents the time t = 0 min) with the AxioVert 200 microscope (Zeiss, Germany).

The cells were viewed with 40 x objective in transmitted light. Phase contrast images were acquired with cooled CCD video camera VisiCam 1280 (Visitron, Germany) and PC software MetaMorf 7.0 (Molecular Devices, USA). After the first image was acquired, 3,3 ml hypoosmolar buffer was added to the cells (in the control samples isoosmolar buffer was used). The osmolarity of buffer used was 100 mOsm. Images of the cells were then taken at various time intervals up to 30 min after buffer exchange (every 15 s until 2 min and in minute steps after that). One to three sequences of each experiment were performed with current cell passage. During each sequence resting cell suspensions were kept at +4 °C.

The cell diameters of 5-10 cells per image sequence (37 images in total) were determined at each time interval. Cell size dynamic was defined as relative change of cell diameter $\nu = d/d_0$, where d is actual cell diameter and d_0 is the initial cell diameter (at t = 0 min). The mean ν values (± STD) for a given experiment (cell line) were calculated from at least three independent experiments and plotted against time.

3.4 FUSION DETECTED BY FLUORESCENT STAINING ASSAY FOR DIFFERENT CELL PAIRS

3.4.1 Cell sizes and concentrations for monolayer determinations

Cell diameters for NS1, huNS1 and lymphocytes B were obtained from measurements on at least four sets of 50 - 100 cells by phase contrast microscopy at 40 × objective magnification. Cell concentrations that result in monolayer formation were determined experimentally for NS1 cells. 20 µl drops of cells in different concentrations (from 25 x 10^6 cells/ml to 1 x 10^5 cells/ml) were plated in a 24 multiplate wells. The optimal concentration was determined where cells were as close as possible to each other and almost no cells were above the monolayer. Concentrations for huNS1 cells and for combination of huNS1 and lymphocytes B were then calculated based on respective cell diameters.

3.4.2 Cell growth characteristics

Human myeloma cell line HuNS1 was chosen for the production of human – human hybridoma cells. It has been shown that myeloma cells are more appropriate for fusion than lymphoblastoid cell lines since they are in a more appropriate state of differentiation for high antibody secretion and have abundant polyribosomes and extensive Golgi apparatus.

HuNS1 cells were purchased from ATCC and were first investigated to determine optimal growth conditions for this cell line. Cells were plated in 24 well multiplates. 10^4 cells were plated in each well at day 0. Each day cells from one well were harvested and counted in growth medium containing nigrasin. Dead cells were observed as dark blue cells under the

phase contrast microscope since nigrasin only enters the dead cells. Numbers of cells (dead, alive and total) were determined from day 0, when the cells were plated, to day 14, when all the cells were dead. Three independent experiments were performed. Growth curve for human myeloma cell line huNS1 was obtained. Results are average of three experiments.

3.4.3 Fusion yields detected by fluorescent staining assay for different cell pairs

3.4.3.1 Loading of cells with fluorescent dyes

Fused cells detection and quantification method utilized two different cell trackers and fluorescence microscopy. The dyes used were green CMFDA (excitation/emission = 492 nm/517 nm) and orange CMRA (excitation/emission = 548 nm/576 nm). Cell tracker CMRA has an overall negative net charge at neutral pH, in contrast with the overall net positive charge of the traditionally used orange cell tracker CMTMR. Thus, unlike CMTMR, cell tracker CMRA remains primarily in the cytoplasm instead of being sequestered inside actively respiring mitochondria. Cell tracker stock solutions (10 mM) were prepared by adding 10,76 µl and 9 µl (for CMFDA and for CMRA, respectively) of DMSO solutions (Sigma-Aldrich, Germany) to 50 µg of the dye.

The stock solutions can be stored in a refrigerator at +4 °C for a few months. Loading solutions (7 µM) were prepared by mixing 2,1 µl of each stock solution with 3 ml of

bicarbonate-free Krebs-Hepes buffer (Salvi et al., 2002) and warmed to +37 °C. While preparing dye solutions direct light on them was avoided.

Cells were grown in two 25 cm^2 culture flasks. NS1 cells were washed with bicarbonate-free Krebs-Hepes buffer (3 ml) after which green and orange cell tracker loading solutions were added (one to each flask). HuNS1 cells and lymphocytes B were centrifuged and cell pellets were resuspended in dye solutions. Cells were placed in an incubator for 30 minutes.

During that time, dyes entered the cells and they became fluorescent due to enzymatic cleavage (we were able to see slight green and orange color of loading solutions under room light). After 30 minutes, loading solutions were replaced by culture media and cells were placed in an incubator for additional 2 hours. During second incubation, dyes were transformed into cell-impermeant reaction products. Finally, differently labeled cells were mixed together at 1:1 ratio.

Mouse NS1 cells were divided in two halves and separately labeled with CMRA or CMFDA, respectively. After that, they were fused together. The same was done for human NS1 cells. Finally, human NS1 cells were labeled with CMRA and fused with human lymphocytes B that were labeled with CMFDA.

3.4.3.2 Electrofusion

Prior to electrofusion experiments, cell concentrations that resulted in a single monolayer of spherical cells on the plate surface in close contact were determined (see cell density determinations). These concentrations were $1{,}5\times10^5$ for NS1 cells, 3,2 x 10^5 cells/ml for huNS1 cells and 2,5 x 10^5 cells/ml for combination of huNS1 cells and lymphocytes B.

20 µ1 drops of cell suspension were plated to the middle of 24-well multiplate (TPP, Switzerland). Close cell to cell contacts were established by means of modified adherence method. Cells in drops were incubated for 20 minutes in an incubator, allowed to slightly adhere to the surface of the plate and form spontaneous contacts.

Before electroporation, 350 µl of hypoosmolar buffer was added. Two minutes after hypoosmolar treatment, electric pulses were delivered using two parallel electrodes (Pl/Ir = 90/10) with 5 mm (or 2 mm) gap connected to the electric pulse generator Cliniporator (IGEA, Italy). Based on electroporation experiment, we used electric field amplitudes for efficient electroporation of all cells in hypoosmolar buffer (1600 V/cm and higher). For control samples no pulses were delivered.

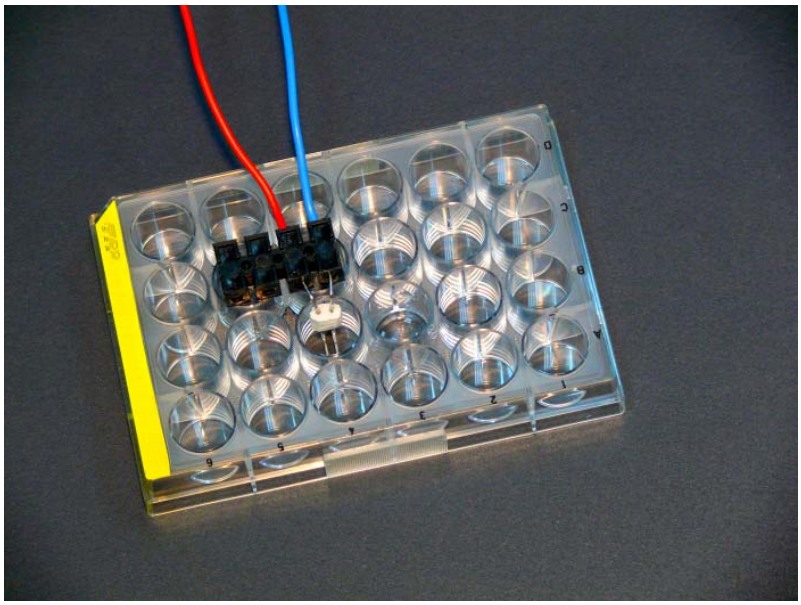


Figure 1: A photograph of a 24 well plate with the electrodes placed in a position for electroporation. Custom made electrodes - two parallel electrodes (Pl/Ir = 90/10) with 5 mm (or 2 mm) gap - were used, connected to the electric pulse generator Cliniporator (IGEA, Italy).

Slika1: Fotografija ploščice s 24 vdolbinicami z elektrodami v položaju za elektroporacijo. Elektrode, izdelane v laboratoriju (Pt/Ir : 90/10) in imajo med žičkama 5 mm (oz. 2 mm) razdalje. Elektrode povežemo z generatorjem električnih pulzov Cliniporator (IGEA, Italy)

3.4.3.3 Detection of fusion

Fusion yield was determined 10 minutes after the pulse application, by dual color fluorescence microscopy using two emission filters (one centered at 535 nm (HQ535/30m, for CMFDA) and the other centered at 510 nm (D510/40m, for CMRA) and monochromator (Polychrome IV, Visitron, Germany). Cells were observed under the Axiovert 200 microscope (Zeiss, Germany) with 20 × objective magnification.

Three images (phase contrast, orange and green fluorescence) were acquired from five randomly chosen fields in each well using cooled CCD video camera VisiCam 1280 (Visitron, Germany) and PC software MetaMorph 7.1 (Molecular Devices, USA). Three channel images were created from each image triplet (phase contrast, orange and green fluorescence) using macro written in free image processing software ImageJ (NIH Image, USA).

In order to improve the visual quality of the images three preprocessing steps were applied to original images: a) background subtraction, b) contrast enhancement (both already implemented in ImageJ) and image smoothing by Sigma filter plus plug-in (filter smoothes an image while object edges are preserved) with default parameter sets. Finally the three channel images were composed using RGB to Gray plug-in. On each composed image cells were manually counted and fraction of double fluorescent cells was calculated as:

$$F_{dual}=N_{dual}/(N_{red}+N_{green}+N_{dual})\times 100, \quad (2)$$

where F_{dual} is fraction of double fluorescent cells in [%], N_{dual} is number of dual labeled cells; N_{red} and N_{green} are numbers of red and greed labeled cells, respectively. Fusion yield was calculated as

$$FY=F_{dual}\times 2, \quad (3)$$

where FY is fusion yield, since half of the fusion cells cannot be detected (in instances when cells of the same color are fused). The mean FY values (± STD) for a given cell line and amplitude were calculated from at least three independent experiments.

3.5 HYBRIDOMA YIELD: FUSION OF LYMPHOCYTES B AND MYELOMA CELLS

3.5.1 Fusion of cells for production of hybridoma cells

20 µ1 drops of cell suspension were plated to the middle of 24 well multiplate (TPP, Switzerland) the same way as in electrofusion protocol with fluorescently labeled cells. Lymphocytes B were mixed in ratio 1:1 with NS1 or huNS1 cells, respectively.

Before electroporation, 50 µl of hypoosmolar buffer was added. Two minutes after hypoosmolar treatment, electric pulses were delivered using two parallel electrodes (Pl/Ir = 90/10) with 5 (or 2) mm gap and electric pulse generator Cliniporator (IGEA, Italy). 1 minute after electroporation, 1 ml growth medium without phenol red and 50 µl (8×10^5) feeder layer cells were added to each well. 15 minutes later, cells from each well were gently mixed and transferred in 10 wells of 96 well multiplate (100 µl in each well).

When the hybridoma clones appeared (after approximately 7 to 10 days), they were counted in each well. Numbers of clones produced in the wells from one electrofusion treatment were summarized. Fusion yield is defined as number of clones divided by all lymphocytes B that were treated.

3.5.2 Post – fusion cell processing: selection of hybridoma cells

The hybrid cells were grown in selective hypoxanthine aminopterin thymidine (HAT) medium. HAT medium contains a drug aminopterin, which blocks the main pathway for nucleotide synthesis, making the cells dependent on another pathway that needs HGPRT (Hypoxanthine-Guanine Phosphoribosyl Transferase) enzyme. Thymidine and hypoxanthine are needed for the synthesis of nucleotides through alternative pathway. Since myeloma fusion partner cells are HGPRT deficient, they will die unless they fuse with B cells. B cells that do not fuse will also die because they lack tumorigenic property

of immortal growth. HAT medium thus allows selection of hybridoma cells, which inherit HGPRT gene from B cells and tumorigenic property from myeloma cells.

Selective HAT medium was prepared from the growth medium (RPMI – 1640 with all additional components) with the addition of the HAT solution. Selective HAT medium contains 0,136 mg hypoxanthine, 0,019 mg aminopterin and 0,39 mg thymidine. OPI solution was added to HAT medium. After approximately three weeks of growing hybridoma cells in HAT medium, HAT medium was replaced with HT medium that lacks aminopterin. Cells were grown in multi-well plates with 96 wells, with 24 wells and in 25 cm^2 culture flasks (all plastic labware: Corning, Costar).

