Human connexin 32 overexpression for 3D crystallization

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Introduction

The genome sequencing projects reveal that integral membrane proteins represent up to one third of the predicted proteins of all organisms examined so far. Furthermore, membrane proteins are targets of the large majority of the drugs that are currently in use. About 80% of all cellular responses are thought to occur through the proteins linked to the cell membrane. An ultimate understanding of their function depends on detailed structural data for each class of membrane proteins, such as transporters, receptors, channels and pumps. The structural information could also greatly improve the efficiency of drug discovery [1–4]. To date, the 3D structures of only a tiny fraction of membrane proteins (around 218) are known in atomic detail, though structures of around 50000 soluble proteins have already been solved [5,6].

Difficulties to determine 3D structures of integral membrane proteins are most notably related to their hydrophobic/hydrophilic nature and inhomogeneity due to post-translational modifications. They are expressed at rather low levels and usually constitute less than 30% of a total cellular protein [7]. This requires developing an efficient overexpression system to produce sufficient amounts of protein for crystallization experiments. In general, the term “overexpression” is used to describe the production of proteins at a level that exceeds the endogenous one, e.g., 10-100 mg of protein per liter of culture. In spite of recent advances in nano volume crystallization technics and methods, still, for systematic 3D crystallization experiments, milligrams of highly pure protein are required. However, only a few membrane proteins are abundant enough in natural sources. An expression level of 0.2 mg per 1 liter of culture will already be limiting. Indeed, one should take into account that the overall yield of the purification procedure is generally very low.

The second limitation for the study of membrane proteins is extreme hydrophobicity due to hydrophobic nature of their membrane embedded part. This makes them refractory to direct manipulation in aqueous solutions and necessitates the use of detergents for their functional solubilization from the membrane and maintenance of their solubility throughout the purification process.

Many eukaryotic membrane proteins are post-translationally modified. Since modifications are usually incomplete, this is a source of inhomogeneity that interferes with successful protein crystallization. Overexpression systems allow genetic engineering of the target protein in order to prevent the modification that usually yield more homogeneous protein and preserve protein function.
The choice of the expression system is critical to obtaining a functional protein. An overexpression system can be either homologous or heterologous. Both systems have their specific advantages and limitations, like efficient overexpression of a given target membrane protein, the genetic characterization, the amenability to manipulation and the cost. The organisms commonly used for protein overexpression are bacteria (*Escherichia coli*), yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*), baculovirus/insect cells, and mammalian cells.

*E.coli* is a convenient host for heterologous protein expression. Its advantages include well-established protocols, fast growth, high levels of heterologous gene expression and scalability of experiments and low cost. It is also important to mention that 80% of all protein structures deposited in the protein data bank were obtained with the proteins overexpressed in *E.coli* [8]. However, heterologous gene expression in *E.coli* can lead to the production of misfolded, aggregated, and/or nonfunctional target proteins. This is often due to the absence of cofactors or lack of post-translational modifications required for function, stability and folding.

Taken together these features make the development of methods for the study of membrane proteins lag far behind those of soluble proteins. Finally, one has to point out that large amounts of pure and homogeneous protein are absolute prerequisites to begin with crystallization experiments.

Connexins belong to a very important class of membrane proteins. Till now, 21 human, 20 mouse, and 20 rat connexin genes have been identified. In living organisms connexins are the principal protein component of gap junctions – specialized cell-cell junctions that directly link the cytoplasm of adjacent cells. They mediate the direct transfer of low-molecular-weight (<1.2 kDa) metabolites and ions, including second messengers. It has been hypothesized that gap junctional intercellular communication plays a crucial role in the maintenance of homeostasis, morphogenesis, cell differentiation, and growth control in multicellular organisms. Recently it was shown that connexin 43 plays an important role in transformation of somatic cells to pluripotent ones. Discoveries of human genetic disorders due to mutations in gap junction protein genes and experimental data on connexin knockout mice provide the direct evidence that gap junctional intercellular communication is essential for tissue functions and organs development, and that its dysfunction causes diseases. At least nine human maladies are currently associated with mutations in genes encoding connexins. Also there are strong evidences that connexins function as malignant tumor suppressors [9].
Taking into account their relevance and impact connexin studies have been started long time ago and connexins are still actively explored. At present a lot of information about connexin physiology, permeability and disease-causing mutations was collected. But still atomic resolution structure of gap junction (formed by human connexin 26) is the only available [10]. This is mostly explained by high complexity of overexpression and purification of connexins in sufficient for crystallization amounts in mammalian or insect cells, which are commonly used for functional expression of mammalian proteins. The second problem is that connexins are subjected to post-translational modifications, which need to be removed to proceed with crystallization. And each additional purification step is crucial for final protein yield. On the other hand expression of recombinant proteins in E.coli eliminates most post-translational modifications and therefore would have an important advantage. Furthermore, it was previously shown that overexpression of connexins 26 and 43 in E.coli is possible and functional protein can be purified (able to form hexameric hemichannels and to bind ligands) [11–13].

Human connexin 32 is the major component of the peripheral myelin and is expressed in hepatocytes, secretory acinar cells, and Schwann cells as well. Among other members of connexin family it is one of the best studied. A lot of data concerning its conductance and permeability properties are available. Furthermore, connexin 32 was previously successfully produced and purified from mammalian cells [14], in vitro (using mammalian cells extract) [15], and from baculovirus/insect cells [16]. That is why there is a possibility to compare functional properties of this connexin overexpressed in different systems. However, previous attempts to express and purify full-length connexin 32 from E.coli were not successful [17]. In our work, we show that this is indeed possible. We developed the protocol of expression and purification of functional protein, able to form hemichannels. It is important to stress that there is no experimental atomic resolution structure of connexin 32 that can help to understand the molecular mechanism of its function. Two structural models of human connexin 32 were proposed [18,19]. In both cases they were created on basis of cryo-electron microscopy maps obtained for connexin 43 [20]. However, a high-resolution X-Ray structure of connexin 32 is to be obtained. We recently expressed the connexin in E.coli, solubilized and purified the protein and performed its first functional tests. The amount of protein is sufficient to start crystallization trials.

To summarize, in this work we overexpressed connexin 32 using membrane-targeting expression tag. Membrane-integrating protein MstX from Bacillus subtilis was attached to N-terminus of Cx32 and helped to increase tremendously expression yield. Since addition of N-
terminal extensions suppresses connexins functions all excessive amino acids must be removed. We demonstrated that MstX is resistant to various cleavage conditions including different proteases usage and variations of linker in between. Also we expressed Cx32 without any expression tag in vitro using E.coli S30 extract and refolded purified connexins into hemichannels. Then, since expression yield was still not high enough (around 0.2 mg of Cx32 per 1 ml of in vitro reaction mixture), we performed a broad screening of cell free reaction conditions and examined influence of short peptides addition on N-terminus. Next, we managed to express and purify connexin 32 from E.coli using certain gene sequence optimization. A refolding procedure was developed and Cx32 functionality was confirmed by assembling it into hemichannels. Further this protein was used for crystallization experiments.
Main results

Human connexin 32 gene was optimized for *E. coli* expression. hCx32 was produced in *E. coli* without any expression enhancers. The same purification and refolding protocol was used as for in vitro expressed connexin. Electron microscopy and dynamic light scattering confirmed proper oligomerization state of resulting Cx32 refolded in amphipol. This protein was used for *in meso* crystallization.

Human connexin 32 was expressed *in vitro* using *E. coli* S30 extract. A broad screening of cell free reaction conditions was carried out in order to increase protein yield. For the same reason influence of short peptides fused with N-terminus of Cx32 was examined. Finally, a suitable purification protocol was derived with the yield of Cx32 of 0.2 mg per 1 ml of *in vitro* reaction mixture. Protein was purified to homogeneity in NLS and SDS. The procedure resulting in assembling of Cx32 into connexons was suggested.

We also performed a number of other studies before we found an efficient approach to expressing hCx32. Among them we would like to mention that:

Human connexin 32 was successfully overexpressed using membrane-targeting expression tag in *E. coli*. Membrane-integrating protein MstX from *Bacillus subtilis* was attached to N-terminus of Cx32 and helped to increase tremendously expression yield. Since addition of N-terminal extensions suppresses connexins functions all excessive amino acids must be removed. We demonstrated that MstX-Cx32 was resistant to various cleavage conditions including different proteases usage and variations of linker in between.
1. Literature overview

1.1. The family of connexin genes

1.1.1. Introduction and nomenclature

Connexins constitute a large family of trans-membrane proteins that allow intercellular communication by forming gap junction channels [21–24]. The first members of this family were isolated by screening of complementary DNA (cDNA) libraries using antibodies directed against purified gap junctions to detect expressed fusion proteins [21] or by hybridization with oligonucleotides corresponding to amino acid sequences derived from the purified proteins [22]. Subsequently, additional members were identified from cDNA and genomic DNA based on their sequence similarities to those of known connexins [24–26].

Though connexin genes have been found only in deuterostomes other intercellular channels and gap junction structures are identified in many lower multicellular organisms. Proteins named innexins are forming gap junctions in most invertebrates. They show membrane topology similar to that of the connexins, but their gene sequences differ from the connexins a lot [27–29]. Also there are three genes, called pannexins, in genomes of higher vertebrates that exhibit high level of predicted amino acid sequences identity to the innexins [30]. Although pannexins can form channels connecting the cytoplasm and extracellular space, like connexin hemichannels, it was not proven that they form intercellular channels in living nature except in certain overexpression systems [31–33].

Till now 21 human genes and 20 mouse genes for connexins have been identified [34]. Each connexin is expressed in specific tissues or cell types and many cell types express more than one connexin (Table 1.1). Some connexins are expressed in cells of many types, but others are expressed in very limited organs and cells. Even in the same tissue, the expression pattern of each connexin shows cell-type specificity and developmental changes, suggesting the presence of distinct but tight control mechanisms for regulation of connexin gene expression.

All members of the connexin protein family are denoted using a standard, operational nomenclature that utilizes the word connexin (abbreviated Cx) followed by a suffix indicating the predicted molecular mass of the polypeptide in kilodaltons [35]. There also exists another nomenclature for identification of connexins. This system was initially developed when vertebrate connexin genes were separated into subgroups, α, β and γ, on the basis of overall sequence similarities and length of the cytoplasmic loop [36,37].
### Table 1.1. Connexin genes and their expression.

<table>
<thead>
<tr>
<th>Human</th>
<th>Mouse</th>
<th>Major expressed organ or cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCx23</td>
<td>mCx23</td>
<td>–</td>
</tr>
<tr>
<td>hCx25</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>hCx26</td>
<td>GJB2</td>
<td>13q11–q12, mCx26</td>
</tr>
<tr>
<td>hCx30</td>
<td>GJB6</td>
<td>13q12, mCx30, Breast, cochlea, placenta, hepatocytes, skin, pancreas, kidney, intestine</td>
</tr>
<tr>
<td>hCx30.2</td>
<td>GJE1</td>
<td>7q22.1, mCx29, Brain, spinal cord, Schwann cells</td>
</tr>
<tr>
<td>hCx30.3</td>
<td>GJB4</td>
<td>1p35–p34, mCx30.3, Skin, kidney</td>
</tr>
<tr>
<td>hCx31</td>
<td>GJB3</td>
<td>1p34, mCx31, Cochlea, placenta, skin</td>
</tr>
<tr>
<td>hCx31.1</td>
<td>GJB5</td>
<td>1p35.1, mCx31.1, Skin</td>
</tr>
<tr>
<td>hCx31.9</td>
<td>GJC1 (GJA11)</td>
<td>17q21.1, mCx30.2, –</td>
</tr>
<tr>
<td>hCx32</td>
<td>GJB1</td>
<td>Xq13.1, mCx32, Hepatocytes, secretory acinar cells, Schwann cells</td>
</tr>
<tr>
<td>hCx36</td>
<td>GJA9</td>
<td>15q13.2, mCx36, Neurons, pancreatic β-cells</td>
</tr>
<tr>
<td>hCx37</td>
<td>GJA4</td>
<td>1p35.1, mCx37, Endothelium, granulosa cells, lung, skin</td>
</tr>
<tr>
<td>hCx40</td>
<td>GJA5</td>
<td>1q21.1, mCx40, Cardiac conduction system, endothelium, lung</td>
</tr>
<tr>
<td>hCx40.1</td>
<td>–</td>
<td>–, mCx39, –</td>
</tr>
<tr>
<td>hCx43</td>
<td>GJA1</td>
<td>6q21–q23.2, mCx43, Many cell types</td>
</tr>
<tr>
<td>hCx45</td>
<td>GJA7</td>
<td>17q21.31, mCx45, Cardiac conduction system, smooth muscle cells, neurons</td>
</tr>
<tr>
<td>hCx46</td>
<td>GJA3</td>
<td>13q11–q12, mCx46, Lens</td>
</tr>
<tr>
<td>hCx47</td>
<td>GJA12</td>
<td>1q41–q42, mCx47, Brain, spinal cord</td>
</tr>
<tr>
<td>hCx50</td>
<td>GJA8</td>
<td>1q21.1, mCx50, Lens</td>
</tr>
<tr>
<td>hCx59</td>
<td>GJA10</td>
<td>1p34, –, –</td>
</tr>
<tr>
<td>hCx62</td>
<td>–</td>
<td>6q15–q216, mCx57, Retinal horizontal cells</td>
</tr>
</tbody>
</table>

1.1.2. Domain structure of the connexins

Connexin genes are translated to proteins that form hexameric structures in the plasma membrane called hemichannels or connexons, harbouring a central pore that permit the passage of ions and small molecules between cytoplasm and extracellular surroundings. Based on hydropathy plots topological model for location of different domains of connexin relative to the plasma membrane (Figure 1.1) was predicted [39–41]. It shows that the connexin polypeptide contains a short cytoplasmic amino-terminal domain (NT), four transmembrane domains (M1 to M4) separated by one cytoplasmic loop domain (CL) between M2 and M3, two extracellular loops (E1 and E2), and a carboxyl-terminal cytoplasmic domain (CT) [35]. The two extracellular loops are highly conserved and necessary for docking of two hemichannels of adjacent cells to form gap junctions. A set of three cysteine residues in each of the extracellular loops may help to maintain the tertiary...
structure necessary for this docking of two hemicannels [42]. The length and sequence on
the NT, M1-M4 are relatively conserved among all connexins. The CT has the greatest
variability in size among connexins, from short 10-12 amino acids in Cx26, Cx30.2 and
Cx31.1 to more than 310 amino acids in Cx59 and Cx62. The CL length also varies quite a
lot, connexins even can be divided into three categories [43]: those that have small (30-35 aa),
medium (50-55 aa), or large (80-105 aa) CL domains.

Figure 1.1. Membrane topology of connexins. The
cylinders, marked M1-M4, represent corresponding
transmembrane domains. The loops between the first
and the second, as well as the third and fourth
transmembrane domains, are predicted to be
extracellular (E1 and E2, respectively), each with
three conserved cysteine residues. Both the N- and C-
termini are facing the cytoplasm [44].

1.1.3. Connexin gene structures

Recent studies identified 21 human and 20 mouse connexin genes in human and
mouse genomes [45–47]. Various connexin genes are found in many different chromosomes,
but there is a cluster of connexins in region of human chromosome 1 [48]. Genes for many
connexins were cloned and studied by polymerase chain reaction. Collected information
shows that many connexin genes have a similar organization and virtually all have only single
copies in the haploid genome [47].

The general genomic structure of connexins is simple and consists of an untranslated
5′-exon (exon 1) separated from a second exon (exon 2) by an intron of variable size. Exon 2
includes the complete connexin coding region and the 3′-untranslated region (UTR) (Figure
1.2). Recent investigations suggest that the use of multiple alternative exons to generate the
5′-UTR may be a feature of many connexin genes, for example Cx32 and Cx36 genes [49].
The human Cx32 gene contains alternative first exons (containing only 5′-UTR) whose use is
tissue specific [50,51]; some bovine Cx32 transcripts have three exons, two of which contain
only 5′-UTR [52]. The Cx36 gene contains two exons, both of which contain untranslated and
translated sequences. The coding region is interrupted by an intron [53–55]. It has been
suggested that these variations in 5′-UTRs may cause differences in mRNA translation or
stability [56,57].
Figure 1.2. Structures of the connexin genes. Diagrams depict the structures of different connexin genes with the coding regions in dark gray boxes and the noncoding regions in white boxes. (A) Common gene structure. The 5′-untranslated exon 1 (5′-UTR) is separated by an intron of variable length from exon 2 harboring the complete coding region (shaded box) and the subsequent 3′-UTR. (B) Different 5′-UTRs can be spliced alternatively and/or consecutively to that exon carrying the coding region. (C) In mCx36, hCx36, mCx39, hCx40.1 and mCx57, the coding region is spliced.

1.1.4. Transcriptional and translational regulation of connexin expression

The transcription of connexin DNAs is regulated by a variety of factors, and a lot is known about this process [35,38,49]. In most connexin genes the basal promoter located less then 300 bp upstream from the transcriptional initiation site contains binding sites for cell type–independent transcription factors including TATA box-binding protein, Sp1/Sp3, and activator protein-1 (AP-1). Binding of these factors may play a critical role in dramatic changes of connexins expression. For example, production of c-fos, which interacts with the AP-1 sites may cause the giant increase in myometrial expression of GJA1 (Cx43) mRNA [58].

Treatment of cells with a variety of chemicals or biological substances (including cyclic adenosine monophosphate [cAMP], phorbol esters, and retinoids) or manipulation of signal transduction pathways (such as the Wnt/β-catenin/T-cell factor (TCF)/lymphocyte enhancer binding factor (LEF) pathway) also may regulate connexin transcription, as was showed before [35,38].

The connexin gene promoters also contain binding sites for cell type-specific transcription factors. For example, multiple factors, including Nkx2.5, GATA4, and Tbx5, regulate GJA5 (Cx40) expression in the heart [59–61]; Nkx2.5 is an important regulator of genes for three connexins (Cx40, Cx43, and Cx45) in the cardiovascular system [62].

Importance of different transcription factors can be also shown on example of GJB1 (Cx32). In liver and pancreas promoter P1 (8Kb upstream the start codon) is used, which
contains binding sites for cell type-independent transcription factors (like Sp1/Sp2 and nuclear factor-1 [63–65]) and cell type-specific factors (like hepatocyte nuclear factor-1 [65]). In Schwann cells cell-specific promoter P2, containing the early growth response gene-2 (Egr2/Knox20) and SOX10 cell-specific factors, is used [14,66].

1.1.5. Connexin biosynthesis and posttranslational modifications

Like all others eukaryotic trans-membrane proteins connexins are synthesized by ribosomes that are bound to the endoplasmic reticulum (ER) membrane [67]. They encode hydrophobic domains (internal trans-membrane segments) that are recognized by a signal recognition particle (SRP). SRP binding is necessary for docking of the SRP/ribosome/nascent-polypeptide-chain/mRNA complex to a protein-channel in the ER membrane [68]. Interesting fact is that connexins with their large hydrophobic domains are initially localized in the hydrophilic lumen of the translocon channel [69,70]. Once synthesis complete, connexins are translocated out of the channel lumen into the hydrophobic ER membrane environment. During this integration folding of connexin proteins is achieved. Different studies have demonstrated that connexin biosynthesis and membrane translocation follow the pathway that has been described for membrane proteins with cytoplasmically located N-termini [71,72].

Connexin oligomerization into connexons was initially reported to occur in the ER (Figure 1.3), however, with some exceptions [73,74]. For example, it has been showed that Cx43 assemble in hexamers only upon arrival in the distal regions of the Golgi complex. Recent data has shown that connexin oligomerization is a sequential process, which starts in the endoplasmic reticulum and ends upon arrival in the Golgi [75,76]. An exception to this pathway is Cx26, which may be either co- or post-translationally transported into the ER or directly to the plasma membrane, without passing through the Golgi apparatus [77].

When connexin oligomerization is finished connexon hemichannels are packaged into vesicles and transferred to the membrane. This connexon transport may either be microtubule dependent or independent [77]. It has been suggested that connexon insertion in the plasma membrane is random. However recent studies indicate that microtubules may be responsible for targeting of these gap junction hemichannels to specific membrane domains or even regions [78].

Upon various types of post-translational connexin modifications (like hydroxylation, nitrosylation, acetylation, disulfide binding and etc.) phosphorylation is the most studied [52-55]. Phosphorylation may occur on various connexin residues and is important for proper
formation and functioning of gap junction channels. Although most connexins undergo these modifications (including Cx32) some of them, for example Cx26, remain unphosphorylated.

**Figure 1.3.** Multiple trafficking and assembly routes of gap junctions and dual intercellular communication pathways. In route A, Cx32 and Cx43 are co-translationally inserted into the endoplasmic reticulum, where they oligomerize into connexons and are then trafficked via the Golgi to the plasma membrane. Apposition of two connexon hemichannels results in docking to generate a gap junction. In route B, an alternative route used by Cx26 does not involve trafficking through the Golgi and possibly involves post-translational insertion into either endoplasmic reticulum or directly into plasma membranes [79].

Phosphorylation is involved in the regulation of gap junctional communication at several stages of the connexin life cycle including hemichannel oligomerization, export of the protein to the plasma membrane, hemichannel activity, gap junction assembly, gap junction channel gating, and connexin degradation [80–82]. The effect of phosphorylation on channel gating is very specific, in case of Cx43 phosphorylation on different residues by the same kinase may lead to opposite effects. It may whether enhance or inhibit gap junction function and gap junction intercellular communication [77]. The phosphorylation may also affect other cellular functions of connexins such as control of growth and proliferation.

1.1.6. **Connexin mutations and disease**

Many mutations of several different connexins are associated with a wide variety of inherited diseases, including neuropathies, deafness, epidermal diseases, cataracts, and oculodentodigital dysplasia (Table 1.2). The inheritance of these diseases may be autosomal dominant, autosomal recessive, or X-linked [35].
Disease-causing mutations have been reported to occur anywhere in the connexin genes. Most of identified connexin mutations are located within the protein-coding region. While affecting synthesis, assembly, channel function, and degradation of connexins these mutations may cause disease through a variety of mechanisms. Mutations can be found also in noncoding regions. Most probably they disrupt elements regulating transcription or interfere with proper splicing to generate mature connexin mRNAs. Several such mutations were found in Schwann cells [14,83] in GJB1 and were associated with X-linked Charcot-Marie-Tooth disease. More information about Cx32 mutations you can find in chapter 1.7.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Inheritance pattern</th>
<th>Connexin Protein</th>
<th>Connexin Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oculodendrodigital dysplasia</td>
<td>AD (AR)</td>
<td>Cx43</td>
<td>GJA1</td>
</tr>
<tr>
<td>Cardiovascular diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>AD/ND</td>
<td>Cx40</td>
<td>GJA5</td>
</tr>
<tr>
<td>Visceroatrial heterotaxia</td>
<td>ND</td>
<td>Cx43</td>
<td>GJA1</td>
</tr>
<tr>
<td>Cataract</td>
<td>AD</td>
<td>Cx46</td>
<td>GJA3</td>
</tr>
<tr>
<td>Myelin-related diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-linked Charcot-Marie-Tooth disease (CMTX)</td>
<td>XR</td>
<td>Cx32</td>
<td>GJB1</td>
</tr>
<tr>
<td>Pelizaeus–Merzbacher-like disease</td>
<td>AR</td>
<td>Cx46/6</td>
<td>GJA12/GJC2</td>
</tr>
<tr>
<td>Hearing loss (non-syndromic or associated with skin disorders)</td>
<td>AR/AD</td>
<td>Cx30/30</td>
<td>GJB6/GJB3</td>
</tr>
<tr>
<td>Skin diseases</td>
<td>AD</td>
<td>Cx26</td>
<td>GJB2</td>
</tr>
<tr>
<td>Keratitis ichthyosis deafness syndrome</td>
<td></td>
<td>Cx26</td>
<td>GJB2</td>
</tr>
<tr>
<td>Volwinkel syndrome</td>
<td></td>
<td>Cx30/30</td>
<td>GJB6/GJB3</td>
</tr>
<tr>
<td>Clouston syndrome</td>
<td></td>
<td>Cx30</td>
<td>GJB6</td>
</tr>
<tr>
<td>Erythrokeratodermia variabilis</td>
<td></td>
<td>Cx30/30</td>
<td>GJB4/GJB3</td>
</tr>
</tbody>
</table>

AD, autosomal dominant; AR, autosomal recessive; XR, X-linked recessive; ND, not determined.

Table 1.2. Connexin-associated diseases and corresponding connexin proteins and genes [44].

1.2. Gap junctions structure

1.2.1. Organization levels of gap junctions

Based on the first studies of gap junctions, although they were limited to relatively low resolution, a model of hemichannel structure was proposed. It suggests that the intercellular channel is formed by the end-to-end interaction of two oligomers termed connexons [84]. Within these oligomers, each connexin subunit is thought to traverse the membrane bilayer four times, placing the amino- and carboxyl-termini on the cytoplasmic membrane surface [39,41,85]. Moving of transmembrane domains with respect to each other
may control channel opening [86]. In nature multiple gap junction channels cluster in the membrane and form gap junction plaques (Figure 1.4).

Figure 1.4. Molecular organization and schematic topology of a gap-junctional plaque. (a): Hemichannels in apposed plasma membranes of neighboring cells can dock to each other and form gap junction channels. Three different types of gap junction have been reported - homomeric/homotypic (1), heteromeric (2) and heterotypic (3) - depending on their molecular composition. Homotypic or heterotypic gap junctions comprise two identical or two different types of hemichannel, respectively. Homomeric or heteromeric hemichannels are composed of one or more connexin (or possibly pannexin) isoforms, respectively. Each hemichannel represents an assembly of six connexin protein subunits. (b): Connexin protein subunits are tetra-spanning membrane proteins that share three conserved extracellular cysteine residues, which are crucial for docking. The subunits vary mainly in their cytoplasmic loop and C-terminal region. S–S represents conserved disulphide bonds in the extracellular domains of connexins [87].

There is a growing evidence that a single gap junction channel can be made of different types of connexins, i.e., two connexons each consisting of different types of connexins can form a heterotypic gap junction channel, whereas one connexon containing different types of connexins can form a heteromeric gap junction channel (Figure 1.4a). This hypothesis was proved by studies of fractions containing detergent solubilized gap junctions [16,88–90]. In general, heteromeric gap junction assembly occurs only between connexins of the same classes. For example, Cx32 and Cx43 translated in vitro do not form mixed gap junction hemichannels, but when two different connexins within the same class are cotranslated, they interact to form heteromeric hemichannels.
Distinct electrophysiological and ion selective properties have been shown not only for homotypic gap junction channels made of different connexins but also between homotypic and heterotypic gap junction channels. The net result of such diversity could provide communication compartments that enable a group of cells to be regulated by changes in the concentration of a specific second messenger or metabolite. More details can be found in [38,42,79,91].

1.2.2. Electron microscopy studies of gap junction structure

Till now electron cryomicroscopy was one of approaches that enriched our knowledge of gap junctions more than any other method. Recently Unger et al [20] determined the structure of a recombinant cardiac gap junction channel formed by Cx43 mutant (most of the COOH-terminal domain was truncated) by electron crystallography at resolutions of 7.5 Å in the membrane plane and 21 Å in the vertical direction. They showed that the recombinant gap junction channel formed by the Cx43 mutant was shaped like a sand glass with a thickness of 150 Å. The outer boundary of the map revealed a tripartite arrangement that consisted of two membrane domains that were separated by an extracellular domain.

![Molecular organization of a recombinant gap junction channel](image)

*Figure 1.5. Molecular organization of a recombinant gap junction channel. (a) A full side view is shown, and (b) a top view looking toward the extracellular gap is shown. For clarity, only the cytoplasmic and most of the membrane-spanning regions of one connexon are shown. The 24 well-resolved rod-shaped features reveal the packing of the transmembrane α-helices, and four have been arbitrarily labeled M1, M2, M3, and M4. Similarly, M1’ and M2’ identify putative a helices that are identical to M1 and M2 because of the six fold symmetry of the channel [20].*

The outer diameter within the membrane region was 70 Å and in the extracellular portion of the channel outer diameter decreased abruptly by 20 Å (Figure 1.5a). Aqueous pathway narrowed from 40 to 15 Å (neglecting the contributions of amino acid side chains) in
proceeding from the cytoplasmic to the extracellular side of the bilayer. Which was caused by the tilt of the channel-lining M3 α-helix (Figure 1.5b). The aqueous pathway then widened again to a diameter of 25 Å within the extracellular vestibule.

Because the connecting loops between the transmembrane α-helices could not be revealed with such low resolution (in-plane resolution of 7.5 Å), there was ambiguity in assigning the molecular boundary of the connexin subunit. But earlier it was already demonstrated that extracellular loops E1 and E2 are involved in initiating the interaction between connexons in adjacent cells (while using site directed mutagenesis to shift four of the six conserved cysteines in the extracellular loops of Cx32 individually, in all possible pairwise combinations, and even in some quadruple combinations Foote et al studied connexons coupling [92]).

1.2.3. X-Ray diffraction analysis of gap junction structure

More precise knowledge was obtained from X-Ray diffraction patterns of Cx26 crystals with resolution 3.5 Å by Maeda et al in 2009 [10]. They confirmed size dimensions had been received earlier by electron crystallography having in mind that side chains were not visible with 7 Å resolution.

Connexin 26 consists of four transmembrane segments (M1-4), two extracellular loops (E1 and E2), a cytoplasmic loop, an N-terminal helix (NT), and a C-terminal segment (Figure 1.6). Any pair of adjacent helices of Cx26 is antiparallel, forming a typical four-helix bundle. M1 and M2 face the interior, whereas M3 and M4 face the hydrophobic membrane environment. M1 is identified as a major pore-lining helix (before there has been a disagreement, one was assuming M3 playing role of major pore-lining helix [18,93], while others – M1 [94,95]). Pore diameter narrows from the cytoplasmic to the extracellular side of the membrane because M1 is canted. First transmembrane domain ends in a short 310-helix. M2 is kinked at Pro87 (Pro87Leu mutation has been shown to cause an abnormal gating [96]) and M2 and M3 protrude into the cytoplasm. M4 is tilted from the molecular axis by about 30°. This results in extension of connexon diameter on the intracellular side.

The extracellular loop E1 contains a 310-helix at the beginning and a short α-helix in its C-terminal half. E2, together with E1, contains a short antiparallel β-sheet and stretches over E1, forming the outside wall of the connexon. Six conserved cysteine residues, three in each loop, form intramolecular disulphide bonds between E1 and E2. The N-terminal half of E2 is flexible and its amino-acid sequence varies greatly among connexins [10]. The C-
terminal half of E2 begins with a $3_{10}$ turn and is followed by a conserved Pro-Cys-Pro motif that reverses its direction back to M4.

![Figure 1.6. Topological diagram of the Cx26. Open boxes indicate regions with a helical secondary structure denoted as NT, M1, M2, M3, M4, $3_{10}$-helix and short helix in E1. The membrane region is depicted in light blue. Red lines indicate ten-amino-acid increments. Color code: white letters in black circles: disordered residues; white letters in grey circles: residues whose side-chains are not included in the model; green: methionine residues; yellow: extracellular cysteine residues that form disulfide bonds; purple: residues mediating intra-connexin interactions; red: residues mediating inter-connexin interactions; blue: residues mediating inter-connexon interactions [10].

Most of the prominent intra-connexin interactions are in the extracellular part of the transmembrane region. Arg32 (M1) interacts with Gln80 (M2), Glu147 (M3), and Ser199 (M4). Two hydrophobic cores around Trp44 (E1) and Trp77 (M2) stabilize the connexin structure. Ala39 (M1), Ala40 (M1), Val43 (E1) and Ile74 (M2) contribute to the first hydrophobic core around Trp44, and Phe154 (M3) and Met195 (M4) form the second core with Trp77 (Figure 1.7). In the intracellular part of the transmembrane region, Arg143 (M3) forms hydrogen bonds with Asn206 (M3) and Ser139 (M3) (Figure 1.7).

Interactions between connexins in single hemichannel are mostly located in the extracellular half of M2 and M4 and in the extracellular loops. Glu47 (E1), Gln48 (E1), Asn62 (E1), Asp66 (E1), Tyr65 (E1), Arg75 (TM2) and Ser72 (E1) from one connexin, and Asp46 (E1), Asp50 (E1), Arg184 (E2) Thr186 (M4) and Glu187 (M4) from the adjacent connexin form the core of the inter-connexin interactions. Although M3 is evolutionarily more variable than the other three helices, every third or fourth residue in M3 is aromatic, generating an aromatic face that is conserved among connexins. Each helix in a connexin contributes to an aromatic cluster in the groove between two adjacent connexins. Most of the
residues involved in intra- and inter-connexin interactions are conserved within the connexin family [10], and mutations of these residues are associated with different disorders [97]. Most probably these mutations affect proper folding and oligomerization of connexins.

**Figure 1.7.** Intra-connexin interactions in the Cx26. The Cα trace is shown in ribbon or line representation and the side-chains in the close-up views in the boxes are shown as sticks. Hydrogen bonds or salt bridges are shown as dotted lines [10].

Both E1 and E2 are involved in the interactions between the two adjoining connexons of the gap junction channel (**Figure 1.8**). In E1, Asn54 forms hydrogen bonds with Leu56 in the opposite connexin, and Gln57 forms symmetric hydrogen bonds with the same residue of the diagonally opposite connexin. Both residues are highly conserved among connexins. In E2, Lys168, Asp179, Thr177 and Asn176 form hydrogen bonds and salt bridges with the opposite connexin. Forming a tight double-layered wall these interactions connects the two adjoining hemichannels and separates the channel pore from the extracellular environment.

The pore of a gap junction channel consists of an intracellular channel entrance, a pore funnel and an extracellular cavity. Intracellular parts of M2 and M3 form the channel entrance. The short NTs of the six connexins are imbedded into the pore, forming the funnel. N-terminal residues Asp2, Trp3, Thr5, Leu6 and Ile9 line the funnel surface. Asp2 forms hydrogen bonds with Thr5 from the neighboring connexin (**Figure 1.9**). Trp3 forms hydrophobic interactions with Met34 (M1) of the neighboring connexin, which draws the NT to the inner wall of the channel. This interaction maintains the funnel in the open state, with
an inner diameter of 14 Å. While Met34Thr mutation is reported to form structures indistinguishable from wild type gap junctions, it decreases electrical current [98,99] by disrupting the interaction of the NT with Trp3 and narrowing the funnel [100].

Cx26 is known to be closed by an inside positive potential. On the other hand Cx32 has Asn at position 2 and closes after an inside negative potential [101]. A cytoplasmic movement of the N-terminus, where the voltage sensor is believed to reside, has been suggested to initiate voltage-dependent gating [101–103]. Although NT plays a major role in this model of voltage-dependent gating (in agreement with previous physiological studies [102–104]) it is possible that the invisible cytoplasmic loop or the C terminus might contribute as a component of the plug, as was described earlier in case of Cx43 [105–107].

**Figure 1.8.** Intercellular interactions. The connexins forming the gap junction channel are labeled A to F and A’ to F’. The right top and bottom boxes show intercellular interactions in E1 and E2, respectively [10].