3.5.3 Growth and testing of hybridoma cells

Hybridoma cells were cultured in CO_2 incubator in humidified atmosphere at +37 °C and 5 % CO_2 in HAT medium. The medium was replaced every two to three days. After approximately 20 days, HAT medium was replaced with HT medium. First clones were detected microscopically after 7 to 9 days. After three weeks, the clones were big enough, so that we were able to test the presence of specific antibodies (we chose blood group system AB0 as a model) in the supernatant. ELISA and agglutination tests were used. The purpose of screening tests was detection of the hybridoma cell lines that produced the desired antibodies among the large number of the hybridoma cell lines that were produced with fusion.

3.5.3.1 Agglutination test

For agglutination test a 1,5 % suspension of fresh erythrocytes were used (50 µl of concentrated erythrocytes in 3 ml of saline). Erythrocytes were added to 100 µl of samples. After centrifugation at 900 g at room temperature for two minutes, agglutinates were resuspended by shaking. The results were determined macroscopically.

3.5.3.2 ELISA

Enzyme-Linked Immuno Sorbent Assay, or ELISA, is an immunological technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. In indirect ELISA, which we used, an amount of antigen is adsorbed to a surface of a microtiter plate with high capacity for protein adsorption. After washing, a sample containing antibody is applied to the plate so that it can bind to the antigen. After washing, a secondary antibody linked to an enzyme is added into the wells. In the final step, a substrate is added, so that the enzyme can catalyze the reaction to a detectable signal, which is measured spectrofotometrically.

In our test, antigens A and B (saliva from secretors of substance A and substance B) were coated to microtiter plates (Corning, Costar) in carbonate/bicarbonate buffer (15 mM $NaHCO_3$, 15 mM Na_2CO_3, 3 mM NaN_3, pH = 9,6) at +4 °C over the night. Following the washing with 0,05 % Tween 20 in phosphate buffer (7,5 mM Na_2HPO_4 x $2H_2O$, 2,5 mM NaH_2PO_4 x $2H_2O$, 0,9 % NaCl, pH = 7,2), the plates were blocked with the phosphate buffer containing 1 % bovine serum albumin (BSA), pH = 7,2. After incubation for 30

minutes at +37 °C and consequent washing, samples (supernatants from the wells with hybridoma cells) were added and microtiter plates were incubated for 90 minutes at +37 °C. After washing, the plates were incubated with horseradish peroxidase (HRP) labeled goat anti-human IgG (Fc specific) and IgM (0.5 μg/ml, Jackson Immuno Research Lab, Dianova, Germany) and incubated for 90 minutes at +37 °C. Following subsequent washing, the enzymatic reaction was conducted with a substrate solution containing ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] – diammonium salt) that yields a green end product after 15 minutes at +37 °C. The green product has two major absorbance peaks, at 410 nm and 650 nm. The optical density was measured at 405 nm with an auto-plate reader (Tecan, Switzerland).

4 RESULTS

The results are listed in the following order: first, the results of the experiments of the efficiency of electroporation for different cell lines and second, the influence of hypoosmolar medium. The results of fusion are presented separately: the results, detected with fluorescent dyes, and those obtained with hybridoma technology. New pipette tip chamber for electroporation is presented at the end.

4.1 THE EFFICIENCY OF CELL ELECTROPORATION

4.1.1 Electroporation efficiency of different cells in mildly hypoosmolar (200 mOsm) medium

The efficiency of electroporation was determined using different values of electric field amplitude for different cell types in mildly hypoosmolar (200 mOsm) medium. Electroporation curves were obtained with application of trains of eight pulses with 100 μs duration and frequency of 1 Hz in all the experiments. Electroporation curve for lymphocytes B differs from mouse and human NS1 cells. Higher values of electric field are needed to electroporate lymphocytes B than NS1 cells. On the other hand, electroporation curves for mouse and human NS1 cell lines are similar and do not reflect the differences in cell sizes between these two cell lines (Fig. 2).

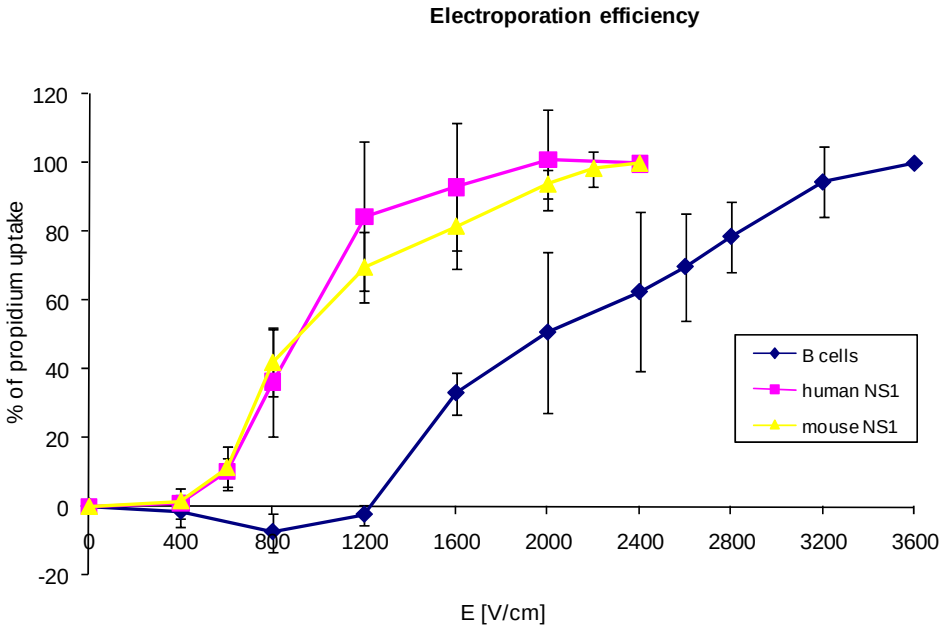


Figure 2: Electroporation efficiency curves for three different types of cells in 200 mOsm medium. Human NS1 (r = 5,25 µm), mouse NS1 (r = 7,75 µm) and primary lymphocytes B from human peripheral blood (r = 3,6 µm) from healthy volunteers were analyzed. The efficiency of electroporation is represented as relative intensity of the propidium iodide fluorescence.

Slika 2: Krivulje učinkovitosti elektroporacije za tri različne tipe celic v 200 mOsm mediju: človeške NS1 (r = 5,25 µm), mišje NS1 (r = 7,75 µm) in primarne limfocite B iz človeške periferne krvi (r = 3,6 µm), ki smo jo dobili od zdravih prostovoljcev. Učinkovitost elektroporacije je predstavljena kot relativna intenziteta fluorescence propidijevega iodida.

Phase contrast microscopy was used to determine the diameters of the cells. All diameter measurements were made using objective magnification 40×. Diameters of the cells are listed in a table 2.

Table 2. Cell sizes in isoosmolar growth medium

Preglednica 2. Velikosti celic v izoozmolarnem rastnem mediju

	cell diameters ± STD [μm]
mouse NS1	15,5 ± 0,5
human NS1	10,5 ± 0,5
lymphocytes B	7,7 ± 0,7

4.1.2 Electroporation efficiency of mouse myeloma cell line NS1 in hypo- and isoosmolar medium

We determined the electroporation efficiency for mouse NS1 cell line in mildly hypoosmolar (200 mOsm) and isoosomolar medium. Both curves are similar.

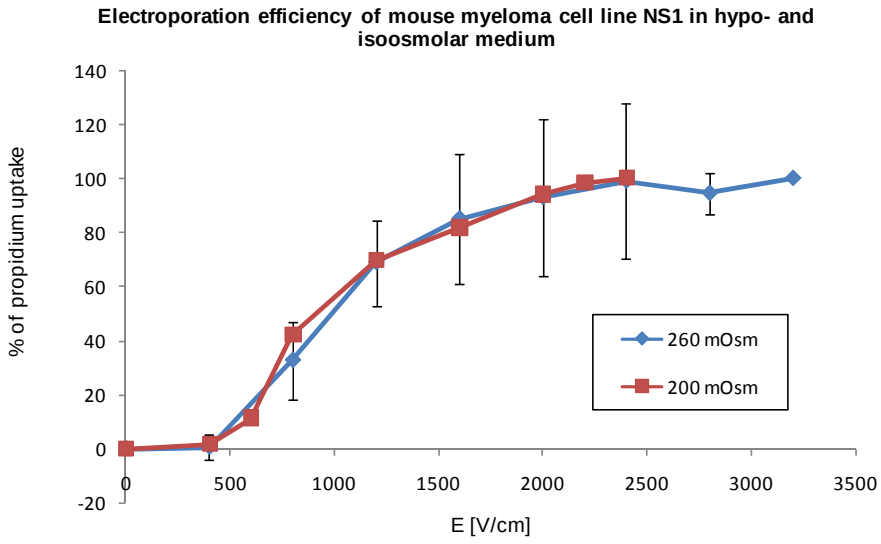


Figure 3: Electroporation efficiency curves for mouse NS1 cell line in two media of different osmolarity (200 mOsm in 260 mOsm). The efficiency of electroporation is represented as relative intensity of the propidium iodide fluorescence.

Slika 3: Krivulji učinkovitosti elektroporacije za mišje NS1 celice v dveh medijih z različno ozmolarnostjo (200 mOsm in 260 mOsm). Učinkovitost elektroporacije je predstavljena kot relativna intenziteta fluorescence propidijevega iodide.

4.2 BEHAVIOR OF CELLS IN STRONGLY HYPOOSMOLAR (100 MOSM) MEDIUM

4.2.1 Behavior of CHO, V79 and B16F1 cells in strongly hypoosmolar (100 mOsm) medium

Behavior of cells in hypoosmolar medium was determined for different cell lines. The results show that cells of different cell lines differ in their response to hypoosmolar medium (Ušaj, et al., 2009b).

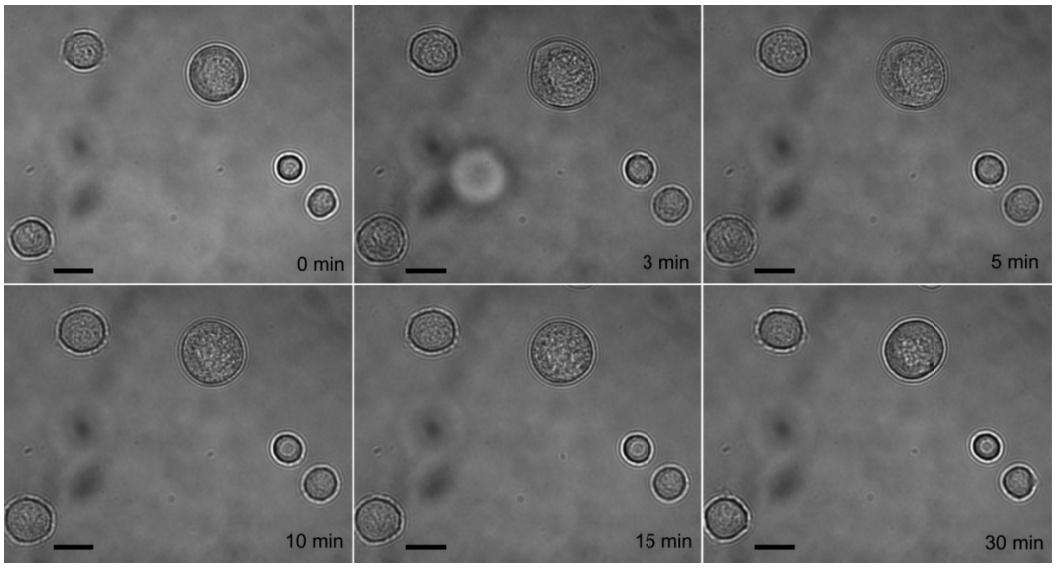


Figure 4: Swelling and regulatory volume decrease (RVD) of B16-F1 cells in 100 mOsm medium. The images show the same cells before (0 min) and after hypoosmolar osmotic stress at the indicated time intervals. Upon medium replacement at zero time, the cells swelled within 5 min, then shrank gradually within the observed time (5-30 min).