**Figure 1.9.** The six NTs form a funnel structure, which is stabilized by a circular network of hydrogen bonds between Asp2 and the main chain of Thr5. The connexins are shown in line and the NTs in ribbon representation superposed on a surface representation. The close-up view shows the interaction between the ring of Trp3 and the methyl group of Met34 (M1) in the adjacent connexin (hydrophobic interaction: orange broken line; hydrogen bond: red broken line) [10].
1.3. Gating mechanisms of gap junctions and their permeability

1.3.1. Connexins voltage sensitivity

Like many others ion channels gap junction channels are voltage dependent, but the magnitude of the dependency varies greatly among connexin family. Connexin channels exhibit complex channel sensitivity, the conductance \( G_j \) of most of them is sensitive to transjunctional voltage \( V_j \), the voltage difference between two cell interiors coupled by gap junctions), but many are also sensitive to membrane potential \( V_m \), a cell absolute inside-outside voltage) [108]. It is hypothesized that this dual voltage regulation is due to the existence of two different gates, each of which specifically senses one type of voltage [109]. The \( G_j \) of most homotypic connexin channels is typically maximal at \( V_j =0 \) \( (G_{j_{\text{max}}}) \) and it decreases symmetrically for positive and negative \( V_j \) pulses to non-zero conductance values. Transitions between the main open state and the closed state could be either fast or slow. Accordingly, these two gating processes have been termed “fast \( V_j \)-gating” and “slow \( V_j \)-gating” respectively. Little is known about the mechanisms responsible for slow \( V_j \)-gating but there are evidences that the C-terminal domain is involved in the fast \( V_j \)-gating, as it is abolished when this domain is truncated [110] or fused to a large molecule like GFP [111], and it is recovered when truncated connexins are coexpressed with C-terminal domains [112]. It is hypothesized that the fast \( V_j \) gating can be explained by the “ball-and chain” model, where the displacement of the C-terminal domain toward the inner mouth of the channel pore would physically close the pore, a model that had already been proposed for the closing state triggered by pH [113], Insulin and IGFs [114]. Nano metric data using AFM also support this model [115].

Connexins sensitive to \( V_m \) have also a slow \( V_m \)-gating mechanism. This mechanism would regulate electrical coupling when \( V_j =0 \), especially in excitable cells. The slow \( V_m \)-gating has been also related to the C-terminal domain, but to the residues close to fourth transmembrane domain [116]. These findings suggest that an outwardly directed movement of the voltage sensor, which would lead to conformational changes that close the pore, mediates slow \( V_m \)-gating.

On the other hand recent structural studies of Cx26 have proposed \( V_j \)-gating model in which N-terminus is a major sensing particle and is forming the plug to clothe the pore (Figure 1.10) [10]. Although this model is not valid for other voltage-sensitive ion channels containing the S4 helix as a voltage sensor [117], it is in good accordance with previous physiological studies [102–104]. Also it has been reported that the Cx37 channels with
truncated N-terminus showed no electrical function [118]. At present there is no appropriate structural model about how such Cx37 channels are totally closed and more detailed structural information for gap junctions is required to explain voltage gating of the whole connexin protein family.

**Figure 1.10.** Modified plug-gating model for voltage-dependent gating of the gap junction channel. Left: Model of the Cx26 gap junction channel in the open state at \( V_j = 0 \). NT (red) is attached to the channel (blue) by the hydrophobic interaction between Trp3 and Met34, keeping the channel open. Right: Model of the Cx26 gap junction channel in the closed state. Relative potential gradient is depicted as \( V_j > 0 \) when the upper cell is more positive than the lower. The NTs of the connexon at the inside positive potential are released from the hydrogen bond network and the Met34 hook shown in the left side, resulting in the formation of a plug [10].

### 1.3.2. Chemical gating of gap junctions

\( Ca^{2+} \) was the first cytoplasmic factor showed to be involved in the regulation of gap junction function [119]. Later DeVries and Schwartz [120] reported that cultured solitary horizontal cells of the catfish retina express an endogenous current mediated by hemichannels that open upon reduction of the extracellular \( Ca^{2+} \). The current is reduced by external \( Ca^{2+} \) higher than 1 mM, treatment with dopamine, or a weak acid that corresponds to the blockade of Lucifer yellow uptake. Subsequently, the electrophysiological characterization of macroscopic and single hemichannel currents has been reported for various connexins [49]. Based on electron microscopic studies it has been proposed that \( Ca^{2+} \) induces a regional closure of the pore [121].

Agonists of hemichannel opening have also been described. For example antimalaric drugs quinidine and quinine enhance hemichannel-mediated currents in retinal horizontal cells bathed in low extracellular \( Ca^{2+} \) [122]. Also alendronate (used in the treatment of bone diseases) induces opening of hemichannels formed by Cx43 [123].
Opening of Cx43 hemichannels can be blocked by activation of a PKC-dependent pathway [124,125]. This proves that protein phosphorylation is involved in gating process. Closure of hemichannels has been observed after extracellular application of lanthanide cations, La$^{3+}$ [126], or Gd$^{3+}$ [127], or treatment with gap junction channel blockers, such as octanol, heptanol, carbenoxolone, oleamide, halothane, and 18-α- and 18-β-glycyrrhetinic acid.

Extracellular acidification (pH reduction) also closes hemichannels formed by various connexins [49]. Cx43 is the best-studied example of pH gating. In its case it have been shown that gating in response to pH changes is caused by interactions between C-terminus and specific segment of CL [113]. Expression of truncated Cx43 (Cx43M257Δ) prevents acidification-induced uncoupling, while if the truncated portion of the CT is coexpressed as a separate peptide uncoupling could be restored [128]. Based on these data the model has been proposed where the CT domain acts as a gating particle. At normal pH, the gating particle is away from the pore and the channel is open. Upon acidification, the particle binds to a separate region of the protein (a receptor) affiliated structurally or functionally with the pore. This particle-receptor interaction leads to channel closure.

NMR studies of Cx43CT indicated reduced mobility of this domain at low pH due to its oligomerization [129]. Further studies demonstrated that the Cx43CT formed dimer in a pH-dependent manner [130]. These results led to the suggestion that dimerization of the CT may be one of the structural changes involved in the pH regulation of Cx43. Additionally, the change in oligomeric state of the CT may play a role in modulating the molecular partners that associate with Cx43 under a given condition, thus acting as a switch for modifications in channel function.

Unfortunately this particle-gating model cannot be accepted for the whole connexin family. It was shown that pH sensitivity varies greatly among connexins [112]. Differences were attributed to the diversity of the primary sequence, particularly in regulatory domain regions like the CT. Although some connexins follow the basic particle-receptor model of pH gating [112] there are exceptions - Cx26, Cx32, and Cx46. All three are not affected by the truncation of their CTs. Studies on Cx46 by Trexler et al suggest that pH gating of that connexin involves direct protonation of the connexin molecule [131].

In case of Cx26 and Cx32 pH-dependent closure was shown when hemichannels were exposed to aminosulfonate buffers (HEPES, MES) [132]. However, pH-dependent channel gating was not seen when non-aminosulfonate buffers were used. At constant pH, increased aminosulfonate concentration caused hemichannel closure. Similar effects were seen with
taurine, the most common cytoplasmic aminosulfonate, at physiological concentrations. Thus, the possibility arises that acidification-induced effects on these channels may actually be mediated through protonation of cytoplasmic regulators.

High-resolution atomic force microscopy showed that the Cx26 pore diameter increased in response to pH growing in HEPES buffer [133]. Pore diameter incensemment was caused by approximately 6.5° rotation in lobes at the extracellular surface of the hemichannels. These transitions suggest a modification of the Cx26 pore structure during pH gating.

### 1.3.3. Permeability of connexin channels

Connexin channels like many other channels possess an aqueous pore through with ions can flow. It can be stated that the molecular weight of substances being allowed to cross the junction should be less than 1kD as suggested by certain experiments with dye molecules [134]. The permeability of gap junctions is widely non-selective allowing the pass of small molecules (charged or not), second messengers and small metabolites. On the other hand gap junctions are impermeable to nucleic acids and proteins [37]. In summary, their pore selectivity is not uniquely defined in contrast to ion channels.

Using standard electrophysiological techniques unitary conductance of gap junctions was studied. It was varying greatly among connexin family members starting from 9 pS in case of Cx31.9 up to 350 pS for Cx30 [35]. Since connexin channels are generally permeable to molecules at least 8 to 10 Å in minimal diameter it have been presumed that connexin channels would not have significant charge selectivity. Direct measurements showed opposite picture (Table 1.3) [35]. The vastly different unitary conductance’s and charge selectivity’s suggest that the pores of connexin channels have diverse properties - structural and electrostatic.

<table>
<thead>
<tr>
<th>Connexin</th>
<th>Conductance ratio cation/anion</th>
<th>Permeability ratio cation/anion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26</td>
<td>2.6</td>
<td></td>
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<tr>
<td>Cx32</td>
<td>0.94</td>
<td>0.77</td>
</tr>
<tr>
<td>Cx37</td>
<td>2.3-3.4</td>
<td></td>
</tr>
<tr>
<td>Cx40</td>
<td>4.5</td>
<td>6.2-6.9</td>
</tr>
<tr>
<td>Cx43</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>Cx45</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cx46</td>
<td></td>
<td>7-10.3</td>
</tr>
<tr>
<td>Cx50</td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.3.** Charge selectivity to atomic ions [35]
In several studies the limiting pore width of connexin channels was measured by observation of relatively unreactive molecules (e.g., sugars, polyethylene glycols) propagation. These studies showed that PEGs 400 Da and larger could not enter the pores but PEGs 300 Da and smaller were able to enter the pores [135]. In case of Cx32 channels this cutoff size was between PEG400 and PEG300 [136]. Based on these studies the ranking of limiting pore diameter can by build: Cx43 (90 pS) > Cx32 (50 pS) > Cx26 (130 pS) = Cx26/Cx32 (48 to 89 pS) > Cx37 (300 pS) and Cx43 (90 pS) ≈ Cx46 (140 pS) > Cx40 (180 pS) [137,138]. To summarize it is obvious that channels with the narrowest pores are more charge selective than channels with wider pore diameters.

It was showed that some types of connexin channels can transfer second messengers, amino acids, nucleotides, Ca^{2+}, and glucose and its metabolites [35,139,140]. However permeability for big molecules (both biological and non-biological) also varies a lot for different connexins [141,142]. From these studies it is clear that gap junction channels can have some affinity selectivity mechanisms that are highly specific for certain permeants, and whose properties cannot be extrapolated in a straightforward way from estimates of pore size, conductance or charge selectivity, or even from known permeants [143,144].

1.4. Charcot-Marie-Tooth disease and connexin 32 mutations

Charcot-Marie-Tooth (CMT) disease was first described by neurologists Jean Martin Charcot, Pierre Marie and Howard Henry Tooth in 1886. This is a group of inherited peripheral neuropathies that affect both motor and sensory nerves and has a high prevalence among the population (1:2500). Although CMT is characterized by distal muscle weakness and atrophy and foot deformities as claw toes (Figure 1.11), it is nowadays classified into different variants according to clinical, electrophysiological, histopathological and genetic features [108].

Figure 1.11. The foot of a person with Charcot–Marie–Tooth disease. The lack of muscle, a high arch, and claw toes are signs of this genetic disease.
The X-linked form of CMT disease (CMTX) is the most common form of hereditary sensory and motor neuropathy [145,146]. The clinical course is characterized by slowly progressive symptoms, such as distal muscle weakness and atrophy, areflexia, and variable sensory abnormalities. While affected males have moderate to severe symptoms, heterozygous females are usually mildly affected or even asymptomatic. In 1993 Bergoffen et al. [147] first associated mutations in GJB1, the gene encoding the gap junction protein connexin 32, with CMTX. Since then more than 290 different disease-causing mutations of Cx32 have been described. CMTX patients have nerve conduction deficits [148] due to axonal loss in peripheral nerves, partially failed regeneration, and myelin abnormalities [149–151]. Although mutant proteins are expressed in Schwann cells but not in axons, it is suggested that CMTX develops because these Schwann cell cannot provide metabolic support for normal axonal function.

Figure 1.12. The structure of the human Cx32 protein and some disease-causing mutations. The consequences of the known CMTX mutations are shown schematically, along with the localization of 51 different Cx32 mutants in transfected mammalian cells [152].
Different types of Cx32 mutations may cause CMTX, including missense (amino acid substitutions), nonsense (premature stop), deletions, insertions, and frame-shift mutations (Figure 1.12). Most of these mutations lead to loss of Cx32 function due to absence of entire Cx32 coding region [153], changes in cell-cell channel gating and permeability [154–156] or loss of channel-forming ability [146,152,157].

In the meantime some several CMTX mutations were showed to lead to toxic gain of Cx32 function. For example S85C [158] and F235C [159] mutants of Cx32 form abnormally active functional hemichannels in *Xenopus oocytes* that may lead to loss of metabolites and ions in Schwann cells.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8-35</td>
<td>amphipol A8-35</td>
</tr>
<tr>
<td>AcP</td>
<td>acetyl phosphate, potassium salt</td>
</tr>
<tr>
<td>AIM</td>
<td>auto-induction media</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>BR</td>
<td>bacteriorhodopsin</td>
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<tr>
<td>Brij</td>
<td>nonionic polyoxyethylene surfactant</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
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<td>n-Dodecyl-β-D-Maltopyranoside</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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</tr>
<tr>
<td>MTAB</td>
<td>myristyltrimethylammonium bromide</td>
</tr>
<tr>
<td>NLS</td>
<td>sodium lauroyl sarcosinate</td>
</tr>
<tr>
<td>OG</td>
<td>octyl-β-glucopyranoside</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenol-pyruvic-acid, mono-potassium salt</td>
</tr>
<tr>
<td>RNasin</td>
<td>RNase inhibitor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
</tbody>
</table>
2. Materials and Methods

2.1. Materials

2.1.1. Organisms

Used *E. coli* strains are listed in the Table 2.1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> TOP10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BL21 (DE3)</td>
<td>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamH1o ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td><em>Escherichia coli</em> C43 (DE3)</td>
<td>F - ompT gal dcm hsdSB (rB- mB-) (DE3)</td>
<td>Lucigen</td>
</tr>
<tr>
<td><em>Escherichia coli</em> Lemo21</td>
<td>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS/ pLemo(CamR) λ DE3 = λ sBamH1o ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5 pLemo = pACYC184-PrhaBAD-lysY</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td><em>Escherichia coli</em> SE1</td>
<td>F-, CmR, ompT, lon, hsdSB (restriction-, modification-), gal, dcm, DE3 (lacI, T7polymerase under the control of the PlacUV5 promoter), ccdB+</td>
<td>Delphi genetics</td>
</tr>
</tbody>
</table>

Table 2.1. List of organisms used in this work.

2.1.2. Vectors

Used vectors with main features are listed in Table 2.2. Below, on Figure 2.1, you can find their maps.

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Relevant features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSCodon1.2</td>
<td>T7 promoter, His C-terminal tag, ccdA, Amp resistance, lacI repressor</td>
<td>Delphi genetics</td>
</tr>
<tr>
<td>pivex2.3d</td>
<td>T7 promoter, His C-terminal tag, high copy number, Amp resistance</td>
<td>5 Prime</td>
</tr>
<tr>
<td>pivex2.4d</td>
<td>T7 promoter, His N-terminal tag, high copy number, Amp resistance</td>
<td>5 Prime</td>
</tr>
<tr>
<td>pKM586</td>
<td>tetO promoter, TEV protease S219D mutant, Kn resistance, 10 copies per cell</td>
<td>Addgene</td>
</tr>
<tr>
<td>pRK603</td>
<td>tetO promoter, TEV protease S219D mutant, Kn resistance, 30 copies per cell</td>
<td>Addgene</td>
</tr>
</tbody>
</table>

Table 3.2. List of vectors with main features used in this work.
2.1.3. Oligonucleotides used for Cx32 cloning

Cloning of Cx32 into an expression vectors was based on a site-directed mutagenesis strategy. Human Cx32 gene was amplified from pMA_Cx32 plasmid (obtained from Dr. Carsten Zeilinger) by PCR and cloned into pSCodon1.2 vector containing already MstX gene (Membrane-integrating protein MstX from *Bacillus subtilis*). Modified pSCodon1.2 recipient vectors were earlier produced by Taras Balandin and Maria Silacheva in our laboratory. Optimized Cx32 gene for *E.coli* expression was synthesized by MWG, Ebersberg, Germany.

The following primers were used to generate constructions used in this work:

frwBamHI_NdeIhCx32 5’-ttgatccatatgaactggacagttgtacacctg-3’
2.1.4. Chemicals

Non-organic salts and acids were bought from Sigma-Aldrich, Applichem and Merck. Detergents and lipids – from Sigma-Aldrich, Affimetrix and Avanti Polar Lipids. Components of cell free expression system – from Sigma-Aldrich, Roth and Roche. DNA modifying enzymes – from Thermo. Factor Xa protease – from Qiagen, enterokinase – from New England Biolabs, thrombin – from Sigma-Aldrich.

2.2. Methods

2.2.1. Molecular biology methods

2.2.1.1. Transformation of plasmids into E.coli cells

Competent cells were prepared as described earlier [160]. Briefly TOP10 E.coli cells were grown in 50 ml of LB medium (1% Trypton, 0.5% Yeast extract, 1% NaCl, pH 7.0) in 500 ml flask at 37°C with vigorous shaking to the early exponential phase (OD600 0.3-0.4). Then the cells were pelleted by centrifugation at 1000×g for 10 min at 4°C and resuspended at one-tenth of their original volume in ice-cold TSS solution (LB broth with 10% PEG8000, 5% DMSO, 50mM MgCl2, pH 6.5). 0.1-ml aliquots of the cells were transferred into cold
polypropylene tubes and frozen in liquid nitrogen. The frozen competent cells were stored at -80°C.