Slika 4: Napihovanje in regulirano zmanjšanje prostornine B16F1 celic v 100 mOsm mediju. Slika prikazuje iste celice pred (0 min) in po izpostavitvi hipotoničnemu mediju ob opisanih časovnih intervalih. Po menjavi medija ob času 0 minut so se celice prvih 5 minut napihovale , potem pa so se postopno skrčile v času opazovanja (5 do 30 minut).

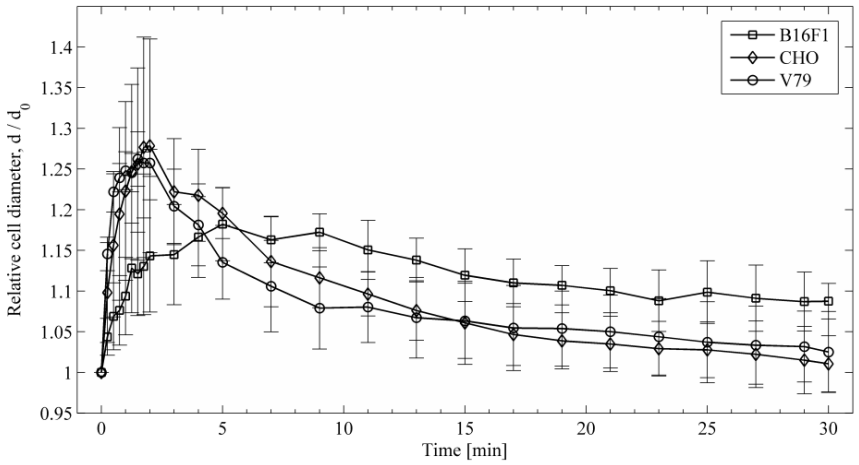


Figure 5: Time course of relative cell diameter (d/d_0) after the osmotic stress for three different cell lines. d is cell diameter and d_0 is the initial cell diameter in isoosmolar medium (t = 0 min). Cell diameter was obtained by directly measuring the size of the cells (approximately 10 to 20 cells) under phase contrast microscope. Each data point represents the mean ± STD of at least three experiments.

Slika 5: Časovno spreminjanje relativnega premera celic (d/d_0) po osmotskem stresu za tri celične linije, kjer je d premer celice in d_0 začetni premer celice v izoozmolarnem mediju. Premer celic smo določili z neposrednim merjenjem velikosti celic (približno 10 do 20 celic) pod fazno kontrastnim mikroskopom. Vsaka točka predstavlja povprečje ± STD vsaj treh poskusov.

4.2.2 Behavior of mouse NS1 cells in strongly hypoosmolar (100 mOsm) medium

Our cells of interest - fusion partner NS1 cells - differ in their behavior from other three model cell lines that we investigated. NS1 cells did not increase their volume to the peak value at specific time and then regulate it back as the other cells. Instead, they started to burst and expressed non-spherical shapes.

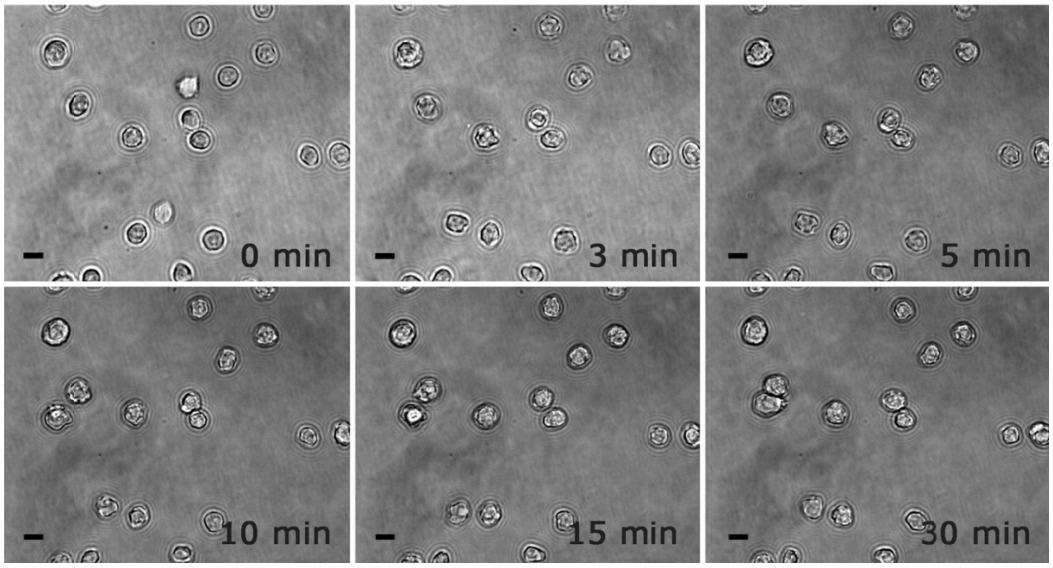


Figure 6: The behavior of mouse NS1 cells in 100 mOsm medium. The image shows the behavior of the cells during 30 minutes after hypoosmolar challenge. These cells did not exhibit any regular size alterations. Instead, NS1 cells started to burst and expressed non-spherical shapes.

Slika 6: Obnašanje mišjih NS1 celic v 100 mOsm mediju. Slike prikazujejo obnašanje celic med tridesetimi minutami izpostavitva hipotoničnemu mediju. Te celice ne kažejo vzorca napihovanja in reguliranega krčenja, ampak na površini tvorijo mešičke in intenzivno spreminjajo svojo obliko.

4.3 FUSION DETECTED BY FLUORESCENT STAINING ASSAY FOR DIFFERENT CELL PAIRS

4.3.1 Cell sizes and concentrations for monolayer determinations

Mouse NS1 cells were investigated to determine optimal concentration for monolayer formation. Cells in different concentrations are shown in Figure 7.

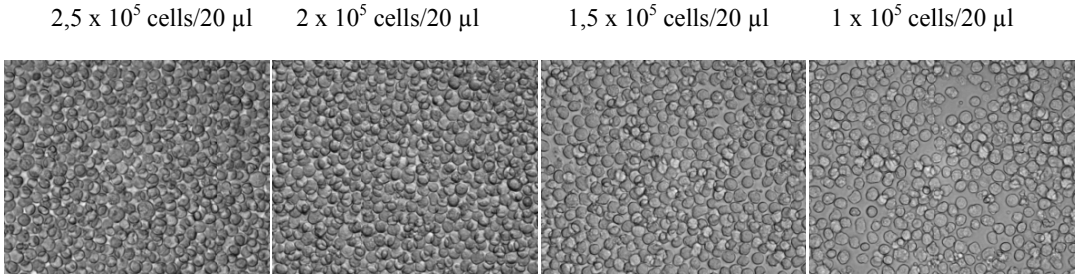


Figure 7: Determination of the optimal concentration for cell monolayer formation. Images show the density of the cells at different cell concentrations in a cell droplet. Cell concentrations are from 2,5 x 10^5 cells/20 µl to 1 x 10^5 cells/20 µl.

Slika 7: Določitev optimalne koncentracije celic za tvorbo monosloja. Slike kažejo gostoto celic nasajenih v kapljicah z različnimi koncentracijami. Koncentracije celic so prikazane od 2,5 x 10^5 cells/20 µl do 1 x 10^5 cells/20 µl.

Optimal concentration for mouse NS1 cell line was determined to be 1,5 x 10^5 cells/20 µl. Optimal cell densities for different combination of cells used in our experiments (human

NS1 cells, human NS1 cells with human lymphocytes B) were estimated from optimal values for mouse NS1 cell line, taking into account differences in size between the three cell types. Estimated values for cell concentrations in different combinations are listed in table 3.

Table 3. Optimal cell concentrations for monolayer formation at different cell combinations. Cell numbers are estimated for 20 µl droplets.

Preglednica 3. Optimalne koncentracije celic za tvorbo monosloja celic pri različnih kombinacijah celic. Število celic je določeno za 20 µl kapljico.

cell combinations:	N of cells: mouse NS1	N of cells: human NS1	N of cells: lymphocytes B
mouse NS1& mouse NS1	1,5 x 10^5	/	/
human NS1& human NS1	/	3,2 x 10^5	/
human NS1&lymphocytes B	/	2,5 x 10^5	2,5 x 10^5

4.3.2 Cell growth characteristics

Human NS1cells were first investigated to determine optimal growth conditions for this cell line since these cells were bought from ATCC and used in our laboratory for the first time. The results are shown in Figure 8. Depending on the results, experiments with these cells were performed in order to insure that harvesting and subculturing time provided cells that were in upper region of the log growth phase. Cells were seeded at 1 or 2 x 10^5 cells/ml and harvested at 1 to 1,5 x 10^6 cells/ml two or three days later to obtain cells in their log phase of growth.

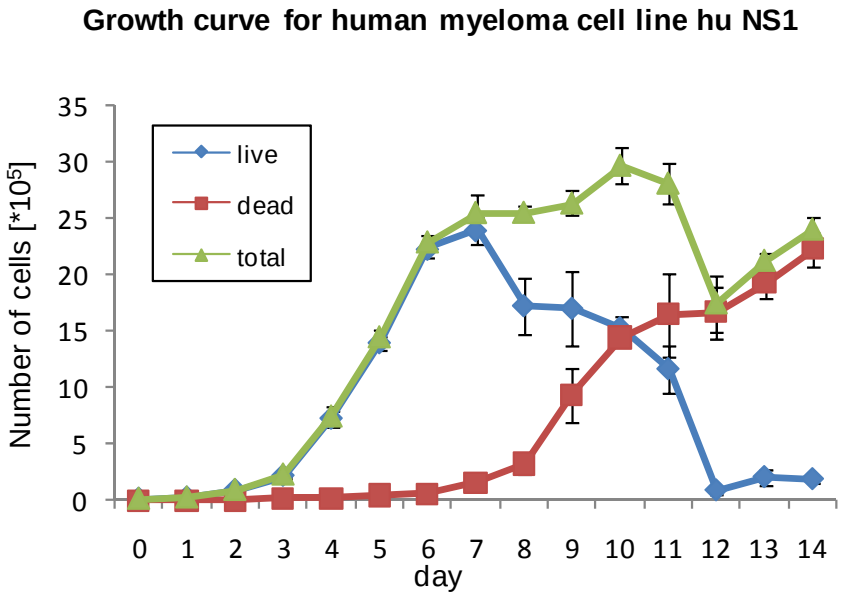


Figure 8: Growth curve for human myeloma cell line NS1. The results are the average of three experiments. The number of cells was determined each day from day 0, when the cells were seeded to a 24 well multiplate at the concentration of 10^4 cells per well to day 14, when all cells were dead.

Slika 8: Rastna krivulja za človeške mielome NS1. Rezultati so povprečje treh poskusov. Število celic je bilo določeno vsak dan od dneva 0, ko so bile celice nasajene v ploščico s 24 vdolbinicami s koncentracijo 10^4 celic na vdolbinico do dneva 14, ko so bile vse celice mrtve.

4.3.3 Fusion yields detected by fluorescent staining assay for different fusion partners

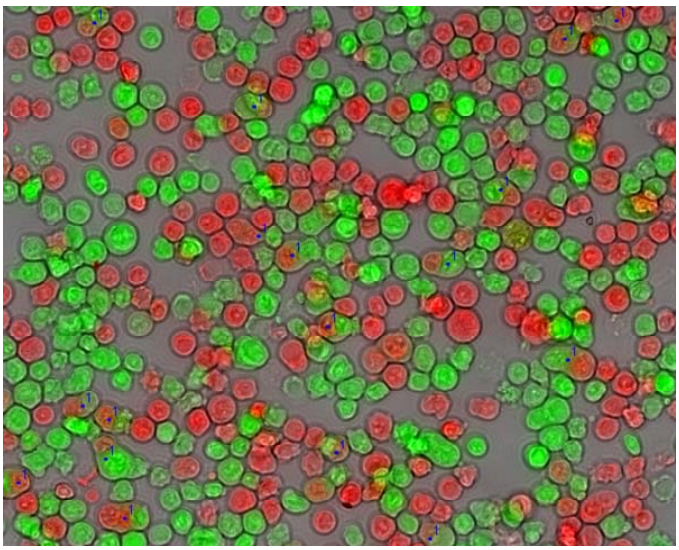
Different cells were fused and fused cells were detected by fluorescent staining. Experiments were performed at different values of electric field amplitudes. No

statistically significant differences between numbers of double labeled cells obtained in experiments with different parameters were observed.