For transformation a plasmid solution (2 µl) or ligation mixture (10 µl) were added to 0.1-ml aliquot of competent cells and kept on ice for 1 h. Then the heat pulse was applied at 42°C for 1 min with gentle stirring. After that the cells were incubated on ice for additional 15 min. Next, 0.5 ml of LB broth was added, and the cells were grown at 37°C with shaking (180 rpm) for 1 h to allow expression of the antibiotic-resistance gene. The bacteria were streaked on agar plate containing appropriate antibiotics (1% Trypton, 1% Yeast extract, 0.9% NaCl, 2% glucose, 1.5% agar, pH 7.0 and 50-100 mg/ml appropriate antibiotic). The plate was incubated overnight at 37°C.

2.2.1.2. Plasmid DNA isolation

For plasmid DNA isolation the separate colony was grown overnight in 20 ml of LB medium in 100 ml flask at 37°C with shaking (180 rpm). The bacteria were pelleted by centrifugation at 5000×g for 10 min at 4°C and resuspended in 0.5 ml Buffer A1 from commercial NucleoSpin® Plasmid purification kit (Macherey-Nagel, Dueren, Germany). Next, the bacteria were processed by SDS/alkaline lysis (addition of 0.5 ml Buffer A2). High-salt Buffer A3 (0.5 ml) was added to neutralize the lysate and to create appropriate conditions for DNA binding to the silica membrane. After centrifugation at 10000×g for 10 min at 4°C the clear supernatant was loaded onto a NucleoSpin® Plasmid spin column. Contaminations like salts and macromolecular cellular components were removed by simple washing with 0.6 ml ethanol-containing Buffer A4. After that additional washing with 0.6 ml Buffer AW was done. The plasmid DNA was eluted in 50 µl slightly alkaline Buffer AE (5 mM Tris-HCl, pH 8.5) and stored at -20°C.

2.2.1.3. Plasmid DNA purification for cell free protein synthesis

For in vitro protein expression high quantity of pure plasmid matrix is needed. That is why the special protocol was used. Briefly, the separate colony was grown overnight in 35 ml of LB medium in 500 ml flask at 37°C with shaking (180 rpm). The bacteria were pelleted by centrifugation at 5000×g for 10 min at 4°C and resuspended in 4 ml Buffer P1 from commercial Qiagen® Plasmid Plus Midi Kit (Qiagen, Hilden, Germany). Next, 4 ml of lysis Buffer P2 was added and the mixture was incubated for 3 min at room temperature. High-salt Buffer S3 (4 ml) was added to neutralize the lysate and to create appropriate conditions for
DNA binding to the silica membrane. The mixture again was incubated for 10 min at room temperature and then filtered through QIAfilter Cartridge. 2 ml of Buffer BB was added to the clear lysate. The mixture was loaded onto Qiagen Plasmid Plus spin column. To wash the DNA 0.7 ml Buffer ETR and 0.7 ml Buffer PE were used. The pure plasmid DNA was eluted in 100 µl Buffer EB and stored at -20°C.

2.2.1.4. Amplification of Cx32 gene.

DNA fragments were amplified by PCR. 50 µl of the reaction mixture contained 50 ng of DNA matrix, 150 pmol of each primer, 20 nmol of each dNTP’s, 0.5 U Phusion Hot Start II DNA Polymerase (Thermo, USA) and 10 µl of Phusion HF Buffer or Phusion GC Buffer. In case of whole plasmid PCR, additives were used – 2% DMSO and 75 nmol MgCl₂.

The amplification of DNA in the thermocycler was done in two-step manner following next scheme:

- 2’ at 98°C
- 20” at 98°C
- 30” at primers’ melting temperature minus 3-5°C – repeat 5 times
- 40” at 72°C
- 20” at 98°C
- 30” at primers’ melting temperature – repeat 35-40 times
- 40” at 72°C
- 10’ at 72°C
- Stay at 4°C

In case of whole plasmid PCR elongation time at 72°C was increased to 2 min in each cycle.

2.2.1.5. PCR products analysis and purification

The PCR products were analyzed by horizontal agarose gel-electrophoresis. DNA probes, mixed with DNA loading buffer (Thermo, USA) in 1:5 dilution, were loaded onto 0.7-1.5% agarose gels running in TAE buffer (40 mM Tris-base, 1 mM EDTA, acetic acid till pH reaches 8.0). Staining of DNA bands was done by adding GelRed (Biotium) in 1:10000 dilution to TAE buffer. The DNA fragments were separated by applying 9 V/cm voltage to the gel. The bands of interest were catted out and NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Dueren, Germany) was used for DNA extraction. Briefly, the agarose gel slice was dissolved in high-salt Buffer NT and applied to a NucleoSpin® Gel and PCR Clean-
up Column followed by centrifugation and a subsequent washing step with ethanol-containing Buffer NT3. The pure DNA was eluted under low ionic strength conditions with slightly alkaline Buffer NE (5 mM Tris-HCl, pH 8.5).

2.2.1.6. DNA restriction and plasmids linearization

PCR products and plasmids were cut with corresponding restriction enzymes by 1 h incubation at 37°C. Usually FastDigest® restriction enzymes from Thermo were used together with FastDigest® buffer. Restriction enzymes were inactivated by 20 min incubation at 60-85°C.

Plasmids, used further for ligation, were incubated with 1 U Thermosensitive Alkaline Phosphatase (Thermo, USA) for 30 min at 37°C after restriction enzymes inactivation.

2.2.1.7. Ligation of DNA

For ligation of the DNA fragments T4 DNA Ligase (Thermo, USA) was used. 100 ng of recipient vector DNA were mixed with excess amount of insert DNA (5-10 times excess) and 1 U of T4 DNA Ligase in ligase buffer. The ligation mixture was incubated overnight at 20°C and then used for transformation or stored at -20°C.

2.2.2. Cell free (CECF) expression of Cx32

2.2.2.1. E.coli S30 extract preparation

_E.coli_ S30 extract was prepared by Vitaly Shevchenko in our laboratory by the procedure described earlier [161,162]. Briefly, fresh overnight culture from _E.coli_ strain BL21 Star in TB medium (1.2% Trypton, 2.4% Yeast extract, 0.5% Glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄) was used to inoculate 10 L of TB medium in a fermenter at a ration 1:100. The cells were incubated at 37°C with vigorous stirring and good aeration till they reached the mid-log phase corresponding to an OD₆₀₀ of 3.5. Fermenter heating was switched off and the broth was chilled down to 6°C as quickly as possible (around 30 min). Final OD₆₀₀ of the broth after cooling was around 4.5. The cells were harvested by centrifugation at 4°C for 10 min at 5000×g in precooled beakers. The pellet was resuspended in 100 ml of S30-A buffer (10 mM Tris-acetate, 14 mM Mg(OAc)₂, 6 mM β-mercaptoethanol, 0.6 mM KCl, pH 8.2) precooled to 4°C. The suspension was centrifuged at 8000×g at 4°C for 10 min. Washing step was repeated two more times. After that the pellet of wet bacterial cells was resuspended in equal volume (for 65g cells 65 ml of buffer were used)
of S30-B buffer (10 mM Tris-acetate, 14 mM Mg(OAc)$_2$, 0.6 mM KCl, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, pH 8.2) precooled to 4°C. The cells were disrupted by passing through the microfluidizer (M110-P, Microfluidics, Newton, USA) precooled at 4°C with one pass. The cell debris was pelleted by centrifugation at 30000×g at 4°C for 30 min. Upper two-third of the supernatant was transferred in a clean tube and centrifugation step was repeated. Again upper two-third of the supernatant was removed, NaCl was added till final concentration of 400 mM and the solution was incubated at 42°C for 45 min in a water bath. Next the solution was dialyzed (dialysis membrane cut-off 14 kDa) at 4°C against 60 vol of S30-C buffer (10 mM Tris-acetate, 14 mM Mg(OAc)$_2$, 0.5 mM DTT, 0.6 mM KOAc, pH 8.2). After 2 h the dialysis buffer was exchanged and dialysis was continued overnight. After that the extract was centrifuged at 30000×g at 4°C for 30 min. The clear supernatant was removed; 0.5-ml aliquots were transferred into cold polypropylene tubes and frozen in liquid nitrogen. The frozen extract was stored at -80°C.

2.2.2.2. T7 RNA polymerase expression and purification

Since T7 RNA polymerase is the most expensive component of cell protein synthesis reaction and has to be applied at relatively high amounts it was produced in our laboratory by the simplified protocol described earlier [161]. Briefly, 4 L of LB medium with 100 mg/l ampicillin was inoculated with a fresh overnight LB culture of BL21 Star containing pAR1219 plasmid at a ratio 1:100. The bacteria were cultured in 2 L flasks at 37°C with vigorous shaking (120-150 rpm) until OD$_{600}$ of 0.6 was obtained. Then T7 RNA polymerase expression was induced with IPTG of 1 mM final concentration and the bacteria were further incubated at 37°C for 5 h. The cells were harvested by centrifugation at 5000×g for 20 min at 4°C and resuspended in 120 ml of T7 buffer (30 mM Tris, 10 mM EDTA, 50 mM NaCl, 5% Glycerol, 10 mM of β-mercaptoethanol, pH 8.0). The cells were disrupted by passing through the microfluidizer precooled at 4°C with one pass. The cell debris was pelleted by centrifugation at 20000×g for 30 min at 4°C. The supernatant was adjusted to a final concentration of 2% streptomycin sulfate by gentle stirring and dropwise addition of a 10% streptomycin sulfate stock solution. Precipitated DNA was removed by centrifugation at 30000×g for 30 min at 4°C.

T7 RNA polymerase was further purified by anion exchange chromatography. Q-Sepharose column (around 20 ml of resin) was equilibrated with T7 buffer. The supernatant was loaded with a flow rate of 1 ml/min. The polymerase was eluted by salt gradient from 50 mM NaCl to 500 mM NaCl in 300 ml in T7 buffer at a flow rate 3 ml/min. Fractions were
analyzed by SDS-PAGE gel electrophoresis. Fractions containing polymerase were further
dialyzed against 100 volumes excess of buffer: 10 mM Tris, 1 mM EDTA, 10 mM NaCl, 1 mM DTT, pH 8.0. T7 RNA polymerase was stored in the same buffer with addition of 50% Glycerol at -20°C.

2.2.2.3. Pipetting scheme for “analytical” and “preparative” scale CF

The pipetting scheme was prepared for each experiment according to Table 2.3. All stock solutions were prepared as described previously [161]. For Mg\(^{2+}\) optimization screens the scheme from Table 2.4 was used.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Analytical scale</th>
<th>Preparative scale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stock</td>
<td>Final MM (µl)</td>
<td>FM (µl) RM (µl)</td>
</tr>
<tr>
<td>NaN(_3) (%)</td>
<td>10</td>
<td>0.05 32</td>
<td></td>
</tr>
<tr>
<td>PEG8000 (%)</td>
<td>40</td>
<td>2 323</td>
<td></td>
</tr>
<tr>
<td>KOAc (mM)</td>
<td>4000</td>
<td>150.8 243</td>
<td></td>
</tr>
<tr>
<td>Mg(Oac)(_2) (mM)</td>
<td>1000</td>
<td>7.1 46</td>
<td></td>
</tr>
<tr>
<td>HEPES buffer (M)</td>
<td>2.5</td>
<td>0.1 237</td>
<td></td>
</tr>
<tr>
<td>Complete (%)</td>
<td>50</td>
<td>1 129</td>
<td></td>
</tr>
<tr>
<td>Folinic acid (mg/ml)</td>
<td>10</td>
<td>0.1 65</td>
<td></td>
</tr>
<tr>
<td>DTT (mM)</td>
<td>500</td>
<td>2 26</td>
<td></td>
</tr>
<tr>
<td>NTP mix (×)</td>
<td>75</td>
<td>1 86</td>
<td></td>
</tr>
<tr>
<td>PEP (mM)</td>
<td>1000</td>
<td>20 129</td>
<td></td>
</tr>
<tr>
<td>AcP (mM)</td>
<td>1000</td>
<td>20 129</td>
<td></td>
</tr>
<tr>
<td>Amino-acid mix (mM)</td>
<td>4</td>
<td>0.5 806 750</td>
<td>2250 2125</td>
</tr>
<tr>
<td>RCDWMDE (mM)</td>
<td>16.7</td>
<td>1 386</td>
<td></td>
</tr>
<tr>
<td>Detergent (%)</td>
<td>10</td>
<td>1 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Σ 2637</td>
<td></td>
<td>Σ 9211</td>
</tr>
<tr>
<td>Mastermix</td>
<td></td>
<td>2452 184</td>
<td>8699 512</td>
</tr>
<tr>
<td>S30 buffer (%)</td>
<td>100</td>
<td>35 2100</td>
<td>5950</td>
</tr>
<tr>
<td>Pyruvat kinase (mg/ml)</td>
<td>10</td>
<td>0.04 2</td>
<td></td>
</tr>
<tr>
<td>TRNA (mg/ml)</td>
<td>40</td>
<td>0.5 6</td>
<td></td>
</tr>
<tr>
<td>T7RNAP (U/ml)</td>
<td>420</td>
<td>6 6</td>
<td></td>
</tr>
<tr>
<td>RNasin (U/ml)</td>
<td>40</td>
<td>0.3 4</td>
<td></td>
</tr>
<tr>
<td>DNA (µg/ml)</td>
<td>200</td>
<td>0.015 34</td>
<td></td>
</tr>
<tr>
<td>S30 extract (%)</td>
<td>100</td>
<td>35 158</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>226 24</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>2637 5302 394 9211 17000 1000</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.3.** General pipetting protocol for CECF reactions.
<table>
<thead>
<tr>
<th>Number of reaction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Mg(^{2+}) concentration (mM)</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Preparation of FM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\mu)l of FM</td>
<td>883.7</td>
<td>883.7</td>
<td>883.7</td>
<td>883.7</td>
<td>883.7</td>
<td>883.7</td>
</tr>
<tr>
<td>(\mu)l of 100 mM Mg(OAc)(_2)</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>(\mu)l of water</td>
<td>116.3</td>
<td>106.3</td>
<td>96.3</td>
<td>86.3</td>
<td>76.3</td>
<td>66.3</td>
</tr>
<tr>
<td>Preparation of RM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\mu)l of RM</td>
<td>65.7</td>
<td>65.7</td>
<td>65.7</td>
<td>65.7</td>
<td>65.7</td>
<td>65.7</td>
</tr>
<tr>
<td>(\mu)l of 100 mM Mg(OAc)(_2)</td>
<td>0</td>
<td>0.75</td>
<td>1.5</td>
<td>2.25</td>
<td>3</td>
<td>3.75</td>
</tr>
<tr>
<td>(\mu)l of water</td>
<td>9.3</td>
<td>8.55</td>
<td>7.8</td>
<td>7.05</td>
<td>6.3</td>
<td>5.55</td>
</tr>
</tbody>
</table>

Table 2.4. Example of Mg\(^{2+}\) optimization screen.

### 2.2.2.4. Reaction setup

“Analytical scale” reactions were carried out in containers showed on **Figure 2.2a** at 32°C overnight with gentle shaking (120 rpm) with dialysis membrane cut-off 14 kDa. For “preparative scale” reactions slide-A-lyzer setup (with the same membrane cut-off) was used (**Figure 2.2b**) and the mixture was incubated at 32°C overnight.

![Figure 2.2. Design of CECF expression reaction containers. (a): Plexiglas reaction chamber for “analytical scale” reactions, and (b): slide-A-lyzer setup for “preparative scale” reactions. RM compartments are separated from FM compartments by dialysis membranes. Filling openings for RM and FM are sealed by appropriate lids and parafilm to prevent evaporation.](image)

#### 2.2.3. *E.coli* culture and Cx32 overexpression

Preliminary *E.coli* overexpression was done following standard expression protocol in LB broth. Briefly, 6 L of LB medium with 100 mg/l ampicillin was inoculated with a fresh overnight LB culture of appropriate bacteria strain with plasmid encoding Cx32 at starting OD\(_{600}\) around 0.2. Bacteria were cultured in 2 L flasks (500 ml of media per 1 flask) at 37°C with vigorous shaking (120 rpm) until OD\(_{600}\) of 0.6-0.8 was obtained. Then, connexin expression was induced with IPTG of 1 mM final concentration and bacteria were further incubated at 37°C for 5 h. The cells were harvested by centrifugation at 5000×g for 30 min at 4°C. After that they were either resuspended in appropriate buffer for further total membrane isolation or frozen in liquid nitrogen and stored at -80°C.
In next experiments auto-induction media (AIM) was used for connexin expression [163]. One colony of freshly transformed bacteria was grown overnight at 37°C in 20 ml of LB broth with 1% glucose and appropriate antibiotic in 100 ml flask. Next morning 0.5 L of AIM without 5052 but with 1% glucose and antibiotic was inoculated with this overnight culture and was grown at 37°C for approximately 5 h in 2 L flask. 6 L of AIM with appropriate antibiotic were inoculated with starting OD$_{600}$ around 0.2. The bacteria were cultured in 2 L flasks at 37°C with vigorous shaking (120 rpm). Glucose level in media was controlled by commercial glucose tests (Merckoquant®). When glucose was depleted, culture was either cooled to 20°C for overnight incubation or left at 37°C for 5-7 h. The cells were harvested by centrifugation at 5000×g for 30 min at 4°C and then either immediately resuspended for further protein purification or frozen in liquid nitrogen and stored at -80°C.