Figure 9: Fusion yield for fusion of cells in different combinations. Contact between cells was achieved with modified adherence method. Trains of eight pulses of 100 µs duration and different amplitudes were used. Fusion was detected by means of fluorescent dyes red CMRA and green CMFDA. The results are the average of one to five experiments.

Slika 9: Izkoristek zlivanja za zlivanje celic v različnih kombinacijah. Stik med celicami smo vzpostavili z modificirano adherentno metodo. Uporabili smo vlake osmih pulzov, dolgih 100 µs z različnimi amplitudami. Zlite celice smo detektirali s pomočjo fluorescentnih barvil CMRA (rdeče) in CMFDA (zeleno). Rezultati so povprečje enega do petih poskusov.

a)



b)

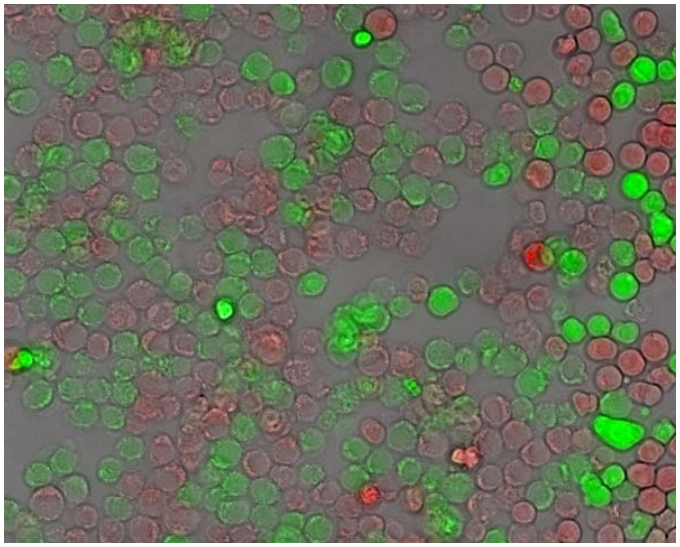
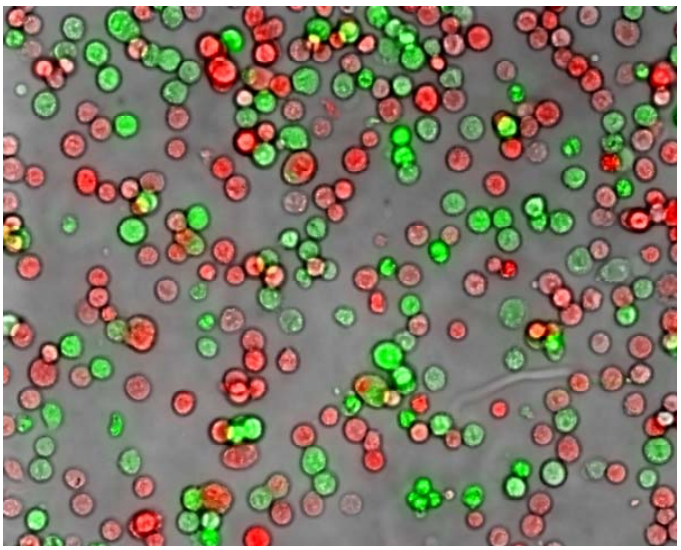


Figure 10: Three channel microscopy image of mouse NS1 cells after electrofusion for 1600 V/cm and 4000 V/cm. Image is composed of phase contrast, CMRA fluorescence (excitation at 548 nm) and CMFDA fluorescence (excitation at 492 nm), objective magnification 20×. Contact between cells was achieved by means of modified adherence method. Electric field amplitudes were (a) 1600 V/cm and (b) 4000 V/cm. Double stained cells are marked with blue marks.

Slika 10: Trikanalna mikroskopska slika mišjih NS1 celic po elektrofuziji za 1600 V/cm in za 4000 V/cm. Slika je sestavljena iz slik faznega kontrasta, fluorecence CMRA (vzbujanje pri 548 nm) in fluorescence CMFDA (vzbujanje pri 492 nm) pri 20 kratni povečavi objektiva. Stik med celicami smo vzpostavili z modificirano adherentno metodo. Amplitudi uporabljenih električnih pulzov sta bili (a) 1600 V/cm in (b) 4000 V/cm. Dvojno obarvane celice so označene z modro oznako.

a)



b)

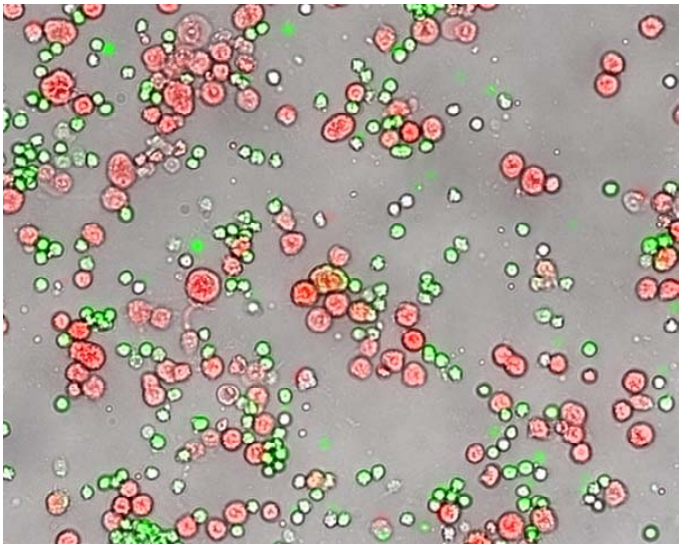
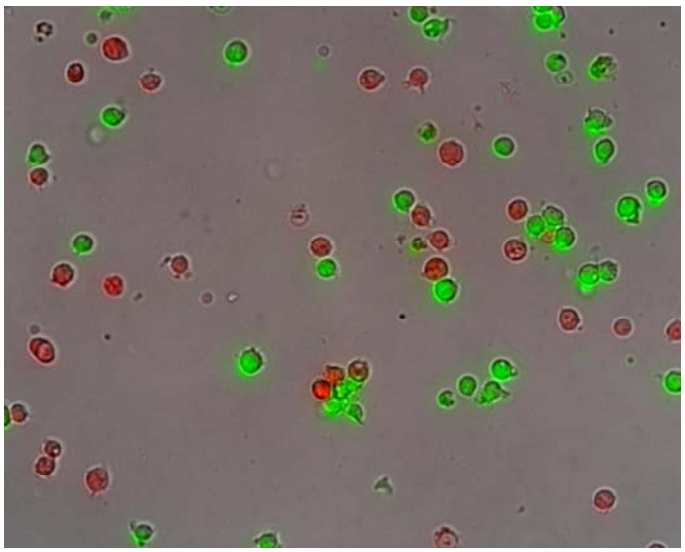


Figure 11: Three channel microscopy image of cells after electrofusion of human NS1 cells with human NS1 cells and human NS1 cells with lymphocytes B. Image is composed of phase contrast, CMRA fluorescence (excitation at 548 nm) and CMFDA fluorescence (excitation at 492 nm), objective magnification 20×. Contact between cells was achieved by means of modified adherence method. Electric field amplitudes were 2400 V/cm. The image shows a fusion of (a) human NS1 cells with human NS1 cells and (b) human NS1 cells with lymphocytes B. Double stained cells are marked with white arrows.

Slika 11: Trikanalna mikroskopska slika celic po elektrofuziji človeških NS1 celic s človeškimi NS1 celicami in človeških NS1 celic z limfociti B. Slika je sestavljena iz slik faznega kontrasta, fluorecence CMRA (vzbujanje pri 548 nm) in fluorescence CMFDA (vzbujanje pri 492 nm) pri 20 kratni povečavi objektiva. Stik med celicami smo vzpostavili z modificirano adherentno metodo. Amplituda uporabljenih električnih pulzov je bila 2400 V/cm. Na sliki je prikazano zlivanje (a) človeških NS1 celic s človeškimi NS1 celicami in (b) človeških NS1 celic z limfociti B. Dvojno obarvane celice so označene z belo puščico.

a)



b)

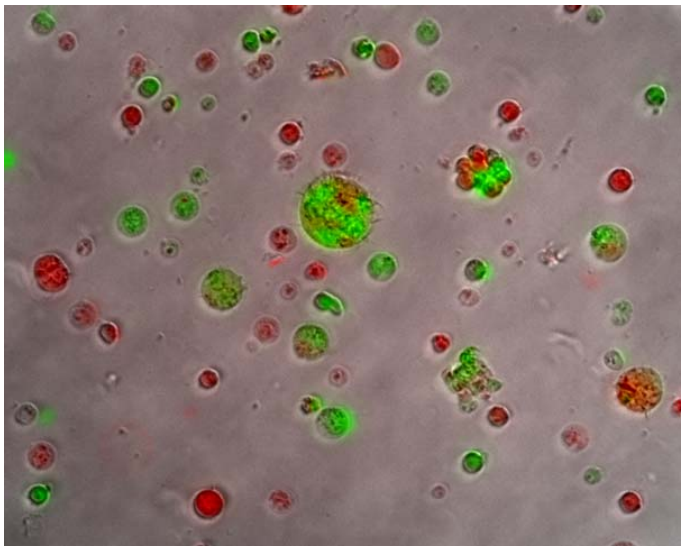


Figure 12: Three channel microscopy image of human NS1 cells after electrofusion of human NS1 for control and for 2500 V/cm. Image is composed of phase contrast, CMRA fluorescence (excitation at 548 nm) and CMFDA fluorescence (excitation at 492 nm), objective magnification 20×. Contact between cells was spontaneous, since these cells grow in clusters. Image a) control – no electric pulses. Image b) electric field amplitude was 2500 V/cm. Double stained cells are marked with white arrows.

Slika 12: Trikanalna mikroskopska slika celic človeških celic NS1 po elektrofuziji za kontrolo in 2500 V/cm. Slika je sestavljena iz slik faznega kontrasta, fluorecence CMRA (vzbujanje pri 548 nm) in fluorescence CMFDA (vzbujanje pri 492 nm) pri 20 kratni povečavi objektiva. Stik med celicami je bil spontan, saj smo celicam dovolili da so se spijele v gruče. Na sliki (a) je prikazan kontrolni vzorec, pri katerem nismo dovedli električnih pulzev. Slika (b) kaže celice po obdelavi z osmimi 100 µs pulzi z amplitude 2500 V/cm. Dvojno obarvane celice so označene z belo puščico.

4.4 HYBRIDOMA YIELD: FUSION OF LYMPHOCYTES B AND MYELOMA CELLS

4.4.1 Hybridoma yield: fusion of lymphocytes B and mouse NS1 cells

Hybridomas (hybrid cells) were obtained from mouse myeloma cell line NS1 and lymphocytes B, isolated from peripheral blood of human donors. Experiments were performed at different values of electric field amplitudes and also at different number and length of electric pulses. There are, however, no statistically significant differences between numbers of hybridoma cells obtained in experiments with different parameters.

In the experiments where 8 pulses of 100 μs length were used, the tendency of reducing the number of the obtained hybridomas can be seen at higher values of electric field amplitudes. At the experiments where 3 pulses of 15 μs length were used, the results at different values of electric field amplitudes were comparable.