For “analytical scale” expression same procedure was used. Briefly, single colony was cultured in 20 ml of AIM with antibiotic at 37°C overnight. Next morning the cells were pelleted down by centrifugation, resuspended in appropriate buffer and further used for expression level analysis.

For 1 L of AIM were mixed 930 ml of ZY broth (1% Trypton, 0.5% Yeast extract), 2 ml of 1 M MgSO$_4$ stock, 20 ml of 50×5052 stock (25% glycerol, 2.5% glucose, 10% α-lactose), 50 ml of 20×NPS stock (0.5 M (NH$_4$)$_2$SO$_4$, 1 M KH$_2$PO$_4$, 1 M Na$_2$HPO$_4$). ZY broth and NPS stock were autoclaved; MgSO$_4$ and 5052 stocks were sterile filtered.

2.2.4. Biochemical methods

2.2.4.1. Gel electrophoresis – denaturing SDS-PAGE

The separation of denaturated proteins with sodium dodecyl sulfate (SDS) was performed using linear gradient gels. These gels have two considerable advantages over uniform concentration gels: they fractionate proteins over a wider range of molecular weights than any uniform concentration gel; the gradient in pore size causes significant sharpening of protein bands during migration [164]. The solutions used for gel preparation are given in Table 2.5.

The separation gel was casted by mixing “heavy” and “light” gels using the gradient mixer (Bio-Rad, Germany) as described previously [164]. A thin layer of isopropanol was overlaid, to fasten the polymerization by avoiding contact with oxygen. The separation gel polymerized overnight in the electrophoresis apparatus. Next morning after rinsing with deionized water, the stacking gel was poured and polymerized for 3 h.
Table 2.5. Resolving gel mixture for gradient gels.

* mixture of 30% Acrylamide and 0.8% Bisacrylamide

<table>
<thead>
<tr>
<th>Compound</th>
<th>16.2% “heavy” gel, ml</th>
<th>7.8% “light” gel, ml</th>
<th>4% stacking gel, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>6 g</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA/BAA* 30%/0.8%</td>
<td>16</td>
<td>6.5</td>
<td>3</td>
</tr>
<tr>
<td>Water</td>
<td>4</td>
<td>15</td>
<td>14.5</td>
</tr>
<tr>
<td>3 M Tris pH 8.8</td>
<td>3.125</td>
<td>3.125</td>
<td>-</td>
</tr>
<tr>
<td>1 M Tris pH 6.8</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>0.250</td>
<td>0.250</td>
<td>0.200</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>TEMED 100%</td>
<td>4 µl</td>
<td>8 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>APS 20%</td>
<td>8 µl</td>
<td>16 µl</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

For protein samples preparation the loading buffer was added (25 mM Tris-HCl 50% Glycerol, 500 mM DTT, 10% SDS, 0.1% bromophenol blue, pH 6.8) in sample:buffer ration 5:1. Mixture was incubated for 15 min at room temperature and loaded onto acrylamide gel.

The separation was carried out by applying an external voltage of 150 V for 1.5 h. Anode and cathode buffer: 25 mM Tris-base, 200 mM Glycine, 0.1% SDS.

2.2.4.2. TCA protein precipitation

Protein precipitation by TCA is widely used to concentrate samples for gel analysis in a polyacrylamide gel and to remove detergents, which can have negative influence on protein’s migration in the SDS PAGE [165]. Brief procedure: to the protein sample 100% trichloroacetic acid (TCA) was added in ratio 1:10 (protein solution:TCA), to get a 10% final concentration. The mixture was incubated for 15 min at –20°C or 30 min on ice. Then the sample was centrifuged for 15 min at 16000×g and 4°C. The supernatant was carefully discarded; the pellet was retained and resuspended in 50-100 µl 0.1 M NaOH. The gel-loading buffer was added (as described before) and sample was further analyzed by acrylamide gel.

2.2.4.3. Staining of proteins on gels

The protein bands were visualized by staining the gel with Coomassie Brilliant Blue staining solution (0.12% Coomassie Brilliant Blue G250, 10% H₃PO₄, 10% (NH₄)₂SO₄, 20% methanol) for 2 h. For destaining 3% acetic acid solution was used.
2.2.4.4. Western-Blot analysis

Proteins were transferred from gel onto PVDF membrane (Macherey-Nagel, Dueren, Germany) in the blotting tank apparatus (Bio-Rad, Germany) for 1h at 100 V. The transfer buffer (48 mM Tris-base, 38.6 mM Glycine, 0.04% SDS, 20% Methanol) was used to pretreat the membrane, the gel and the filter paper. After transfer, the membrane was briefly rinsed with TBST buffer (20 mM Tris-HCl, 100 mM NaCl, 5% Glycerol, 0.05% Tween20, 0.02% NaN₃, pH 8.0), and afterwards incubated in TBST-milk buffer (20 mM Tris-HCl, 100 mM NaCl, 5% Glycerol, 0.05% Tween20, 0.02% NaN₃, 5% dry milk, pH 8.0) overnight. Next morning it was incubated for 1.5 h with the primary antibodies (connexin 32 mouse monoclonal IgG, Santa Cruz, Dallas, USA), diluted (1:2000) in 8 ml TBST-milk buffer. After two 5 min washes with the same buffer the membrane was incubated with the secondary antibodies (goat anti-mouse IgG-AP, Santa Cruz, Dallas, USA) again in 8 ml TBST-milk buffer (with 1:2000 dilution). Finally, the membrane was washed two times with TBST buffer. Substrate NBT/BCIP solution (Thermo, USA) was used to elicit the signal by chemiluminescence, yielding an intense, insoluble black-purple precipitate when reacted with alkaline phosphatases.

2.2.5. Protein purification

2.2.5.1. Isolation of E.coli total membranes

Cell pellets were resuspended in ice-cold homogenization buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.5) in ratio 1:5 (1 g of cells per 5 ml of buffer). Deoxyribonuclease I from bovine pancreas (Sigma-Aldrich, Germany) was added to remove DNA from the cell suspension after lysis (5 mg of DNAse per 30 g of cells). The cells were disrupted by passing through the microfluidizer (M110-P, Microfluidics, Newton, USA) precooled at 4°C with three passes. The suspension was loaded onto glycerol gradient 30-100% and centrifuged for 3 h at 4°C in the swinging bucket rotor (SW-32Ti, Beckmann, USA). For gradient preparation glycerol dilutions (0.5 ml 100%, 0.5 ml 90%, 1 ml 80%, 1 ml 60%, 5 ml 30%) in homogenization buffer starting from the heaviest were pipetted into thick walled centrifugation tubes. After each dilution tubes were placed at -80°C for 10 min until the layer is either very viscous or frozen. With this procedure a very sharp gradient was created. On top of the last frozen layer 20 ml of the sample was pipetted.
After centrifugation fractions containing 60-100% glycerol were collected and dialyzed (dialysis membrane cut-off 14 kDa) against 1 l of ice-cold homogenization buffer overnight.

Next morning the dialysis was terminated, 1% of Triton X100 was added to the suspension in order to wash the membranes. The mixture was incubated for 1 h at 4°C under gentle rotation. Then the membranes were sedimented by centrifugation at 120000×g for 1 h at 4°C. The supernatant was discarded; the pellet was resuspended in 150 ml of ice-cold solubilization buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.5).

2.2.5.2. Protein solubilization

For solubilization screening the suspension of crude total membranes in solubilization buffer was incubated overnight at 4°C under gentle rotation within 1% of different detergents (DM, DDM, OG, FOS10, FOS12, FOS14, Brij35, NLS). Then the suspension was centrifuged at 120000×g for 1 h at 4°C to remove insoluble material. The pellets and supernatants were further analyzed by SDS PAGE gel electrophoresis.

For “preparative scale” purification combination of DM and NLS was used. First 1% of DM was added to the membrane suspension. The mixture was incubated at 4°C for 1 h with moderate stirring. Next 1% of NLS was added and the suspension was further incubated on stirrer overnight. Insoluble proteins and lipids were removed by centrifugation with the same parameters. The supernatant was further used for connexin 32 purification.

In later experiments solubilization of Cx32 was carried out by adding 2% of NLS to the membrane suspension. The rest of the procedure was unchanged.

2.2.5.3. Affinity chromatography

Ni²⁺-NTA beads (Qiagen, Germany) were first washed with 5 column volumes (CV) of deionized water and then pre-equilibrated with the same volume of solubilization buffer containing 1×CMC of different detergents.

In case of “analytical scale” purification binding of the solubilized material was done using gravity flow columns. 600 µl of the supernatant after the last centrifugation was loaded onto 200 µl of prepared Ni²⁺-NTA beads. After washing the resin with 1 ml of equilibration buffer bound proteins were eluted with 600 µl of elution buffer (20 mM Tris-HCl, 100 mM NaCl, 50 mM EDTA, 1×CMC of different detergents). All manipulations were done at room temperature.
Given “preparative scale” purification the supernatant containing solubilized proteins was first diluted with solubilization buffer to reduce NLS concentration to 0.5% and then incubated with pre-equilibrated Ni\(^{2+}\)-NTA beads (with solubilization buffer containing 0.5% NLS) at 4°C for 5 h under gentle rotation. Usually for 1 g of initial bacterial cells 0.2 g of dry Ni\(^{2+}\)-NTA resin was taken. Then resin was loaded onto chromatographic column (GE Healthcare, USA) and further washed with 5×CV of washing buffer (20 mM Tris-HCl, 100 mM NaCl, 30 mM Imidazole, 0.5% NLS, pH 7.5) using AKTA purifier chromatographic system (GE Healthcare, USA). Next washing step was carried out to exchange NLS to less “harsh” detergent. For this purpose the column was washed additionally with 5×CV of washing buffer, containing 0.2% DDM or 0.2% SDS. Finally, bound proteins were eluted in 2×CV of elution buffer (20 mM Tris-HCl, 100 mM NaCl, 400 mM Imidazole, pH 7.5) with appropriate detergent. If SDS was used in washing and elution buffer the purification on chromatographic system was done at room temperature, in other case – at 4°C.

Different chromatography parameters were checked for optimization of the purification: the beads volume, the concentration of detergents, the concentration of imidazole at washing stage and the effect of urea. All these trials were done using the “analytical scale” purification protocol.

### 2.2.5.4. Anion-exchange chromatography

MonoS\(^{\text{TM}}\) and MonoQ\(^{\text{TM}}\) ion-exchange columns (GE Healthcare, USA) were used to separate connexin 32 with cleaved and not MstX in case of in vivo cleavage with TEV protease.

Due to high cationic strength of MonoS\(^{\text{TM}}\) resin, the eluate from Ni\(^{2+}\)-NTA in 0.2% Brij35 was dialyzed (membrane cut-off 14 kDa) overnight against 100-fold excess of column equilibration buffer (20 mM Tris-HCl, 0.2% Brij35, pH 6.0) at 4°C. Next morning dialysis was terminated and the protein solution was centrifuged at 20000×g for 20 min at 4°C to remove aggregates. The clear supernatant was loaded with a flow rate of 0.1 ml/min onto pre-equilibrated with 5×CV of equilibration buffer MonoS\(^{\text{TM}}\) column. Protein was eluted by salt gradient (in equilibration buffer) up to 1 M NaCl in 20×CV at the same flow rate. Fractions were collected and further analyzed by SDS-PAGE and Western-blot.

In case of MonoQ\(^{\text{TM}}\) column the same protocol was used with deviations only in the equilibration buffer (20 mM Tris-HCl, 0.2% Brij35, pH 8.0).

In several experiments 6 M Urea was added to the equilibration buffers to improve the separation.
2.2.5.5. Size-exclusion chromatography (gel-filtration)

To control hemichannel formation size-exclusion chromatography was used. Eluates from metal-affinity chromatography were dialyzed (membrane cut-off 14 kDa) overnight against 100-fold excess of elution buffer without imidazole (20 mM Tris, 100 mM NaCl, 0.2% DDM or 0.1% SDS, pH 7.5). Next, the proteins suspension was concentrated using centrifugal filter (Millipore, USA) with membrane cut-off 30 kDa to 0.5 ml. Aggregates were removed by centrifugation at 20000×g for 20 min. Supernatant was either loaded onto Superose® 6HR 24 ml column (GE Healthcare) pre-equilibrated with 30 ml of suitable buffer (20 mM Tris-HCl, 100 mM NaCl and 5×CMC of appropriate detergent, pH 7.5) or used for refolding experiments and only then analyzed by gel-filtration. The column flow rate was adjusted to 0.1 ml/min. The fractions corresponding to peaks on the chromatogram were pooled and further analyzed by SDS-PAGE gel electrophoresis. The column calibration curves are shown on Figure 2.3.

![Superose 6HR 24 ml calibration](image)

Figure 2.3. Calibration curves for Superose 6HR 24 ml column. Globular proteins were used as standards.

2.2.6. Refolding of denatured protein

2.2.6.1. Refolding by dialysis

Amphipol (A8-35) mediated refolding of Cx32 solubilized in SDS was carried out as described previously [166]. Briefly, to Cx32 after elution from Ni²⁺-NTA column and dialysis against buffer without imidazole but with 0.1% SDS amphipol was added at the ratio 10 g of A8-35 per 1 g of SDS-unfolded connexin. The mixture was incubated for 30 minutes at room temperature and then was loaded into slide-A-lyzer (with membrane cut-off 20 kDa) and dialyzed against 100-fold excess of refolding buffer (20 mM Tris-HCl, 100 mM NaCl, 0.2% DDM or 0.1% SDS, pH 7.5).
1 mM KCl, pH 7.5) at 4°C overnight. Aggregates and precipitated SDS were removed by centrifugation at 20000×g for 20 min at 4°C. Further this mixture was concentrated and analyzed by size-exclusion chromatography.

2.2.6.2. SDS precipitation by KCl

In this case refolding of Cx32 form SDS to amphipol was done by stepwise addition of 1 M KCl stock solution to protein suspension. The same procedure as described above was used. Only instead of dialysis after short incubation at room temperature sample was moved on ice and KCl was added by small portions to final concentration equal 100 mM. Precipitated SDS as its potassium salt was removed by centrifugation at 20000×g for 20 min at 4°C.

2.2.7. Characterization

2.2.7.1. MALDI-TOF analysis

For mass spectrometric analysis of Cx32 by MALDI-TOF pure protein solution in DDM obtained by size-exclusion chromatography was used. Measurements were carried out at the mass spectrometry analysis service of Institute of Structural Biology, Grenoble, France.

2.2.7.2. Dynamic light scattering to study samples monodispersity

The measurements were carried out using Dyna Pro-E-20-660 devise (Proterion corp., USA) in quartz cuvettes with 57 µl volume (Helma, z=8.5 mm, path length=3 mm).

2.2.7.3. Electron microscopy analysis of solubilized and reconstituted into liposomes Cx32

Samples of Cx32 in different detergents or reconstituted into liposomes were adsorbed to the clean side of a carbon film formed on mica (the carbon-mica interface) and negatively stained using 1% uranyl acetate. Photographs were taken under low-dose conditions with a JEOL 1200 EXII electron microscope operating at 100 kV at a nominal magnification of 40000×. All measurements were carried out in the electron microscopy laboratory of Institute of Structural Biology, Grenoble, France.
2.2.8. Crystallization of Cx32 in meso

Fractions corresponding by size to hemichannel after size-exclusion chromatography of Cx32 refolded into amphipol were concentrated to final protein concentration 20-30 mg/ml using centrifugal filter with membrane cut-off 30 kDa. Protein concentration was controlled by NanoDrop spectrophotometer (Thermo, USA).

Detergents (OG, DDM, FOS12) were added to the concentrated protein solution till the aimed concentration is achieved. Detergent concentration was checked by IR spectroscopy. The protein solution was added to monooleoyl in a ratio 1:1 (vol:vol) and several passes through interconnected syringes for cubic phase preparation were performed. Cubic phase was ready for usage after 1-2 days.

Crystallization was carried out using robot for in meso membrane protein crystallization (NT 8 Crystallography, Formulatrix, USA). Crystallization probes were visualized and systematically scanned by automated system for imaging crystallization probes (Rock Imager 1000, Formulatrix, USA). All crystallization experiments were done in the membrane protein crystallization platform of Institute of Structural Biology, Grenoble, France.
3. Results and discussion

3.1. Expression and purification of human connexin 32 in *E. coli* using membrane-targeting expression tag (MstX from *Bacillus subtilis*)

For structural studies of integral membrane proteins (IMPs) the production of large amounts of properly folded protein plays crucial role. Highly hydrophobic membrane proteins require auxiliary proteins to prevent their aggregation during the protein folding process. It is thus likely that production of eukaryotic IMPs in bacteria is additionally limited due to the complexity involved in the biogenesis of IMPs, coordinated by the ribosome and the SecYEG translocon assisted by chaperones such as SecB and the ATPase motor SecA, some of which may be needed to be co-overexpressed and/or impose additional specificity [167].

Mistic is a membrane-associated protein originally discovered in *Bacillus subtilis*. It was shown to enhance expression levels of a large number of foreign IMPs at the membrane of *E. coli*, when used as a fusion partner linked to the N-termini of cargo proteins [168–171]. It was showed that Mistic lacks identified signal sequence: no any LSS was identified. Also its overexpression, whether alone or in fusion to other IMPs, apparently prevents the toxicity issues associated with overloading the protein translation machinery [168].

Taking into account previous unsuccessful attempts to overexpress full-length connexins incorporated into cell membranes in *E. coli* using water-soluble fusion partners (thioredoxin [17] and glutathione S-transferase [13]) we decided to fuse Mistic to N-terminus of our target human connexin 32 to enhance membrane-targeted overexpression. Construction without any fusion tag was also generated and used as control of expression yield.

3.1.1. Assembling and expression of human connexin 32 gene in fusion with Mistic and enterokinase cleavage site in between

3.1.1.1. Cloning strategy

Human Cx32 gene was amplified from pMA_Cx32 plasmid (kindly provided by Dr. Carsten Zeilinger) by PCR using two primers - frwBamHEKCx32 and revXhoIhCx32. PCR product was digested with *BamHI* and *XhoI* DNA restriction enzymes and cloned into pSCodon1.2 vector containing already MstX gene followed by enterokinase (EK) cleavage site (the vector has been produced earlier by Taras Balandin in our laboratory). Final construct is illustrated on Figure 3.1 and have molecular weight 45.9 kDa. This construct was designated as H6MstXEKhCx32. Nucleic acid and amino acid sequences of human Cx32 and
Mistic can be found in appendix. E.coli strains (SE1, C43, Lemo21) were transformed with the plasmid and used for expression.

### Figure 3.1
Basic features of the generated construct of human connexin 32 gene in fusion with MstX and enterokinase cleavage site in between.

#### 3.1.1.2. Expression and purification

**Test expression in SE1 E.coli strain in auto induction media (AIM)**

One colony of freshly transformed bacteria (SE1 strain) with a new plasmid construct was cultured for 16 h at 37°C in 25 ml of AIM with 100 mg/L ampicillin. As expression rate control empty pSCodon1.2 vector was used. It was also transformed into SE1 cells.

Next morning OD$_{600}$ was measured. Normalized volumes of two cultures (2 OD$_{600}$×ml) were transferred to tubes and cells were pelleted down by centrifugation. Each pellet was resuspended in 200 µl of 1× gel-loading buffer and analyzed by SDS-PAGE and Western-blot. Results are illustrated on **Figure 3.2**. This indicates that new construct of Cx32 is successfully overexpressed and different oligomeric states are observed. Oligomerization may be caused not only by connexin 32, but also MstX have strong tendency to form dimers and trimmers [172].

#### Figure 3.2
Western-blot of total lysates of cells with new Cx32 construct and with empty pSCodon1.2 vector using Anti-Connexin32 mouse antibody, anti-mouse lgG alkaline phosphatase conjugate and BCIP/NBT enzyme substrate. 5 µl, 10 µl, and 20 µl of samples were loaded. M: size marker. Different bands correspond to different oligomeric states of expressed H$_6$MstXEKhCx32. With arrow monomer band is indicated.

Monomer band, depicted on this picture with arrow, migrate in the gel slightly faster than it should. Such behavior was previously reported for connexins and may indicate that SDS in loading buffer does not fully unfold connexins. Moreover, variety of bands on immunoblot cannot be explained only by oligomerization. This leads to conclusion that
connexins are associated with other proteins in *E.coli* and migrate together in acrylamide gel. Transfer efficiency and primary antibody binding of different oligomers varies significantly which makes total Cx32 yield calculations very complicated. For this further protein purification is essential.

Same procedure was used to examine protein expression rate over time. Each hour culture OD$_{600}$ and glucose concentration were measured. From normalized volumes of cell suspension samples for SDS-PAGE were prepared. Resulting Western-blot is shown on Figure 3.3. This picture illustrates that induction with lactose successively starts after bacteria have consumed all the primary hydrocarbons. It also shows that 16 hours incubation period is sufficient for cells to produce significant amount of Cx32. Longer incubation period leads to proteolysis (data not shown).

<table>
<thead>
<tr>
<th>h</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>16</th>
<th>M (kDa)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>170</td>
<td>130</td>
<td>100</td>
<td>70</td>
<td>55</td>
<td>40</td>
</tr>
</tbody>
</table>

**Figure 3.3.** Western-blot analysis of total lysates of cells with Cx32 construct collected on different time starting from inoculation. M: size marker. Moment when zero glucose concentration has been observed is depicted with arrow.

**Choice of best suitable *E.coli* strain for overexpression**

To find optimal *E.coli* strain for our construct expression we compared protein yields in three strains – C43, Lemo21 and SE1. C43 and Lemo21 were proposed suitable for toxic proteins or/and membrane proteins overexpression while SE1 possess toxin/antidote system which leads to better plasmid stabilization.

pSCodon1.2 containing H$_2$MstXENhCx32 was transformed into these three strains. Expression was carried out in AIM with 100 mg/l ampicillin for 16 h at 37°C. Total cell lysates were loaded onto glycerol gradients 30-100% and fractionated after centrifugation. Fractions corresponding to 80-100% glycerol were diluted to reduce glycerol concentration to 5% and applied to glycerol gradients second time. Centrifugal tubes after both centrifugations are illustrated on Figure 3.4. This picture indicates that in case of SE1 strain membrane fraction is larger, which may lead to better protein yield.
After first centrifugation fractions corresponding to 80-100% glycerol were diluted to final glycerol concentration 5% and loaded onto gradient second time.

To examine this speculation we checked all fractions with SDS-PAGE and Western-blotting Figure 3.5. This analysis confirmed that in case of SE1 strain Cx32 yield was greater and more protein was incorporated into cell membranes or expressed as inclusion bodies in comparison with other two strains.

<table>
<thead>
<tr>
<th>Glycerol Concentration</th>
<th>SE1</th>
<th>Lemo21</th>
<th>C43</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 5%</td>
<td>L</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2 – 30%</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3 – 40%</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4 – 60%</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5 – 80%</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6 – 100%</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

**Figure 3.4.** Photograph of centrifugal tubes with glycerol gradients after first (A) and second (B) centrifugations.

**Figure 3.5.** Western-blot analysis of fractions with different glycerol concentrations after two centrifugations for three *E. coli* strains – SE1, Lemo21 and C43. M: size marker. L: total lysate. Numbers correspond to glycerol concentrations: 1 – 5%, 2 – 30%, 3 – 40%, 4 – 60%, 5 – 80%, and 6 – 100%.

**Detergents screen for Cx32 solubilization**

To study the solubility of the expressed Cx32 in different detergents and to choose the best suitable one for further purification we performed solubilization screen in 7 detergents – Fos10, Fos12, Fos14, DM, DDM, OG, Brij35. Membranes in fraction with 80-100% glycerol after second centrifugation were used. First they were dialyzed overnight at 4°C to remove
glycerol and then mixed with detergents to final detergent concentrations 1%. This mixture was kept overnight at 4°C on magnetic stirrer. Next morning samples were centrifuged and analyzed. Results are shown on Figure 3.6. Fos-cholines were more efficient and less Cx32 was observed in pellets when they were used for solubilization. Inside this family of detergents other tendency may be indicated – longer detergent hydrophobic tail helps to increase solubilization rate.

<table>
<thead>
<tr>
<th>Fos10</th>
<th>Fos12</th>
<th>Fos14</th>
<th>DM</th>
<th>DDM</th>
<th>OG</th>
<th>Brij35</th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td>p</td>
<td>l</td>
<td>M</td>
<td>s</td>
<td>p</td>
<td>l</td>
</tr>
</tbody>
</table>

Figure 3.6. Western-blot analysis of different detergents efficiency for Cx32 solubilization. M: size marker; l: mixture of membranes with detergent before centrifugation; s: supernatant after centrifugation; p: pellet resuspended in corresponding volume of 1× gel-loading buffer; t: membranes suspension before addition of detergent.

**Purification of solubilized in different detergents Cx32 using Ni²⁺-NTA agarose**

Supernatants after solubilization test were further purified using Ni²⁺-NTA agarose and following “analytical scale” purification protocol. Resulting eluates were analyzed by SDS-PAGE and Western-blot (Figure 3.7). Same experiment was carried out with various urea concentrations (0.5–6 M) and various pH of solubilization buffer (pH 7–9). In all cases similar results were observed – only small amount of solubilized protein was found in eluates, around 90% of Cx32 flow through Ni²⁺-NTA agarose; more “harsh” detergents like fos-cholines give better result in terms of Cx32 yield in eluates. We assume that such detergents favor better exposition of polyhistidine-tag, which results into better binding to the affinity resin. Nevertheless, total yield of purified protein was quite high (around 0.5 mg per 1 L of culture, determined from SDS-PAGE using a BSA band with known protein amount as a reference). So we decided to continue experiments with this construct and to proceed with cleaving-off MstX from Cx32.

For further enterokinase cleavage analysis the purification of Cx32 without urea was upscaled. Purification buffer with pH 8.0 was used.
3.1.1.3. **Mistic cleavage tests with enterokinase**

First, eluates after affinity chromatography were dialyzed overnight against 100-fold excess of EK cleavage buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM CaCl₂, pH 8.0 with corresponding detergents – DM, FOS12 or Brij35). Cx32 concentrations in eluates were determined from SDS-PAGE using a BSA band with known protein amount as a reference. Next, samples were diluted with same buffers to final Cx32 concentration 0.2 mg/ml. 20 µl aliquots were mixed with different amounts of enterokinase (final EK concentration 0.1 µg/ml and less) and incubated overnight at 20°C or 4°C. These samples were analyzed further by Western-blotting. **Figure 3.8** shows resulting immunoblot in case of DM (0.2%). No desired cleavage pattern is observed, either EK concentration was to low and no cleavage occurred (rows 3-4) or unspecific proteolysis was observed (rows 1-2). More detailed screening of EK concentration gave the same results. Similar picture was observed for Cx32 purified in Brij35, while in case of Fos12 we did not see any cleavage at all.

![Immunoblot analysis](image)

**Figure 3.7.** Immunoblot analysis of different fractions of Cx32 solubilized in several detergents and purified using Ni²⁺-NTA agarose (all without urea). M: size marker; bc: Cx32 solution in detergent before purification; ft: flow through; el: eluate.

![Western-blot analysis](image)

**Figure 3.8.** Western-blot analysis of Cx32 (solubilized and purified in DM) samples with different enterokinase concentrations incubated overnight at 20°C and 4°C. M: size marker; 1: 0.1 µg/ml EK; 2: 6 ng/ml EK; 3: 0.4 ng/ml EK; 4: 0.02 ng/ml EK; c: control samples without EK; c*: Cx32 sample before incubation. Bands indicating unspecific proteolysis are depicted with arrows.

It was previously reported that detergents have strong influence on proteases activity [173] and in case of “harsh” detergents it decreases dramatically. This explains our observations in case of Fos12. Results for DM and Brij35 indicate that enterokinase cleavage
site is not exposed well enough for efficient EK binding, which prevents MstX cutting-off under low EK concentrations. Construction with longer linker between Mistic and Cx32 was generated and examined for this reason (data not shown). Unfortunately we obtained the same results – either no cleavage was observed under low EK concentrations, or unspecific proteolysis occurred. That is why we decided to replace EK cleavage site with thrombin cleavage site and to try luck with a new construction.

3.1.2. Assembling and expression of human connexin 32 gene in fusion with Mistic and thrombin cleavage site in between

3.1.2.1. Cloning strategy

Cx32 gene was amplified from pSCodon1.2_H6MstXEKCx32 plasmid by PCR using two primers - frwBamHTrhCx32 and revXhoIhCx32. PCR product was digested with BamHI and XhoI DNA restriction enzymes and cloned into pSCodon1.2 vector containing already MstX gene. Final construct is illustrated on Figure 3.9 and has a calculated molecular weight of 45.7 kDa. This construct was designated as H6MstXTrhCx32.

Figure 3.9. Basic features of the generated construct of human connexin 32 gene in fusion with MstX and thrombin cleavage site in between.

3.1.2.2. Expression and purification

Expression and purification were carried out using standart protocol described in methods. We repeated solubilization screening using several detergents (Fos12, Fos14, DM, DDM, OG, MTAB, Brij35). Results for two of them (MTAB and Fos12) are shown on Figure 3.10.

Replacing protease cleavage site had no significant influence on purification process and final Cx32 yield. Still more “harsh” Fos12 gave better results and almost twice more protein could be eluted from Ni2+-NTA column when it is used for solubilization (comparing to “mild” DDM or MTAB detergents). Again, total Cx32 yield was around 0.5 mg per 1 L of culture.
Western-blot analysis of MTAB and Fos12 efficiency for Cx32 solubilization and purification using Ni²⁺-NTA agarose. M: size marker; p: pellet after solubilization and centrifugation; s: supernatant after solubilization and centrifugation; ft: flow through column pre-packed with Ni²⁺-NTA resin; el: eluate from Ni²⁺-NTA column; 1: fraction with less then 30% of glycerol after E.coli total membrane sedimentation; 2: fraction with more then 30% of glycerol; lys: total cells lysate.

Low efficiency of widely used for membrane proteins solubilization “mild” non-ionic detergents (like DM, DDM, or OG) brought as to idea that maybe our Cx32 in fusion with Mistic is overexpressed as inclusion bodies. In this case commonly used approach is to reduce expression rate so that all synthesized target protein have enough time to incorporate into cellular membranes. For this we tried expression at low temperatures starting from 18°C. Cultivation period was increased so that after bacteria had consumed all the primary hydrocarbons (glucose concentration was controlled over time) induction took not less than 6 h. In this case total Cx32 yield reduced dramatically and only around 0.1 mg of target protein per 1 L of culture could be purified. Nevertheless, we observed the same Cx32 behavior – usage of Fos12 allowed us to solubilize around 80% of target protein from E.coli total membranes, while using more “soft” detergents we managed to extract only 20% of Cx32.

That is why we returned to standard 37°C expression and purification protocol, upscaled it and purified our new H₆MstXEKCx32 construct in three detergents – Fos12, DM and Brij35. Further this protein was used for screening of thrombin cleavage conditions.

3.1.2.3. Mistic cleavage tests with thrombin

First, eluates after affinity chromatography were dialyzed overnight against 100-fold excess of thrombin cleavage buffer (200 mM Tris-HCl, 1 M NaCl, 25 mM CaCl₂, pH 8.0 with 0.2% of DM, FOS12 or Brij35) at 4°C. Cx32 concentrations in eluates were determined from SDS-PAGE using band with known BSA concentration as a reference. Next, samples were diluted with same buffers to final Cx32 concentration 0.2 mg/ml. 20 µl aliquots were mixed
with different amounts of thrombin (final thrombin concentration 40 U/ml and less) and incubated overnight at 20°C. Further this samples were analyzed by immunoblotting (Figure 3.11).

Unlike enterokinase thrombin don’t lose its activity in the presence of “harsh” Fos12. This means that higher amounts of target Cx32 can be purified and subjected to Mistic cleavage. But still results were disappointing. Even 1000-fold excess of thrombin amount above recommended by supplier was not enough to cut-off Mistic from all connexin in sample. Unspecific proteolysis again occurred, although its results were not directly visualized by immunoblotting. This we understood comparing integral intensities of bands corresponding to monomer and dimer in the samples with and without thrombin. Summarized integral intensity of all bands in the samples with thrombin (for example, column “1” with DM on the immunoblot) was twice lower than in the control sample (column “c” with DM). More detailed screening of cleavage conditions (protease concentration, temperature, pH of used buffer) gave no significant improvement – higher concentrations of thrombin in the reaction mixtures caused degradation of all connexin 32, while with smaller one not all target protein was cut.

Further work on this direction seemed unreasonable and we decided to proceed using other protease to remove Mistic, FXa protease. Also, taking into account low binding rate of target protein to Ni²⁺-NTA resin when polyhistidine-tag was placed before Mistic, we decided to move it to C-terminus of Cx32. The results are illustrated in the next section.
3.1.3. Assembling and expression of human connexin 32 gene in fusion with Mistic and factor Xa protease cleavage site in between

3.1.3.1. Cloning strategy

Cx32 gene was amplified from pSCodon1.2_H6MstXTrCx32 plasmid by PCR using two primers - frwAgeIFXaCx32 and revBamHICx32. PCR product was digested with AgeI and BamHI DNA restriction enzymes and cloned into pSCodon1.2 vector containing already MstX gene (the recipient vector has been produced earlier by Maria Silacheva in our laboratory). Final construct is illustrated on **Figure 3.12A** and have molecular weight of 48.8 kDa. This construct was designated as MstXTrFXahCx32FXaH₈.

In parallel we generated Cx32 construction without any fusion-tag on N-terminus to compare expression rates and to study Mistic influence on Cx32 yield. For this Cx32 gene was amplified from pSCodon1.2_MstXTrFXahCx32FXaH₈ plasmid by PCR using two primers - frwBamHINdeICx32 and revBamHICx32. PCR product was digested with NdeI and BamHI DNA restriction enzymes and cloned into pSCodon1.2_MstXTrFXahCx32FXaH₈ vector. Final construct is illustrated on **Figure 3.12B**. This construct was designated as hCx32FXaH₈. Its molecular weight is 34 kDa.

![Figure 3.12](image)

**Figure 3.12.** Basic features of generated constructs of human connexin 32 gene in fusion with MstX and factor Xa protease cleavage site in between - MstXTrFXahCx32FXaH₈ (A) and without any fusion-tag on N-terminus - hCx32FXaH₈ (B).

3.1.3.2. Expression and purification

**Test expression of Cx32 in fusion with Mistic and without**

Plasmids with both constructs were transformed into SE1 *E.coli* strain. To compare Cx32 expression yields we did test expression in 25 ml of AIM with 100 mg/l ampicillin. Bacteria were cultured for 16 h at 37°C with vigorous shaking. Next morning OD₆₀₀ was measured. Normalized volumes of two cultures (2 OD₆₀₀×ml) were transferred to tubes and cells were pelleted down by centrifugation. Each pellet was resuspended in 200 μl of 1× gel-loading buffer and analyzed by SDS-PAGE and Western-blotting (**Figure 3.13**).
Figure 3.13. Western-blot of total lysates of cells with new Cx32 constructs (in fusion with Mistic – columns 1, 2; and without – 3, 4) using Anti-Connexin32 mouse antibody, anti-mouse IgG alkaline phosphatase conjugate and BCIP/NBT enzyme substrate. M: size marker; 1, 3: 10 µl of samples was loaded; 2, 4: 20 µl of samples was loaded.