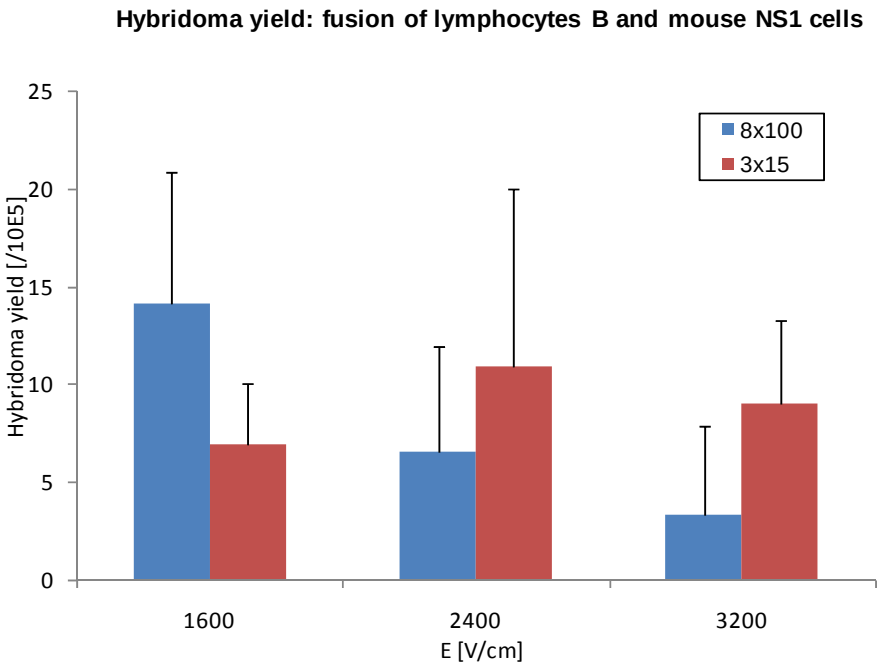
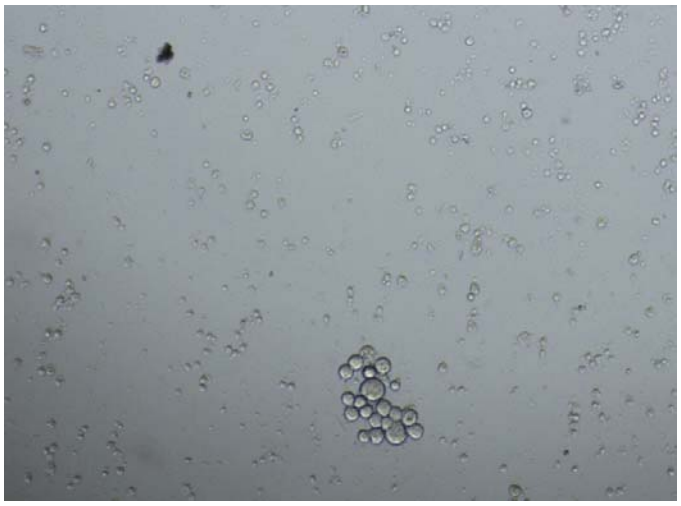


Figure 13: The production of hybridomas from mouse NS1 cells and primary human lymphocytes B from human peripheral blood. The results are the average of three to five experiments. Blue bars represent the experiments where trains of eight pulses of 100 µs duration were used. Red bars represent the experiments where trains of three pulses of 15 µs duration were used.

Slika 13: Proizvodnja hibridomov iz mišjih NS1 celic in primarnih človeških limfocitov B iz človeške periferne krvi. Rezultati so povprečje treh do petih poskusov. Modri stolpci predstavljajo poskuse, kjer smo uporabili vlake osmih 100 µs dolgih pulzov. Rdeči stolpci predstavljajo eksperimente, kjer smo uporabili vlake treh 15 µs dolgih pulzov.

In the experiments, in which fused cells were determined by fluorescence microscopy and in the experiments, where hybridoma cells were obtained, cells were treated in the same way (the protocol was the same) except for the loading of the cells with fluorescent dyes and post fusion treatment of the cells. The number of hybrid cells is lower than the number of dually labeled cells for two orders of magnitude (approximately 1/100 of dually labeled cells versus 1/10000 of hybridoma cells).

a)



b)

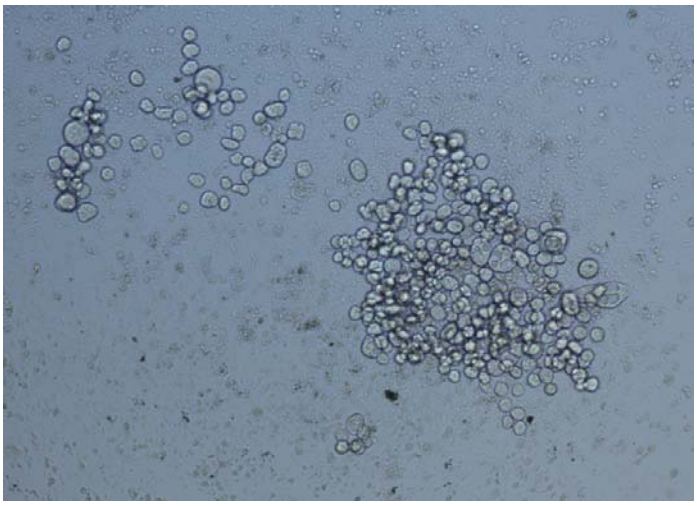


Figure 14: Clones of hybrid cells produced from mouse NS1 cells and human lymphocytes B from human peripheral blood. Images show clones of hybrid cells that can be observed a) after approximately 7 days, b) after additional few days.

Slika 14: Kloni hibridnih celic iz mišjih NS1 celic in primarnih človeških limfocitov B iz človeške periferne krvi. Sliki prikazujeta klon hibridnih celic, kot ga vidimo po (a) približno sedmih dnevih in (b) po še nekaj dodatnih dnevih.

4.4.2 Hybridoma yield: fusion of lymphocytes B and human NS1 cells

We performed the experiments for the human – human hybridomas production using the same parameters of electric pulses and the same experimental conditions as for the mouse – human hybridomas production. The results were negative; no clones of hybrid cells were observed.

4.4.3 Production of antibodies

Hybrid cells were tested for production of antibodies with different methods (see Materials and methods 3.5.3). The results were negative in all experiments.

4.5 PIPETTE TIP CHAMBER

The hypothesis of this dissertation was that cell electroporation and fusogenicity are controlled by the parameters of the electric pulses used (amplitude, number, length, repetition frequency) and that while preserving cell survival level we can improve fusion yield by applying pulses in different directions, as opposed to only one direction. In order to be able to test that hypothesis accurately, we designed a pipette tip with integrated electrodes for electroporation of cells in suspension (Trontelj et al., 2008a).

a) b)

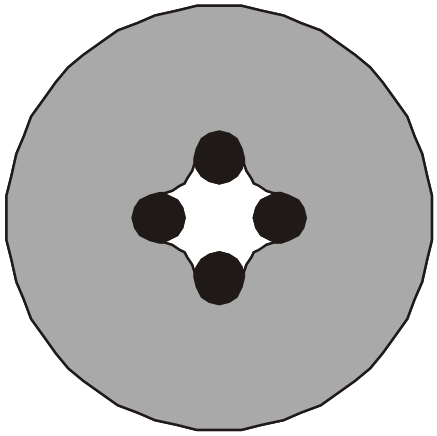


Figure 15: Pipette tip electrode chamber. a) the cross view of the chamber: the electrodes are denoted by black circles. Grey color represents a surrounding material and white color represents the interior of the chamber. b) a photo of a chamber.

Slika 15: Koničasta komora z vgrajenimi elektrodami. Shema na levi prikazuje presek komore: črni krogi ponazarjajo electrode, okolišni material je siv in bela je notranjost komore. Na desni je fotografija komore.

The pipette tip has a specifically shaped interior, so that all the cells within the tip are exposed to the applied electric pulses of different directions, which was not the case for the electrodes used in our previous study. With this special design all the cavities where the electric field was deformed, were eliminated.

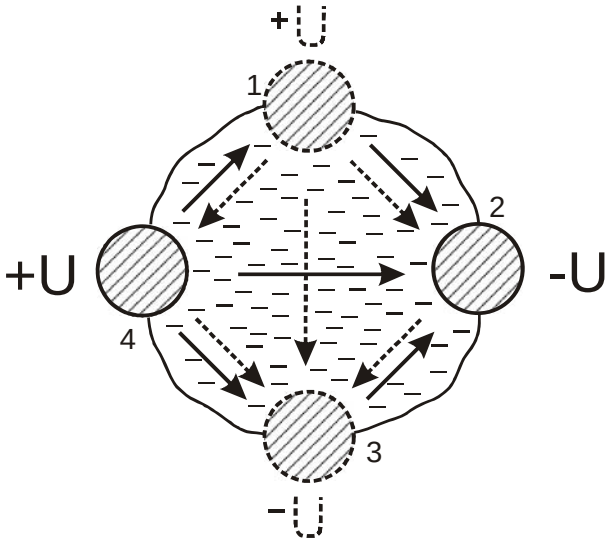


Figure 16: The schematic of the electrodes used in previous experiments, and the drop of cell suspension between them. During the treatment with pulses in perpendicular directions, pulses in two perpendicular directions are applied between electrodes 1 and 3 and 2 and 4. The resulting electric fields are depicted with full and dashed arrows (Trontelj et al., 2008).

Slika 16: Shema elektrod uporabljenih v prejšnjem delu in kapljica celične suspenzije med njimi. Prikazana sta dva pulza, dovedena v pravokotnih smereh. En pulz med elektrodi 1 in 3 (črtkano) in drug med elektrodi 2 in 4 (cela črta). Povzročeno električno polje je prikazano s puščicami (Trontelj et al., 2008).

The pipette tip was successfully used for gene transfection, where pulses applied in different directions improved the transfection rate of cells, compared to pulses in the same

direction (Kandušer et al., 2009). In our fusion experiments, however, no fused cells were detected.

5 DISCUSSION

Different parameters, affecting cell electrofusion were analyzed in this dissertation. The influences of some of them are expected, yet differ for different types of cells and many of them are still not understood. Results are also compared on the basis of the detection method used.

5.1 THE EFFICIENCY OF ELECTROPORATION

5.1.1 Electroporation efficiency of different cells in mildly hypoosmolar (200 mOsm) medium

We hypothesized that because of the mainly experimental nature of determining the optimal electrical parameters of cell electroporation, it is necessary to define optimal conditions for each (fusion partner) cells separately. The efficiency of electroporation was, therefore, determined for different cell types in mildly hypoosmolar (200 mOsm) medium.

The prediction of the electroporation theory is that larger cells are electroporated at lower electric field than smaller cells. The comparison of the cell sizes with their electroporation curves shows that electroporation is size specific although strain dependence was also observed. Electroporation curve for lymphocytes B differs from mouse and human NS1 cells as predicted on the basis of their much smaller size (Fig. 2). Higher values of electric

field are thus needed to electroporate lymphocytes B. These results are in accordance with the results obtained by other researchers (Sixou and Teissié, 1990) and agree with theoretical predictions (Kotnik et al, 1997). On the contrary, electroporation curves for mouse and human NS1 cell lines are similar and do not reflect the differences in cell size between these two cell lines (Fig. 2). In this case, other influences on electroporation can be observed. The values of electrical parameters needed for optimal electroporation was already shown to differ between different cells and depend on cell size and their chemical and/or biological properties (Čemažar et al., 1998; Kandušer et al, 2006).

Suitable electrical parameters for electroporation and electrofusion were thus determined. The highest fusion yield (using the same pulse parameters: 8 pulses of 100 μs duration, at 1 Hz frequency) can be expected at 1600 to 2000 V/cm, where lymphocytes B are already partially electroporated and myeloma cells are not yet irreversibly electroporated. At lower electric field amplitudes cell fusion probably would not occur and at higher electric field amplitudes cell survival is probably very low.

5.1.2 Electroporation efficiency of mouse NS1 cell line in hypo- and isoosmolar medium

One of the earliest approaches that improved fusion yield considerably was the use of hypoosmolar electrofusion medium. When placed in hypoosmolar medium, cell volume increases due to water influx until cells reach their maximum size. Hypoosmolar cell swelling triggers many regulatory volume pathways which then results in immediate

regulatory volume (size) decrease to a more or less original size. Despite this general hypoosmolar cell size dynamics, differences among the cell lines were observed (Ušaj et al., 2009b).

The initial hypoosmolar swelling of different cells is different in its size and is accomplished within different time frames. We determined the electroporation efficiency for mouse NS1 cell line in mildly hypoosmolar (200 mOsm) and isoosmolar medium (Fig. 3). 200 mOsm medium was chosen because mouse NS1 cells do not tolerate lower osmolarities (100 mOsm). Electroporation curves in isoosmolar and in hypoosmolar medium were similar, thus we can conclude that cell swelling is small and the difference in cell sizes between cells in isoosmolar medium (normal cells) and cells in hypoosmolar medium (swollen cells) does not influence electroporation.