Since in case of the construct without Mistic no any band was visualized on immunoblotting we assumed that expression rate of target protein is extremely low and less than 50 ng of connexin were loaded onto the gel. These results are in a good agreement with previously reported [11,12].

“Preparative scale” expression and purification of MstXTrFXahCx32FXaHs. “Two-detergent” approach.

Expression was carried out using the standard protocol “E.coli culture and Cx32 overexpression” described in methods. We again did solubilization screening with several detergents, including sodium lauroyl sarcosinate (NLS) – “harsh” ionic surfactant previously reported to be effective for solubilization of connexins expressed in E.coli [11]. Observed picture was the same as for previous Cx32 constructs in case of all detergents except NLS – “harsh” Fos12 solubilized around 50% of Cx32 from total E.coli membranes, while DM, Brij35 and others – only about 20%. On the other hand NLS solubilized all the Cx32 and no protein was observed in the pellet after centrifugation (data not shown). We carried out “preparative scale” solubilization and purification in 1% NLS and noticed that binding efficiency of MstXTrFXahCx32FXaHs (to Ni\(^{2+}\)-NTA resin) significantly increased and 90% of the solubilized protein was recovered in the eluate. This helped to increase the Cx32 purification yield up to 1 mg per 1 L of culture.

Since previously reported data indicate that proteases activity in NLS-containing buffers tremendously decreases [173] the only possibility for us was to replace it with other, protease-compatible, detergent on the Ni\(^{2+}\)-NTA column. To do this washing the resin with 1% NLS in washing buffer was followed by washing with 1% Brij35. Protein elution was carried out in 0.2% Brij35. Unfortunately, analyzing the eluate we did not find Cx32. We assumed that all the target protein was stuck on the column.
Only combining two detergents on the solubilization stage we recovered the protein in the eluate. First, 1% Brij35 was added to a total E.coli membrane suspension. Mixture was incubated for 3 h at 4°C with moderate stirring. Next, 1% NLS was added and mixture was further incubated overnight. Ni²⁺-NTA resin was equilibrated with a buffer containing 1% of both detergents. Supernatant after centrifugation was loaded on the column; column was first washed with 5×CV of washing buffer with 1% NLS and 1% Brij35 and then with 5×CV of washing buffer containing only 0.2% Brij35. Finally protein was eluted in 0.2% Brij35. This retreat on the purification procedure helped us to recover around 80% of the Cx32 loaded on the column. Figure 3.14 demonstrates a typical elution profile with imidazole gradient 30 to 400 mM.

![Figure 3.14](image)

**Figure 3.14.** Elution profile of MstXTrFXahCx32FXaH₈ from Ni²⁺-NTA column with imidazole gradient (30-400 mM) after two washings: with 5×CV of washing buffer containing 1% NLS and 1% Brij35; with 5×CV of washing buffer containing only 0.2% Brij35. Fraction numbers are depicted above the x-axis.

![Figure 3.15](image)

**Figure 3.15.** Western-blot of different fractions after Cx32 elution with imidazole gradient from Ni²⁺-NTA column. Numbers above lanes indicate certain fractions from elution profile, shown on Figure 3.14. M: size marker. Arrow points to a major contaminant band.

Analysis of corresponding fractions was done by SDS-PAGE and Western-blotting. Results are illustrated on Figure 3.15. Although “two-detergent” approach helped to increase
yield of target protein it did not solve completely a purity problem (one contaminant band is shown by arrow on the picture above). When we collect a middle part of the elution peak only 40% of Cx32 was recovered after purification, if we collect the whole elution peak – Cx32 composes less than 50% of total protein in the eluate. To proceed with crystallization additional purification step is essential. But since we need to cut-off Mistic to allow connexin form proper hexamers we decided first to focus on MstX cleavage.

3.1.3.3. Mistic cleavage tests with factor Xa protease

As in previous cleavage screenings we first dialyzed the eluate after metal-affinity chromatography overnight against 100-fold excess of Factor Xa cleavage buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM CaCl₂, pH 6.5 with 0.2% of Brij35) at 4°C. Next, samples were diluted with the same buffer to a final Cx32 concentration of 0.2 mg/ml. 20 µl aliquots were mixed with different amounts of Factor Xa protease (five-fold dilutions starting with a maximum protease concentration of 40 U/ml) and incubated overnight at either 20°C or 37°C. Further these samples were analyzed by SDS-PAGE and Western-blotting. Results are illustrated on Figure 3.16.

Like with enterokinase or thrombin an excess of Factor Xa protease caused unspecific proteolysis while smaller amounts of the protease in the reaction mixture had no effect. 25-fold excess of Factor Xa protease above the recommended by supplier (1 U per 10 µg of protein to be cleaved) caused complete Cx32 degradation at 37°C and more than 50% degradation at 20°C (column 1). At the same time the recommended amount of the protease had no effect at all (column 3). More detailed screening of protease concentration gave no significant improvement.

Poor cleavage may be caused by steric hindrance when the protease cleavage site is too close to ordered structure in the target protein. To overcome this problem we elongated the linker between Mistic and connexin 32 by addition of the Strep-tag (WSHPQFEK) between the thrombin and Factor Xa cleavage sites (see Figure 3.12A). Then we repeated solubilization and purification procedure following “two-detergent” approach. After dialysis of Cx32 against the same Factor Xa cleavage buffer samples were mixed with dilutions of the protease and incubated overnight at either 20°C or 4°C. Results were visualized by immunoblotting (Figure 3.17).
3.1.4. Assembling and expression of human connexin 32 gene in fusion with Mistic and TEV protease cleavage site in between for in vivo cleavage

All three proteases described above (EK, thrombin and Factor Xa) show good result in case of water-soluble proteins. But there are not so many examples of their successive usage for membrane proteins, especially when fusion partner is also membrane protein. On the other hand recently it was shown that TEV protease could effectively remove Mistic from the fused MstX-NTT1 membrane protein [174].
TEV protease is the common name for the 27 kDa catalytic domain of the Nuclear Inclusion A (NIa) protein encoded by the tobacco etch virus (TEV). Its sequence specificity is far more stringent than that of Factor Xa, thrombin, or enterokinase [175]. For this reason TEV protease is widely used for cleaving fusion proteins [176]. Also important to notice that it can be easily overproduced and purified in large quantities using E.coli as expression system [177].

For these reasons we decided to introduce TEV protease cleavage site between Mistic and our target Cx32 and to try out in vivo cleavage approach.

3.1.4.1. Cloning strategy

Cx32 gene was amplified from pSCodon1.2_ MstXTrFXahCx32FXaH₈ plasmid by PCR using two primers - frwAgeITevhCx32 and revBsrgIhCx32Tev. The PCR product was digested with AgeI and BsrgI DNA restriction enzymes and cloned back into pSCodon1.2_ MstXTrFXahCx32FXaH₈ vector. The final construct is illustrated on Figure 3.18 and has molecular weight 50.5 kDa. This construct was designated as MstXTrTevhCx32FXaH₈.

![Figure 3.18](image)

**Figure 3.18.** Basic features of a generated construct of human connexin 32 gene in fusion with MstX and TEV protease cleavage site in between.

3.1.4.2. Expression and purification

Expression and in vivo cleavage of Cx32 using separate plasmids for TEV protease parallel expression

For expression first pSCodon1.2_ MstXTrTevhCx32FXaH₈ vector was transformed into SE1 E.coli strain. Next, bacteria were cultured and used for preparation of chemically competent cells. Either pRK603 or pKM586 (both are shown on Figure 3.19) containing gene of TEV protease were transformed into these cells. Finally, we got SE1 E.coli bacteria, with two combinations of plasmids: pSCodon1.2_ MstXTrTevhCx32FXaH₈ with pRK603 and pSCodon1.2_ MstXTrTevhCx32FXaH₈ with pKM586.

Both vectors (pRK603 and pKM586) are intended to be used for controlled intracellular processing of fusion proteins (TEV protease substrates) in E.coli [176]. Since SE1 strain does not produce Tet repressor expression of the TEV protease gene is constitutive.
and no induction is needed. Both, pRK603 and pKM586, are low copy plasmids – 10 copies per cell are maintained in case of pKM586 and around 30 copies per cell in case of pRK603.

Test expression was carried out in in 25 ml of AIM with 100 mg/L ampicillin and 50 mg/L kanamycin. Single colonies were transferred to media and bacteria were cultured for 16 h at 37°C with vigorous shaking. Next morning OD_{600} was measured. Normalized volumes of two cultures (2 OD_{600}×ml) were transferred into tubes and cells were harvested by centrifugation. Each pellet was dissolved in 200 µl of 1× gel-loading buffer and analyzed by SDS-PAGE and Western-blotting (Figure 3.20).

Both helper plasmids had no influence on the total expression rate of Cx32. But unspecific cleavage was observed. Meanwhile pRK603 gave around twice more cleaved protein then pKM586. It is perfectly displayed with cleaved monomer bands on Figure 3.20 (depicted by arrows).

Although cleavage was not complete we decided to proceed with pRK603 and to try expression conditions optimization to achieve Mistic cutting-off from all expressed Cx32. For this we tried various cultivation temperatures (20°C, 24°C and 37°C) possessing that cultivation temperature might influence TEV protease expression level and folding. At 20°C we observed that more Cx32 was cleaved, but there was a drawback – unspecific proteolysis also took place and we saw bands corresponding to degraded protein on immunoblots. That is why we had to return to standard expression protocol and to cultivate bacteria at 37°C.
Figure 3.20. Western-blots of total lysates of cells with new Cx32 construct for in vivo Mistic cleavage using Anti-Connexin32 mouse antibody, antimouse IgG alkaline phosphatase conjugate and BCIP/NBT enzyme substrate. M: size marker; 1: 20 µl of sample with pKM586; 2: 20 µl of sample with pRK603; 3: 20 µl of control sample without TEV protease containing vectors. Bands corresponding to cleaved Cx32 monomer are depicted with arrows.

### Purification of solubilized Cx32 using Ni$^{2+}$-NTA agarose

“Preparative scale” expression was carried out as described in methods. Cx32 was solubilized using “two-detergent” approach in 1% Brij35 and 1% NLS. Before applying to Ni$^{2+}$-NTA pre-packed column supernatant after centrifugation was diluted with equal volume of the same buffer to reduce NLS and solubilized proteins concentrations. This helped to increase binding efficiency so that only 20% of Cx32 flowed through the column. Further the column was washed with 5×CV of washing buffer with 1% NLS and 1% Brij35 and then with 5×CV of washing buffer containing only 0.2% Brij35. Finally protein was eluted in 0.2% Brij35. Typical elution profile is illustrated on Figure 3.21.

Figure 3.21. Elution profile of MstXTrTevhCx32FXaH8 coexpressed with TEV protease from Ni$^{2+}$-NTA column with imidazole gradient (30-400 mM) after two washings: with 5×CV of washing buffer containing 1% NLS and 1% Brij35; with 5×CV of washing buffer containing only 0.2% Brij35. Fraction numbers are depicted above the x-axis.

Analysis of eluate fractions demonstrated that Ni$^{2+}$-NTA agarose did not allow separating of Cx32 from not-cleaved Mistic-Cx32. Corresponding Western-blot is showed on the Figure 3.22.
Ion exchange chromatography to separate cleaved and not cleaved Cx32

Since isoelectric point of Mistic (pI=4.5) and connexin 32 (pI=9.2) differs significantly we assumed that ion exchange chromatography would perfectly fit to separate cleaved and not cleaved Cx32. As cation and anion exchangers, MonoS and MonoQ, respectively, were considered. Both of them are incompatible with charged ionic surfactants. For this reason all experiments were carried out with Cx32 solubilized in relatively “soft” detergent – Brij35.

Prior to application to both columns eluates after Ni\textsuperscript{2+}-NTA purification were dialyzed against buffers recommended by supplier containing 0.2% Brij35 (see methods). Protein was eluted in raising concentration of NaCl from 0.05 M up to 1 M in 20×CV. Fractions were collected and further analyzed by SDS-PAGE and Western-blotting. Multiple elution peaks obtained were very similar regarding to ratio between uncleaved Mistic-Cx32 and cleaved out Cx32 product. Connexin with Mistic and cleaved connexin appeared to form complexes (or oligomerized) by means of hydrophobic interactions. And for this reason salt cannot help to separate them.

“Harsh” ionic surfactants (NLS or SDS) can harm our protein and reduce separation efficiency of ion chromatography. For this reason we could not use them to eliminate hydrophobic interactions between cleaved and not cleaved Cx32. Since urea and guanidine hydrochloride are widely used for denaturation of proteins and as a mild solubilization agent we decided to screen their influence in our case. We varied urea (from 1 M up to 8 M) and guanidine hydrochloride (from 0.5 M up to 6 M) concentrations in equilibration and elution buffers. On Figure 3.23 elution profile in case of MonoQ is shown when 6 M urea was added to the equilibration buffer. All fractions were collected and analyzed by immunoblotting (Figure 3.24).
Elution profile of MstXTrTevhCx32FXaH8 coexpressed with TEV protease from MonoQ column with salt gradient (0-1 M NaCl). Loaded protein was preliminary purified using “two detergent” protocol on Ni$^{2+}$-NTA agarose and dialyzed against MonoQ equilibration buffer (20 mM Tris-HCl, 6 M urea, 0.2% Brij35, pH 8.0). Fraction numbers are depicted above the x-axis.

Addition of urea and guanidine hydrochloride had no noticeable influence on separation of processed and not Cx32. Connexin was detected in both states in all eluate fractions. This meant that the only possibility to get connexin without Mistic in case of TEV protease cleavage was to achieve complete processing *in vivo*.

Co-expression of TEV protease and connexin 32

Since helper plasmid pRK603 with higher amount of copies per cell showed better results than pKM586 we decided to introduce TEV protease into pSCodon1.2 (high copy plasmid) already containing Cx32 gene. TEV protease gene was also controlled with *lac* operon, which meant that it would be expressed in parallel to connexin. The resulting construct is illustrated on Figure 3.25.

Unfortunately, TEV protease expression rate turned out to be too high and toxic effect was observed. Also analysis showed that total Cx32 yield was 3 times lower in comparison to usage of helper plasmid.
3.2. Cell free expression of human connexin 32 using S30 E.coli extract

Structural study of membrane proteins is a complex task and problems may appear on each step. Purification of large quantities of membrane proteins always was essential for crystallization experiments. In spite of recent advances in nano-volume crystallization milligram amounts of a purified membrane protein are still required. At present, one of the most preferred heterologous expression system is E.coli, which is both time-consuming and has the limitation of producing membrane proteins, in particular those of eukaryotic origin. Although recent improvements using engineered strains and protein fusion partners have led to improved production of insoluble proteins, such approaches are generally not universal and require optimization in each individual case. Cell-free protein synthesis (CFPS) is emerging as an attractive alternative since it offers a simple, open and flexible approach to rapid synthesis of folded proteins. The use of PCR fragments, avoiding the need for in vivo cloning, gives cell-free systems even greater usefulness for parallel expression of large numbers of different proteins. It is also capable of functionally producing both disulphide-bonded and membrane proteins, providing a platform for generation of “difficult-to-express” proteins [178]. In case of membrane proteins (MPs) CFPS can help to eliminate some principal problems occurring in conventional in vivo systems, like toxicity of the overproduced MPs upon insertion into the cytoplasmic membranes, poor growth of overexpressing strains, proteolytic degradation of the expressed MPs, or generally unfavorable impacts on cellular metabolisms. Also, it was previously reported that in case of CFPS genes with native codon usage had as high a yield as optimized codon usage [179,180]. This means that no codon optimization is required for effective heterologous expression in CF.
Although we showed that overexpression of connexin 32 using Mistic as fusion partner is possible, we did not succeed in its cleavage from N-terminus of our connexin. We demonstrated that MstX is resistant to various cleavage conditions including different proteases usage and variations of linker in between. For this reason appropriate system to produce milligram amounts of Cx32 without any N-terminal additives is needed. And such system was reported to exist – in 2010 G. Chang et al mentioned that connexin 32 might be overexpressed in CFPS using E.coli extract [181]. For these reasons we decided to use cell free protein expression system to overexpress connexin 32.

3.2.1. Cx32 gene optimization

Since CFPS is more expensive than usual in vivo expression every milligram of produced protein is on account. First we decided to perform test expression of several human connexins to know which protein yield we expect.

To do test expression we cloned our Cx32 into pivex2.3d vector, specially designed for CFPS. Briefly, Cx32 gene was digested from pSCodon1.2_hCx32FXaH8 plasmid with Ndel and XhoI DNA restriction enzymes and cloned into empty pivex2.3d vector. Final construct had molecular weight 34 kDa and was designated as pivex2.3d_hCx32FXaH8.

Constructs with 3 other connexins (Cx26, Cx30.2 and Cx40) were generated by Taras Balandin and Dmytro Volkov in our laboratory.

Plasmids were produced and purified in sufficient for CFPS amounts following the procedure “plasmid DNA purification for cell free protein synthesis” described in Methods. The expression was carried out in the “analytical scale” reaction setup with Mg\(^{2+}\) concentration 14 mM, K\(^+\) – 290 mM and 1% Brij58. After overnight incubation at 32°C reactions were terminated. Reaction mixtures were centrifuged at 18000×g for 20 min at 4°C to remove precipitants and insoluble additives. 1 µl of supernatant from each chamber was mixed with 1× gel-loading buffer and analyzed by SDS-PAGE and immunoblotting (to identify bands corresponding to connexins anti-His\(_6\) primary antibodies were used). The resulting gel is shown on Figure 3.26.