5.2 BEHAVIOR OF CELLS IN STRONGLY HYPOOSMOLAR (100 MOSM) MEDIUM

Behavior of cells in hypoosmolar medium (100 mOsm) was determined for different cell lines. The results show that cells of different cell lines differ in their response to hypoosmolar medium. Nevertheless, CHO, V79 and B16F1 cell lines exhibit an expected behavior. They increased their volumes to the peak value at a specific time (2 minutes for CHO and V79 and 5 minutes for B16F1) and then regulated it back (Figs. 4 and 5).

Observed behavior of our cell of interest, mouse myeloma cells NS1 (Fig. 6), however, differs significantly from the behavior of the three cell lines described above. NS1 cells did not exhibit any regular size alterations. Instead, they started to burst and express non-spherical shapes. Similar behavior was reported earlier for L-cells (murine fibroblast) which were converted to flaccid "ghost" by 60 mOsm medium while 150 mOsm medium did not cause this problem. Low hypoosmolar treatment of L-cells consequently led to a drastic decrease in cell viability (Sukhorukov et al., 1993).

Lowering of the osmolarity to 30 mOsm usually led to bursting of HEK293 cells (Zimmermann et al., 2006) while the cells withstood 50 - 75 mOsm medium well. All these results suggest that NS1 cells are not as osmotolerant for low osmolar medium as the other cell lines we used in our study. Decrease in viability of NS1 cells after hypoosmolar treatment for 13 % support explanation of higher sensitivity of NS1 cells to hypoosmolar treatment (Ušaj et al., 2009b).

The behavior of NS1 cells in 100 mOsm hypoosmolar medium can be tentatively explained by physical rigidity of these cells, which could be a consequence of their cytoskeletal structure (Sukhorukov et al., 1993). Higher buffer osmolarities may thus have to be used for NS1 cells in order to obtain spherical cell shape with smooth membrane that are favorable for electrofusion according to Sukhorukov (Sukhorukov et al., 2005).

5.3 FUSION DETECTED BY FLUORESCENT STAINING ASSAY FOR DIFFERENT CELL PAIRS

The hypothesis of this dissertation was that cell electroporation and fusogenicity are controlled by the parameters of the electric pulses used (amplitude, number, length, repetition frequency) and that while preserving cell survival level we can improve fusion yield by applying pulses in different directions, as opposed to only one direction. In order to be able to test this hypothesis accurately, we designed a pipette tip (Fig. 15) with integrated electrodes for electroporation of cells in suspension (Trontelj et al., 2008a). This pipette tip was designed to be used in electrofusion experiments using pulse first protocol, which were described in our previous work (Trontelj et al., 2008) to determine the influence of electrical pulse directions. It was designed to reduce the time needed for cell manipulation and to improve the interior of the chamber.

Duration of the transfer of the cells from the electrode chamber to the centrifuge where contacts between the cells are achieved is important because the fusogenicity of the electroporated cells decreases with time (Teissié and Ramos, 1998). And the shape of the tip interior was designed so that all the cells within the tip are exposed to the applied electric pulses of different directions, which was not entirely the case for the electrodes used in our previous study (Fig. 16). With this special design all the cavities where the electric field was deformed were eliminated (Figs: 15 and 16). The pipette tip was successfully used for gene transfection, where pulses applied in different directions improved the transfection rate of cells, compared to pulses in the same direction (Kandušer et al., 2009). In our fusion experiments, however, no fused cells were detected. Since no

protocol changes have been made since our previous experiments, there are two possible explanations for this: i) changes that fluorescent cell tracker dyes make on the membranes, or ii) only cells labeled with the same dye fused.

Therefore, fusion experiments were performed using another method to achieve cell contact. We used modified adherent method, where cells are allowed to attach only slightly to the surface of the well, so that medium can be exchanged and cells still preserve their spherical shape. This method is simple to perform also in aseptic conditions with the purpose of growing and selecting the resulting fused cells further. Modified adherence method was shown to be efficient for adherent cells that adhere to the surface adequately in short time (Ušaj et al., 2009a). Namely, fusion yields obtained with adherent cells are higher (up to 14 %) in comparison to fusion yields obtained with suspension cells, which do not adhere to the surface of the plate strong enough and are, therefore, displaced already by the application of the electric pulses. Despite the weak adherence of NS1 cells, we were able to produce double labeled fused cells from the suspension cells: mouse NS1 cells (Fig. 10), human NS1 cells (Fig. 11-a) and from the combination of human NS1 cells and human lymphocytes B (Fig. 11-b).

5.3.1 Cell sizes and concentrations for monolayer determinations

Optimal concentrations for monolayer formation in a suspension drop for different cell types were determined prior to fusion experiments (Table 3). Optimal cell density is achieved, when cells are packed closely together yet with almost no cells forming the

second layer since these cells can not adhere to the surface; instead, they form cell clusters after electroporation. Position of cells in a monolayer assures good contact between the cells after electroporation since cells that are in contact (adhered) with the surface, do not move. Also, this allows for the counting of the cells because cell clusters do not appear.

5.3.2 Fusion yields detected by fluorescent staining assay for different fusion partners

Fused cells were detected by double fluorescent staining and counted under the microscope. Experiments were performed at different values of electric field amplitudes. No differences between fusion yield of mouse NS1 cells and human NS1 cells were expected according to their electroporation efficiency curves (Fig. 2). The yields of double stained cells for fusion of these two cell lines (cells of each cell line are fused separately) are in the range of 1 to 5 % of the cells used for the experiment with the highest value at 2400 V/cm where all cells are electroporated (Fig. 9).

Fusion yields for fusion of human NS1 cells with human lymphocytes B are smaller and were observed only at fields' amplitudes of 3200 V/cm and higher (Fig. 9), even though large portions of lymphocytes B are electroporated already at 1600 V/cm (Fig. 2). The reason that fused cells are not observed at lower electric field amplitudes can be the small resolution of our detection method. For precise detection of smaller fusion yields, larger field of view would be needed to analyze more cells.

Beside the small number of cells that can be analyzed under the microscope, another problem appeared concerning detection in this cell combination. As can be seen on Figure 11, lymphocytes B are much smaller than NS1 cells, which makes it difficult to distinguish the double labeled cells from other cells. Therefore, it is possible that some of the fused cells remain undetected.

Another type of experiment was done, where human NS1 cells were fused. In this experiment, the cells were not "sitting" on the plate surface, but were allowed to form clusters of cells. Namely, human NS1 cell line is a suspension cell line and spontaneously form clusters of cells that float in a medium. The experiment showed that the cells in clusters are connected in a way that is very convenient for cell fusion, since large fused cells were obtained after electroporation (Fig. 12).

Fusion yield in these last experiments was apparently much higher than in all other experiments. It was, however, not accurately estimated nor optimized since this kind of contact between cells is specific. It can only be used within this cell line and therefore cannot be used in hybridomas production.

5.4 HYBRIDOMA YIELD: FUSION OF LYMPHOCYTES B AND MYELOMA CELLS

5.4.1 Hybridoma yield: fusion of lymphocytes B and mouse NS1 cells

Hybridomas (hybrid cells) were obtained from mouse myeloma cell line NS1 and lymphocytes B isolated from peripheral blood of healthy human donors. Experiments were performed at different values of electric field amplitudes and also number and length of the electric pulses. There are, however, no statistically significant differences between the numbers of hybridoma cells obtained in experiments with different parameters (Fig. 13).

In experiments where 8 pulses of 100 μs length were used, the tendency of reducing the obtained hybridomas number can be seen at higher values of electric field amplitudes. At the experiments where 3 pulses of 15 μs length were used, the results at different values of electric field amplitudes were mutually comparable. This can be tentatively explained by lower survival of cells at higher amplitudes of electric pulses in experiments where 8 pulses of 100 μs length were used.

In the experiments in which fused cells were determined by fluorescence microscopy and in the experiments where hybridoma cells were obtained, cells were treated in the same way (i.e. the protocol was the same) except for the loading of the cells with fluorescent dyes and post fusion treatment of the cells. Number of hybrid cells is lower than number of

double labeled cells for two orders of magnitude (approximately 1/100 of double labeled cells versus 1/10000 of hybridoma cells).

This difference can be ascribed predominantly to the low survival and proliferation of the fused cells. Different fusion partner cells were used in experiments with fluorescent dyes (human NS1) and in hybridoma experiments (mouse NS1). The reason for that is that fusion of lymphocytes B with human myeloma cells was unsuccessful. Partly the difference in fusion yields could be, therefore, ascribed to the fact that fusion partner cells were different. However, mouse NS1 and human NS1 cells are very similar in their properties (electroporation efficiency and fusion yield) so this contribution can be excluded.

5.4.2 Antibody production

Hybrid cells in all experiments were tested for production of antibodies of blood group system AB0 with different methods (see Materials and methods 3.5.3). The results were, however, negative in all the experiments. This can be due to the small percentage of activated lymphoblasts that produce the particular antibodies in our samples.

Our result for average diameter of lymphocytes B ($d = 7,7 \pm 0,7$ μm) is in accordance with the diameters of naive and memory lymphocytes B (so called »small lymphocytes«), which are in the range of 8 to 10 μm, while the size of the activated or effector lymphocytes B

(also called »large lymphocytes« or lymphoblasts) is in the range of 10 to 12 μm. This led us to conclude that the vast majority of lymphocytes B in our samples were small lymphocytes B that did not produce antibodies.

It is known that many antibody-producing plasma cells migrate to secondary lymphatic organs, where they secret antibodies for long periods of time. Antibodies are then distributed throughout the body via the blood stream (Abbas, 2007, p. 70). The number of lymphocytes B in the peripheral blood is, however, small (5 to 15 %). The frequency of antigen-specific B cells is even smaller (in human peripheral blood it has been estimated to 10^{-4} to 10^{-5}), depending on the immune status of the donor (Ritter and Ladyman, 1995, p.98).

A route of immunization that may be useful when intending to raise human mAb, in cases where it is not feasible to immunize cells *in vivo*, is that of *in vitro* immunization. In this system, splenic or peripheral blood leucocytes (i.e. lymphocytes B, lymphocytes T and antigen-presenting cells) are cultured together with the antigen; following this *in vitro* priming, cells are subsequently fused with myeloma cells in the conventional way. The culture conditions are critical and require much patience to optimize (Ritter and Ladyman, 1995, p.14).

Antigen-induced lymphocyte proliferation normally occurs in the lymphoid tissues and can be visualized in vitro by cultivating lymphoid cells with specific antigens. Even in the

absence of antigens, mitogenic lectins (carbohydrate-binding proteins which stimulate cell division) are capable of the polyclonal stimulation of lymphoid cells. These mitogenic lectins (mitogens) are derived from various plants and bacteria. Lymphocyte activation, by either antigens or mitogens, results in intracellular changes and subsequent development into lymphoblasts. Mitogen stimulation of lymphocytes in vitro is believed to mimic stimulation by specific antigens fairly closely (Roitt, 1993, p. 210).

The lack of well-established techniques for *in vitro* immunization of human lymphocytes B is, perhaps, the most significant obstacle to human Ab production. The success of *in vitro* immunization is influenced by the dose, form and purity of the antigen. In most studies, non-specific mitogens, such as endotoxin, phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) have been used in addition to antigen. Useful strategies for developing human mAbs are using much more complicated and thoroughly optimized protocols for *ex vivo* immunization (Li et al., 2006).

6 CONCLUSIONS

1. We determined electroporation efficiency at different amplitudes of the electric field, for each (fusion partner) cells. The results show that electroporation of our cells is size specific, although strain dependence was also observed. In order to be able to study electrical pulse protocols with pulses in different directions, we designed and used a pipette tip electroporation chamber. Because of the negative results obtained with "pulse first" protocol, the possibility of improving fusion yield while preserving cell survival level was not further investigated.

2. The influence of medium osmolarity on cell fusion was studied on different cell lines and the data was used in cell fusion experiments. The results show large differences between different cell lines. We can tentatively conclude that the influence of medium osmolarity on cell fusion between different cell lines differs as well. However, this remains to be confirmed. Other parameters (temperature and presence of bivalent cations) were not studied in our work since the reproducibility of our fusion method was low and statistically significant differences were not expected to be obtained.

3. We used a double staining method for cell fusion detection. We compared the results obtained with this method to the results obtained by hybridoma technology. The number of hybrid cells is lower than the number of double labeled cells for two orders of magnitude. This difference can be ascribed predominantly to the low survival and

proliferation of fused cells. Cell fusion detection with the new method of cell size measurements was, however, not performed. Namely, size differences between the two cell types that are fused with hybridoma technology are so large that these measurements are not possible.

4. Using electrical parameters assuring efficient electroporation of all cell lines used for production of hybridomas, we were able to efficiently produce heterohybridomas between mouse myeloma fusion partner cell line and human lymphocytes B. The experiments on the production of human hybridomas using human myeloma fusion partner cell line were, however, unsuccessful. MoNS1 and huNS1 cells are both suspension cell lines. However, huNS1 cells grow as floating clusters while moNS1 cells grow slightly adhered to the surface. From that we conclude that an adherent fusion partner cell line could be more successful in our specific modified adherence method. In our hybridomas, no production of the antibodies against the antigens of blood group system AB0 was detected. For future experiments, *in vitro* immunization protocol for lymphocytes B should be developed in order to obtain active lymphoblasts.

5. We designed electroporation chamber (pipette tip) for electroporation of cells in suspension. This chamber enables us to deliver electric pulses to cells in different directions; it also reduces the number of cell manipulations needed and shortens the period from pulsing to achieving cell contact. This electroporation chamber is designed in a way to allow further upgrading and to allow the use of separate treatment of both fusion partner cells at the same time. Pulse first experiments (with pipette tip

electroporation chamber) were, however, unsuccessful. Therefore, a problem of different cell sensitivity to electric pulses (because of the cell size or other reasons, as discussed in chapter 5.1.1) was partially overcomed by using electric pulses with parameters that destabilize both fusion partner cells (determined in the first part of this work).

7 SUMMARY

7.1 SUMMARY IN ENGLISH

Cell fusion is an intensively investigated method since fused cells and their products are used in different areas of biotechnology and medicine. Electrofusion is a common method for the induction of cell fusion, where cells that are destabilized by means of high voltage electric pulses and are at the same time in close contact, fuse. The use of the hybrid cells is defined by the properties of the parental cells; however, it is limited by the yield of the method for cell fusion. In this work we studied some of the many parameters that influence fusion of different cells (electric field parameters and medium osmolarity). Experiments on electrofusion of different cell combinations were performed and fusion yields were determined by means of the double staining method. Results obtained in electroporation and electrofusion experiments were then used in the experiments of hybridomas production.

New pipette tip electroporation chamber was designed for electroporation of cells in suspension with electric pulses delivered in different directions. Furthermore, a contact method (modified adherence method) was developed and used for fusing myeloma fusion partner cells and human lymphocytes B. Mouse – human heterohybridomas were efficiently produced from moNS1 fusion partner cells and human lymphocytes B. Fusion yields obtained by clone counting method in production of hybridomas were significantly smaller than fusion yields obtained with double staining method.

7.2 POVZETEK V SLOVENŠČINI

7.2.1 Uvod – biološka fuzija

Zlivanje oz. fuzija membrane je proces, kjer iz dveh ločenih lipidnih dvoslojev nastane eden. V bioloških sistemih (živih organizmih) je zlivanje membran ključnega pomena za komunikacijo med različnimi deli eukariotske celice (Martens and McMahon, 2008). Primere takega zlivanja opazimo pri eksocitozi na plazemski membrani, med znotrajceličnimi membranami in pri vdoru virusov v citosol (Harrison, 2008).

Zlivanje celic pa je proces, ki obsega združenje dveh ločenih membran v enoten lipidni dvosloj, v katerem sta zaobjeti vsebini obeh celic. Zlivanje celic je zelo razširjen proces v živih organizmih. Ključnega pomena je med razvojem organizma in kasneje pri imunskem odzivu. Pri odraslem organizmu je prisoten tudi pri popravilu tkiv (Alvarez-Dolado et al., 2003; Vassilopoulos in Russell 2003b; Wang et al., 2003). Kljub razširjenosti procesa zlivanja celic, pa je lahko le-ta v nekaterih primerih tudi škodljiv. Ugotovili so namreč, da so nekatere tumorske celice še posebej fuzogene (t.j. dovzetne za zlivanje) (Pawelek in Chakraborty, 2009).

Za zlivanje lipidnih dvoslojev je potrebno premagati energetsko bariero. Energija je potrebna za izpodrinjenje vodnih molekul iz prostora med membranama, za ukrivljanje membrane in tvorbo struktur membrane, različnih od dvosloja, ki služijo kot intermediati za zlivanje. Energijo za te procese zagotavljajo visoko specializirani fuzijski proteini, t.i.

fuzogeni, ki imajo pri fuziji celic vlogo katalizatorjev. Njihove strukture so zelo različne, vendar pa je mehanizem, po katerem poteka proces zlivanja v vseh primerih zelo podoben.

Za večino primerov zlivanja so pokazali, da se začnejo z medceličnim prepoznavanjem, prileganjem in pritrjevanjem. Temu sledi dejansko zlivanje plazemskih membran, v katerem se spremeni konformacija membrane, ki povzroči lokalen stik med zunanjima slojema obeh membrane in njuno zlitje, t.i. hemifuzijo. Za dokončanje fuzije mora priti do odprtja fuzijske pore. Prehod med odpiranjem in zapiranjem majhnih fuzijskih por in nastankom velike stabilne pore pa je verjetno energijsko najpotratnejši del procesa (Chernomordik et al., 2006).

7.2.2 Uporaba fuzije v biotehnologiji in medicini

Celična fuzija v bioloških sistemih večinoma poteka specifično, vodijo jo specializirani proteini. Sposobnost membran, da se zlivajo nespecifično zaradi zunanjega povzročitelja, kot je npr. električno polje, pa je pomembna za biotehnologijo, medicino in za raziskave v biologiji. Omogoča nam pridobivanje zelo uporabnih hibridnih celic in njihovih produktov, kot so npr. monoklonska protitelesa. Omogoča nam tudi študij osnovnih mehanizmov fuzije, saj so si mehanizmi zlivanja različnih membran zelo podobni (Chernomordik et al., 2006).

Postopek za pridobivanje klonske populacije celic (hibridomov), ki izločajo protitelesa definirane specifičnosti, t.i. hibridomno tehnologijo, sta že leta 1975 razvila Köhler in

Milstein in za to leta 1984 prejela Nobelovo nagrado za medicine (Köhler and Milstein, 1975). Bistvo njune »hibridomne tehnologije« je zlivanje limfocitov B, ki proizvajajo protitelesa, z mielomskimi celicami, ki jih lahko gojimo v pogojih *in vitro*. Hibridomi tako rastejo v pogojih *in vitro*, kjer po ustrezni selekciji in kloniranju izberemo takšne, ki proizvajajo protitelesa definirane specifičnosti, t. i. monoklonska protitelesa. Poleg pridobivanja hibridomov, ki proizvajajo monoklonska protitelesa, pa lahko zlivanje celic uporabimo še v druge namene.

Zelo aktualna uporaba zlivanja celic je tudi priprava hibridnih celičnih cepiv za imunoterapijo pri rakavih obolenjih (Trevor et al., 2004; Weise et al., 2004; Yu et al., 2005). Pred nekaj leti se je namreč pokazalo, da se tudi pri obnavljanju tkiv s celično terapijo pojavi mehanizem zlivanja celic. Pri transplantaciji izvornih celic enega tipa tkiva te celice pod ustreznimi pogoji ustvarijo tkivo drugega tipa, prisotnega na mestu transplantacije, kar omogoča ciljano terapijo z namenom obnavljanja organov (Alvarez-Dolado et al., 2003; Vassilopoulos and Russell 2003b; Wang et al., 2003). Zlivanje celic je prav tako uporabna metoda za vnos receptorjev v celično membrano ciljne celice, kar omogoča študij njihove strukture in biološke vloge (Grasso et al., 1989; Heller and Grasso, 1990). Zlite celice so primerne tudi za študij elektrofizioloških lastnosti celic, saj so zaradi povečane velikosti zlite celice, večje tudi vrednosti električnih parametrov, ki na ta način postanejo merljive (Zimmermann et al., 2006).

7.2.3 Elektrofuzija

Že leta 1961 so ugotovili, da se pod določenimi pogoji zlivajo somatske celice *in vitro*. Spontano zlivanje somatskih celic so prvič dokazali, ko so identificirali celice z mešanimi kariotipi po skupnem gojenju različnih celičnih linij v kulturi (Barski et al., 1961). V zgodnjih sedemdesetih letih so uvedli viruse in polietilenglikol kot agensa za zlivanje protoplastov in živalskih celic. V poznih sedemdesetih letih pa so prvič poročali o fuziji, ki jo je povzročilo zunanje električno polje, torej o elektrofuziji (Senda et al., 1979).

Pri z zunanjimi vplivi induciranem zlitju celic nastanejo hibridne hčerinske celice, ki nosijo oba nabora starševskih kromosomov v enem jedru. Za večino celic je to pogubno, iz nekaterih hibridnih celic pa lahko po naslednjih celičnih delitvah nastane stabilna celična linija z naborom kromosomov, ki ji omogoča preživetje in je sestavljen iz kromosomov obeh starševskih celic (Vassilopoulos and Russel, 2003b).

Izkazalo se je, da lahko električno polje povzroči zlivanje zelo različnih celičnih in modelnih membran (lipidnih dvoslojev, liposomov ...). Sklepali so, da so za zlivanje pomembne lastnosti membran, ki so neodvisne od tipa posamezne membrane. Membrane so normalno stabilne strukture, odporne na zunanje razmere, zato je za zlivanje potrebna destabilizacija, to je sprememba molekularne strukture membrane v tako, ki je ugodna za zlivanje. Poleg tega pa je potrebno zagotoviti še stik med membranami, ki bo omogočil zlitje njihovih lipidnih dvoslojev (Dimitrov, 1995).

7.2.3.1 Destabilizacija membrane

Pri elektrofuziji destabilizacijo membrane dosežemo z elektroporacijo. To pomeni, da z uporabo kratkih, visokonapetostnih električnih pulzov v celični plazemski membrani povzročimo nastanek hidrofilnih por. Le-te so lahko reverzibilne in se po določenem času zaprejo (angl. resealing), pri nadkritični membranski napetosti pa pride do ireverzibilnega porušenja, ko se membrana ne more več zaceliti in celica propade (Kotnik et al., 1997; Kotnik et al., 2005).

Poenostavljeno lahko rečemo, da je z elektroporacijo destabilizirana membrana tudi fuzogena (sposobna zlivanja). Velikost transmembranske napetosti, ki je potrebna za zlivanje, je namreč približno enaka kot tista, ki je potrebna za elektroporacijo. Poleg tega sta elektroporacija in elektrofuzija podobno funkcijsko odvisni tudi od drugih električnih parametrov, kot so število pulzov, čas trajanja posameznega pulza in smer dovajanja električnih pulzov (Zhelev et al., 1988; Teissié and Rols, 1993; Trontelj et al., 2008). Poleg parametrov električnega polja, lahko na destabilizacijo membrane in na njeno preživetje pomembno vpliva tudi smer dovajanja električnih pulzov (Reberšek et al., 2007; Valič et al., 2003; Kotnik et al., 2001)

7.2.3.2 Stik med celicami

Do zlivanja celic pride le, ko so njihove destabilizirane membrane dovolj blizu skupaj. Celice se normalno ne približujejo druga drugi na razdaljo, ki bi bila dovolj majhna za fuzijo (≈ 1 nm). Zaradi negativnega naboja, ki ga ima večina celic na svoji površini, se

namreč medsebojno odbijajo. To odbojno silo je treba premagati, če želimo, da se celice dovolj približajo in lahko pride do mešanja lipidnih molekul iz različnih sosednjih membran.

Stik med celicami lahko vzpostavimo na različne načine. V preteklosti so poskušali s številnimi različnimi metodami, ki pa se niso uveljavile. Danes se najpogosteje uporablja dielektroforeza, pri kateri celice z uporabo nehomogenega izmeničnega električnega polja nizkih jakosti uredimo v verižice (angl. pearl chains). Trenutno vsi komercialno dosegljivi sistemi za elektrofuzijo delujejo na tem principu. Ta metoda zahteva uporabo pufrov nizkih prevodnosti, da preprečijo pretirano segrevanje suspenzije zaradi električnega polja, kar ni ugodno za celice (Jaroszeski, 1994b). Po drugi strani pa elektroforetske metode omogočajo razvoj posebnih komor – mikročipov, tudi pretočnih, ki načeloma omogočajo avtomatizacijo in pospešitev procesa (Fox et al., 2006; Cao et al., 2008).

Pri zlivanju celic, ki so pritrjene na podlago in so konfluentne, je stik med njimi spontan (Tessie et al., 1982; Finaz et al., 1984; Blangero et al., 1989). V tem primeru pri elektroporaciji pogosto nastanejo mnogojedrne celice; to pomeni, da se veliko celic zlije v eno samo. Take celice redko preživijo in se delijo, zato ta metoda večinoma ni primerna za pridobivanje zlitih celic. Obetavne rezultate po drugi strani daje modificirana metoda pritrjevanja, kjer celice gosto nasadimo in jih inkubiramo le kratek čas (20 minut). V tem času se celice pritrdijo na podlago dovolj močno, da lahko zamenjamo medij in izvedemo elektroporacijo (Ušaj et al., 2009a). S to metodo večinoma pridobimo celice, ki imajo malo (od dve do pet) jeder in so zato v mnogo večji meri preživijo in so sposobne delitve.

Modificirana metoda pritrjevanja je primerna za celice, ki rastejo pritrjene in se tudi v kratkem času dovolj močno pritrdijo na podlago. Slabše rezultate pa s to metodo dobimo pri suspenzijskih celicah, ki se le rahlo ali sploh ne pritrdijo (usedejo) in se premaknejo že zaradi menjave medija ali dovedenih električnih pulzov. Kljub temu smo s to metodo uspeli pripraviti hibridome med suspenzijskimi mielomskimi celicami NS1 in humanimi limfociti (sliki 13 in 14).

Pri vseh zgoraj naštetih metodah so celice že v stiku, ko jih izpostavimo električnim pulzom. Način, s katerim lahko zagotovimo stik med celicami po elektroporaciji, pa je centrifugiranje celic (Sowers et al., 1986; Teissié in Rols, 1986). To zaporedje postopka elektrofuzije (angl. contact first) načeloma dopušča obdelavo vsake od dveh vrst partnerskih celic z različnimi električnimi pulzi. Posebej je to zaporedje uporabno, ko želimo med seboj zlivati celice, ki za optimalno sposobnost zlivanja potrebujejo obdelavo z različnimi električnimi pulzi.

Za uporabo zlitih celic, moramo celične membrane z elektroporacijo reverzibilno destabilizirati, tako da se membrane zacelijo (okrevajo) in celice preživijo. Optimalne vrednosti električnih parametrov, s katerimi dosežemo reverzibilno elektroporacijo oziroma sposobnost zlivanja, pa so odvisne od velikosti celic (Kotnik et al., 1997) in od bioloških lastnosti posameznih vrst celic (Neil in Zimmerman, 1993; Čemažar et al., 1998; Čegovnik in Novakovič, 2004; Yu et al., 2008). Optimalne vrednosti električnih parametrov so zato za različne vrste celic različne.

V tej nalogi smo določili optimalne vrednosti električnih parametrov za različne vrste celic (slika 2). Primerjava velikosti celic z njihovimi elektroporacijskimi krivuljami je pri primerjavi limfocitov B z mielomskima celičnima linijama pokazala, da je občutljivost celic odvisna od njihove velikosti. Pri primerjavi obeh mielomskih celičnih linij pa smo opazili, da na elektroporacijo vplivajo tudi drugi dejavniki, saj njuna razlika v velikosti ni povzročila razlike v elektroporaciji. Ti rezultati so v skladu z rezultati dobljenimi v drugih raziskavah (Sixou in Teissié, 1990; Čemažar et al., 1998; Kandušer et al., 2006) in tudi s teoretičnimi napovedmi (Kotnik et al., 1997).

Če vzpostavljamo stik med celicami z že destabiliziranimi membranami, je pomembno, da to storimo v čim krajšem času po elektroporaciji, ko so celične membrane še sposobne zlivanja. Ta čas je reda velikosti sekund do minut in je med drugim odvisen od intenzivnosti električnih pulzov in temperature (Dimitrov in Sowers, 1990; Teissié in Ramos, 1998).

V tej nalogi smo izdelali koničasto komoro z integriranimi elektrodami (slika 15) (Trontelj et al., 2008a), ki skrajša čas med elektroporacijo in vzpostavitvijo kontakta med celicami pri "pulse first" protokolu in hkrati omogoča dovajanje pulzov v različnih smereh. Dovajanje pulzov v različnih smereh je bilo pri elektrodah, ki smo jih uporabljali v prejšnji študiji nepopolno (slika 16) (Trontelj et al., 2008). Posebna oblika izdelane koničaste komore z integriranimi elektrodami pa zagotavlja, da so vse celice dejansko izpostavljene električnemu polju v isti smeri (primerjaj sliki 15 in 16). Izdelana koničasta komora je bila uspešno uporabljena za gensko transfekcijo, kjer so pulzi, dovedeni v različnih smereh,

povečali učinkovitost transfekcije v primerjavi s pulzi, dovedenimi v isti smeri (Kandušer et al., 2009). Vsi poskusi fuzije s to komoro pa so bili žal neuspešni.

Pri poskusih elektrofuzije smo zato za doseganje stika med celicami uporabili modificirano metodo pritrjevanja celic. Ta metoda je primerna za pripravo hibridomov, saj je enostavna za izvajanje v sterilnem okolju z namenom nadaljnje uporabe dobljenih hibridnih celic.

Razvita modificirana metoda pritrjevanja je učinkovita za celice, ki normalno rastejo pritrjene na podlago in se v kratkem času dovolj trdno pritrdijo na podlago. Izplen zlivanja, dobljen s takimi celicami, je namreč višji (do 14 %) od izplena zlivanja suspenzijskih celic (Ušaj, 2009a). Slednje se namreč nikoli ne pritrdijo dovolj močno na podlago in jih zato že dovajanje električnih pulzov lahko odlepi. Kljub rahli pritrditvi suspenzijskih NS1 celic pa smo s to metodo uspeli zliti mišje NS1 celice z mišjimi NS1 celicami (slika B), človeške NS1 celice s človeškimi NS1 celicami (slika 11-a) in človeške NS1 celice s človeškimi limfociti B (slika 11-b).

Na učinkovitost zlivanja celic pa poleg parametrov električnega polja vplivajo tudi drugi dejavniki, kot so sestava medija, prisotnost dvovalentnih kationov, temperatura, ozmolarnost medija in ostali še nepoznani dejavniki. Koristnost prisotnosti dvovalentnih kationov je bila dokazana v preteklosti (Matibiri in Mantell, 1995; Rols in Teissié, 1989; Stenger et al., 1991). Uporaba hipoozmolarnega medija pa je najbolj povečala učinkovitost zlivanja celic (Ahkong in Lucy, 1986; Schmitt in Zimmermann, 1989; Zimmermann et al., 1990; Foung et al., 1990).

Pri uporabi hipoozmolarnega medija je pomembna dolžina hipotonične obdelave celic in ozmolarnost uporabljenega medija (Ušaj et al., 2000b). Napihovanje celic zaradi vdora molekul vode v celico, ki ga hipoozmolarni medij povzroči, namreč v celicah sproži proces reguliranega zmanjšanja prostornine (angl. regulatory volume decrease). Če se to zgodi preden izvedemo elektrofuzijo, lahko takšna regulacija prostornine popolnoma izniči siceršnji pozitivni učinek hipoozmolarnega medija. Prostornina celice se namreč začne manjšati, obnovijo se mikrovili, daljša izpostavitev celic hipoozmolarnemu mediju pa negativno vpliva na njihovo preživetje (Shukorukov et al., 2005; Kiesel et al., 2006; Foung et al., 1990; Rehman et al., 1992).

Zato smo določili obnašanje različnih vrst celic v 100 mOsm mediju. Rezultati kažejo, da se različne celične linije med seboj razlikujejo v odzivu na hipoozmolarno obdelavo. Vendarle se celice CHO, V79 and B16F1 obnašajo v skladu z dinamiko napihovanja celic in posledičnega reguliranega zmanjšanja prostornine. Svojo prostornino so povečale do maksimalne vrednosti v določenem času (2 minuti za CHO in V79 ter 5 minut za B16F1), čemur je sledilo zmanjšanje prostornine (sliki 4 in 5).

Obnašanje celic, ki nas zanimajo, mielomske celične linije NS1, pa je odstopalo od zgoraj opisanega vzorca reguliranega manjšanja prostornine (slika 6). Mišje NS1 celice se namreč niso napihovale, pač pa so začele nepravilno spreminjati obliko. Podobna opažanja so zabeležena v literature še za nekaj vrst celic, pri čemer so istočasno izmerili zmanjšanje preživetja (Sukhorukov et al., 1993; Zimmermann et al., 2006). Na osnovi naših opazovanj lahko sklepamo, da NS1 celice niso tako ozmotolerantne kot ostale vrste celic. Tudi

zmanjšanje preživetja celic za 13 % (Ušaj et al., 2009b) si lahko razložimo kot večjo občutljivost celic NS1 na hipoozmolarni medij. Obnašanje NS1 celic lahko poskusimo razložiti s fizično rigidnostjo membran teh celic, ki bi lahko bila posledica njihove strukure citoskeleta (Sukhorukov et al., 1993). Za poskuse elektrofuzije smo zato uporabili 200 mOsm (manj hipoozmolaren) medij.

7.2.3.3 Ovrednotenje izplena zlitih celic

Za zaznavanje in ovrednotenje izplena zlivanja celic se večinoma uporablja dvojno barvanje s fluorescentnimi barvili (slike 10, 11, 12 in 14). Izplen zlivanja se v tem primeru podaja kot delež dvojno obarvanih celic. Izplen zlivanja lahko določimo s štetjem zlitih in nezlitih celic pod mikroskopom ali s pretočnim citometrom. Pri slednjem se je treba zavedati, da dobimo nekoliko višje vrednosti zaradi celičnih skupkov, ki jih meritev prepozna kot zlite celice (Jaroszeski et al., 1994; Gabrijel et al., 2004).

Pri hibridomni tehnologiji izplen zlivanja določimo s štetjem hibridomov, nastalih iz limfocitov B in partnerskih mielomskih celic, ki so preživeli ter se uspešno delijo. Delež pridobljenih hibridomov je tako bistveno nižji kot delež dvojno obarvanih celic.

Pri naših poskusih smo za detekcijo uporabili metodo dvojnega obarvanja s citosolnima fluorescentnima barviloma CMFDA in CMRA. Rezultate smo primerjali s tistimi, ki smo jih dobili z metodo štetja klonov pri hibridomni tehnologiji. Po pričakovanju so bili

rezultati, dobljeni s fluorescentnimi barvili, za dva velikostna razreda večji od rezultatov, dobljenih s štetjem klonov (primerjaj sliki 9 in 13).

Z uporabo električnih parametrov, ki smo jih določili v poskusih določanja učinkovite elektroporacije, smo pridobili hibridome med mišjimi mielomskimi celicami NS1 in človeškimi limfociti B (slika 13). Poskusi zlivanja limfocitov B s človeškimi mielomskimi celicami NS1 pa so bili žal neuspešni. Iz podatkov, dobljenih v naši nalogi, zaključujemo, da bi bila lahko za pridobivanje človeških hibridomov ključna izbira adherentne celične linije za fuzijskega partnerja.

Pridobljene hibridome smo testirali za proizvodnjo protiteles proti antigenom krvnoskupinskega sistema AB0. Vsi testi so bili negativni. Sklepamo, da je neproizvajanje protiteles posledica tega, da so bili naši limfociti B najverjetneje neaktivirani. Dobili smo jih namreč iz periferne krvi, ki ni najboljši vir za limfocite B, ki proizvajajo protitelesa. Za bodoče eksperimente bi bilo zato potrebno pridobiti limfocite B iz ustreznih tkiv ali pa razviti primeren imunizacijski protokol za aktivacijo limfocitov B *in vitro,* kar je tudi etično bolj sprejemljiva in lažje dostopna možnost.

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