It appeared that in case of optimized for E.coli Cx26 gene (oCx26) expression rate was slightly higher then for wild-type gene (Cx26). To find out whether there exists any correlation between free energy of connexin mRNA secondary structure and CFPS yield we built diagram, illustrated on Figure 3.27. Thus, it is obvious that certain connexin yield from CFPS strongly depends on free energy of its mRNA secondary structure. Small deviation in case of Cx30.2 may be explained by big loop in the beginning of its mRNA.
Based on these results we did optimization of connexin 32 gene sequence, which helped us to reduce free energy more than twice (from -222 kkal/mol to -97.1 kkal/mol). Optimized oCx32 gene was synthesized by MWG, Ebersberg, Germany and cloned into pivex2.3d vector using NdeI and XhoI DNA restriction sites. Final construct had molecular weight 34.4 kDa and was designated as pivex2.3d_ohCx32FXaH_{10}.

Despite such significant reduction of free energy control expression experiment with Cx32 and oCx32 showed only 30% yield increase in case of optimized gene (total connexin 32 yield was 0.3 mg of protein per 1 ml of reaction mixture).

3.2.2. N-terminal tags to increase Cx32 yield

In 2012 S. Haberstock *et al* showed that addition of short N-terminal tags (7 amino acids) could result in significant (reported 32-fold) CFPS yield improvement in case of membrane proteins [182]. Observed results were explained by optimization of translation initiation.
To use this approach we first designed construct with N-terminal polyhistidine-tag. Briefly, oCx32 gene was amplified from pixev2.3d_ohCx32FXaH10 vector by PCR using two primers - frwM1GCx32 and revXhoIohCx32. M1G mutation was introduced to improve cleavage with Factor Xa protease in case of successful overexpression. PCR product was digested with SacII and XhoI DNA restriction enzymes and cloned into empty pivex2.4d vector. Final construct is illustrated on Figure 3.28 and have molecular weight 33.8 kDa. This construct was designated as pivex2.3d_H6FXaM1GoCx32.

**Figure 3.28.** Basic features of a new construct for CFPS expression of connexin 32. Cx32 gene sequence was optimized to reduce free energy of mRNA secondary structures. M1G mutation was introduced to improve cleavage with Factor Xa protease.

Short peptides, listed in Table 3.1, were introduced to N-terminus of H6FXaM1GoCx32 by circular PCR using two corresponding primers and pivex2.3d_H6FXaM1GoCx32 as a template. PCR products were purified, ligated and transformed into TOP10 E.coli competent cells. After production and purification of sufficient amounts of resulting plasmids we carried out expression experiment in the “analytical scale” reaction setup with Mg2+ concentration 14 mM, K+ – 290 mM and 1% Brij58. After overnight incubation at 32°C reactions were terminated. The reaction mixtures were centrifuged at 18000×g for 20 min at 4°C to remove precipitants and insoluble additives. The supernatants were analyzed as before by SDS-PAGE (Figure 3.29).

In our case addition of N-terminal expression enhancers had no significant effect. Cx32 yields (monomer bands intensities) were almost the same as for initial construct (H6) with small deviations. Yield in case of C-terminal polyhistidine-tag (H10) was two times higher.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Tag sequence</th>
<th>Primers combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>pivex2.4_AT-H6FXaM1GoCx32</td>
<td>MKYYKYY</td>
<td>frw_AT_H6 and rev_RBS_T7</td>
</tr>
<tr>
<td>pivex2.4_Ser-H6FXaM1GoCx32</td>
<td>MKSSSSS</td>
<td>frw_SER_H6 and rev_RBS_T7</td>
</tr>
<tr>
<td>pivex2.4_G-H6FXaM1GoCx32</td>
<td>MKSKGEE</td>
<td>frw_G_H6 and rev_RBS_T7</td>
</tr>
<tr>
<td>pivex2.4_H-H6FXaM1GoCx32</td>
<td>MKPYDGP</td>
<td>frw_H_H6 and rev_RBS_T7</td>
</tr>
</tbody>
</table>

**Table 3.1.** List of primers combinations and N-terminal tags sequences used to generate new constructions to enhance expression rate of Cx32 in CFPS.
Figure 3.29. Coomassie-stained SDS gel for analysis of N-terminal tags influence on Cx32 expression rates in CFPS. 1 µl of each reaction mixture was loaded. M: size marker; 1, 2: number of reaction chamber (for each construction two reaction chambers were used); AT, Ser, H, G: constructions with N-terminal expression enhancers; H₆: pivex2.3dᵋH₆FXaM₁GoCx32; H₁₀ᵋ: pivex2.3dᵋohCx32FXaH₁₀. With arrow monomer bands are depicted. Positions of monomers bands were identified using immunoblot with Anti-Connexin32 mouse antibodies.

On this point we decided to stop “playing” with DNAs and to proceed to expression conditions screens (vary detergents and add lipids to CFPS reaction mixture).

3.2.3. Detergent screen to increase Cx32 yield

In CFPS membrane proteins can be produced either as a precipitate or as solubilized proteins. The CF expression system is considerably tolerant upon relatively high concentrations of a variety of detergents and lipids [183–185] that usually are provided as a hydrophobic environment to stabilize the synthesized MPs immediately after translation. Alternatively, the vast majority of MPs are produced as precipitates if detergents are not supplemented. However, those precipitates usually can easily be solubilized with suitable detergents without the necessity to apply extensive denaturation and renaturation steps as known from refolding protocols.

Addition of suitable detergent or lipid to CPFS can play important role in terms of MPs yield [183]. To find optimal conditions for Cx32 overexpression we screened several detergents. Concentration of each detergent which did not suppress CF protein production were previously reported in [162]. In our experiment we used 8 detergents: 1.5% Brij35, 1.5% Brij 58, 1% Brij78, 0.4% Digitonin, 0.1% Triton X-100, 0.1% DM, 0.1% DDM, 0.1% Tween20. Reactions were carried out in “analytical scale” setup with Mg²⁺ concentration of 14 mM and K⁺ of 290 mM. After overnight incubation at 32°C reactions were terminated.
The reaction mixtures were centrifuged at 18000×g for 20 min at 4°C to remove precipitants and insoluble additives. As before, the supernatants were analyzed by SDS-PAGE (Figure 3.30A). From intensities of monomer bands we calculated approximate Cx32 yields for each detergent. Normalized expression rates are shown on Figure 3.30B.

![Coomassie-stained SDS gel for analysis of detergents influence on Cx32 expression rates in CFPS. 1 µl of each reaction mixture was loaded. M: size marker; 1: Brij35 1.5%; 2: Brij58 1.5%; 3: Brij78 1%; 4: Digitonin 0.4%; 5: Triton X-100 0.1%; 6: DM 0.1%; 7: DDM 0.1%; Tween20 0.1%; 9: proteorhodopsin expression in Digitonin 0.4%; PM: pellet mod (without addition of any detergent). Cx32 monomer bands are depicted with arrow. (B) Normalized Cx32 yields from CFPS with different detergents. Protein yields were calculated from intensities of monomer bands on SDS-PAGE gel.](image)

Although tremendous detergent influence was not observed, digitonin and Brij78 demonstrated best results – 20% Cx32 yield increase. Expression rates in case of Brij35, Brij58 and Tween were almost the same. DM, DDM and Triton X-100 suppressed CF production of Cx32 more significantly. But because of availability problems for “preparative scale” expression and further studies we had to choose Brij58 (Brij78 was not available in sufficient for “preparative scale” setup quantities in our laboratory; digitonin was too expensive and was increasing the final cost of 1 ml CFPS reaction mixture more than twice).

3.2.4. Lipids and lipid/detergent mixtures in CFPS of Cx32

To study lipids influence on Cx32 yield from CFPS we screened several concentrations of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). Dry POPC powder was dissolved in chloroform and then dried overnight under nitrogen gas. Lipid film was rehydrated in S30 buffer (see methods) at concentration 100 mg of lipid per 1 ml of buffer (10% stock), and the suspension was warmed to 37°C until it became transparent. Next, as in case of detergents, certain amounts of this stock were added to CF reaction
mixtures to obtain desirable final concentration (0.05%, 0.2% and 0.4%). Addition of above 0.4% of lipids was previously reported to significantly suppress CF protein production. Reactions were carried out in “analytical scale” setup with Mg\(^{2+}\) concentration of 14 mM and K\(^+\) of 290 mM. After overnight incubation at 32°C reactions were terminated. The reaction mixtures were centrifuged at 18000×g for 20 min at 4°C. The pellets were resuspended in 50 µl of 1× gel-loading buffer and analyzed by SDS-PAGE (this time we analyzed only pellets, since we were interested in Cx32 incorporated into membranes). The resulting polyacrylamide gel is illustrated on Figure 3.31A.

**Figure 3.31.** Lipids and lipid/detergent mixtures influence on the Cx32 yields from CFPS. (A) Coomassie-stained SDS gel for analysis of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) influence on Cx32 expression rates in CFPS. (B) Coomassie-stained SDS gel for analysis of POPC/Brij58 and POPC/DDM influence on Cx32 expression rates in CFPS. For both gels 1 µl of each reaction mixture was loaded. M: size marker; PM: pellet mode. Cx32 monomer bands are depicted with arrow. With red triangles Cx32 yield behavior is illustrated.

After incubation in reaction mixtures with liposomes we observed noticeable turbidity. This might mean that during reaction multilamellar liposomes were formed. These liposomes excluded certain volume of reaction components from mixture thus resulting into expression efficiency decrease. Observed Cx32 production levels in the samples with lipids were 30% lower than in the control sample with 1.5% Bij58. Increase of POPC concentration in the reactions resulted in the Cx32 yield decrease. In pellet mode (PM), when no detergent or lipid was added to the mixture, protein yield was almost equal to those from mixtures with POPC.

To overcome formation of liposomes in reaction mixtures we added combination of lipids with excessive amounts of detergent. We assumed that such combination would supply lipid/protein interface helping proper Cx32 folding while lipids would be solubilized in detergent micelles and would not form bilayer. 10% POPC stock solution was mixed with
20% detergents stocks (DDM and Brij58) in the same S30 buffer with certain ratio (POPC/Brij58 – 1/1, 0.8/1, 0.6/1, 0.4/1, 0.2/1, 0.1/1; POPC/DDM – 1/1, 0.5/1, 0.25/1, 0.15/1, 0.1/1), extruded though 50 nm filter and added to CF reaction mixtures. Reactions were carried out in analytical scale setup with Mg$^{2+}$ concentration of 14 mM and K$^+$ of 290 mM. After overnight incubation at 32°C reactions were terminated. The reaction mixtures were centrifuged at 18000×g for 20 min at 4°C. This time no turbidity was observed, so only the supernatants were analyzed (Figure 3.31B).

In case of POPC/Brij58 combination the same tendency (as only with POPC) was observed – higher amounts of lipids suppressed Cx32 production more significantly. In case of POPC/DDM mixtures we saw opposite effect, addition of lipids enhanced Cx32 production. This might be explained by higher toxic effect of DDM on CFPS (then Brij58), which was reduced by lipids addition. Nevertheless, both combinations did not show any significant Cx32 yield increase in comparison to addition of only Brij58. For this reason we stopped screening experiments and proceeded to further protein production and purification.

### 3.2.5. Expression and purification of Cx32 from CFPS

For “preparative scale” reactions slide-A-lyzer setup (with 14kDa cut-off membrane) was used (see Figure 2.2b from methods) and the mixture was incubated at 32°C overnight with gentle stirring (120 rpm). For “preparative scale” CF expression of Cx32 we added 14 mM Mg$^{2+}$, 290 mM K$^+$ and 1.5% Brij58 into the mixture. Next morning the reaction was terminated. The reaction mixture was discarded and centrifuged at 18000×g for 20 min at 4°C to remove precipitants and insoluble additives. The supernatant was collected and applied onto PD10 desalting column pre-equilibrated with desalting buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.5 + 1% Brij58). Next, the eluate was loaded onto column pre-packed with Ni$^{2+}$-NTA and pre-equilibrated with the same buffer. The column with bound protein was washed with 5×CV of washing buffer containing 1% Brij58. Cx32 was eluted with elution buffer containing 1% Brij58. First experiments with Brij58 showed that only 20% of Cx32 was recovered in eluate, all the rest was found in the flow through.

To increase binding efficiency we screened three other detergents: quite “harsh” NLS and SDS as well as suitable for further crystallization DDM. 0.5% of each surfactant was added to the removed from slide-A-lyzer reaction mixture. The resulting sample was incubated for 1 h at 32°C with gentle stirring and then centrifuged at 18000×g for 20 min at 4°C. Purification protocol was completely repeated; Brij58 was replaced with 0.5% NLS, SDS or DDM in all buffers. The best result was observed in case of NLS – all Cx32 from the
reaction mixture was recovered in the eluate, no Cx32 was found in the flow through. Final connexin purity was 80%. When we used SDS and DDM only 50% of Cx32 was recovered in the eluates, the rest either unfound or not eluted from Ni^{2+}-NTA resin.

N-lauroyl sarcosine is interfering with further crystallization. To replace it with more suitable DDM or SDS we used gel-filtration. The eluate after metal-affinity chromatography was applied to Superose6HR 24 ml column pre-equilibrated with equilibration buffer containing either 0.2% DDM or 0.2% SDS. The elution profiles are illustrated on **Figure 3.32**. For both detergents only 20% of Cx32 was recovered in the eluates. The rest was either found in the free volume (aggregated) or was stuck on the column. It is important to notice that Cx32 was eluted in SDS in one broad peak at 13.5 ml, which corresponded to the characteristic size of connexin hemichannel (200 kDa). But, since peak was much broader than for globular proteins used for calibration we concluded that variety of oligomeric states were present in the eluate. The similar picture was seen for the eluate in DDM - different oligomeric states were observed although most of Cx32 ran as monomer (peak at 17 ml). Anyway, gel-filtration allowed Cx32 transferring from NLS to neither SDS nor DDM, since too much protein of interest was lost.

![Figure 3.32](image)

**Figure 3.32.** Size-exclusion chromatography to replace 0.5% NLS with 0.2% SDS (red) or 0.2% DDM (blue). Cx32 in 0.5% NLS was loaded onto pre-equilibrated with appropriate detergent Superose6HR 24 ml column and eluted in the same buffer with 0.1 ml/min flow speed. Grey lines correspond to calibration with globular proteins.

The expertise-dependent solution was to try replacing detergent before elution from Ni^{2+}-NTA resin. For this purpose NLS in washing and elution buffer was replaced for either
0.5% SDS or 0.5% DDM. In case of DDM no Cx32 was found in the eluate, which indicated that all connexin stuck on the resin. While in SDS all Cx32 was recovered in the eluate with final purity more than 90% (Figure 3.33). For further experiments purification protocol with elution in 0.5% SDS was chosen.

Figure 3.33. Coomassie-stained SDS gel for analysis of Cx32 purity after metal affinity chromatography. M: size marker; 1, 2 and 3: different eluate fractions. Bands, corresponding to different Cx32 oligomers (monomer, dimer and trimer) are depicted with arrows.

3.2.6. Refolding of Cx32

For usual vapor diffusion crystallization approach homogeneous protein preferably solubilized in suitable detergent, which stabilize its correct folding, is required. Almost the same situation is in case of in meso crystallization, except that membrane protein may be supplied in lipids, i.e. reconstituted into lipids. For connexins, since we are interested in crystallization of hemichannel, homogeneity means not only high protein purity, but also absence in the sample supplied for crystallization other oligomeric states except hexamers.

As was showed above in SDS Cx32 does not tend to form stable hemichannels and variety of oligomeric states can be observed. Also SDS is quite “harsh” detergent and one cannot consider that it’s able to stabilize proper connexin folding. For this reason SDS must be replaced with either lipid or suitable for crystallization “mild” detergent. Lipids have crucial structural and modulatory effect on connexins. Certain phospholipids are tightly associated with connexin channels and are co-purified with connexins when “mild” detergents are used for solubilization. This fact was previously reported and since then confirmed several times [186]. Usage of “harsh” NLS and SDS in our purification protocol allows us to assume that all lipids are washed away from Cx32 and none are associated with protein. But this means that for proper hemichannel formation we possibly need to supply certain lipids to our sample.

But there exits the alternative. Quite recently new polymers were introduced – amphipols (in this study A8-35 was used), amphipathic polymers developed for handling MPs in aqueous solutions under less destabilizing conditions than those of detergent solutions. They have proved to be efficient tools for folding MPs to their native state starting from a
denatured state in SDS or urea [166,187]. Furthermore, in aqueous solutions A8-35 does not form bilayers (like lipids), but forms globular particles that resemble detergent micelles. Upon associating with MPs, it covers their transmembrane surface with an amphipathic layer thus stabilizing MPs. Also, recent studies showed that bacteriorhodopsin (BR) from *Halobacterium salinarum* trapped in A8-35 can be crystallized using in meso approach (unpublished). Unfortunately, A8-35 cannot support *in vitro* MP synthesis [188,189]. This may be related to the observation that charged detergents tend to block MP CFPS [183].

To refold Cx32 solubilized in SDS in A8-35 we first decided to use dialysis. To Cx32 after elution from Ni<sup>2+</sup>-NTA column and dialysis against buffer without imidazole but with 0.1% SDS amphipol was added at the ratio 10 g of A8-35 per 1 g of SDS-unfolded connexin. The mixture was incubated for 30 minutes at room temperature and then was loaded into slide-A-lyzer (with membrane cut-off 20 kDa) and dialyzed against 100-fold excess of refolding buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM KCl, pH 7.5) at 4°C overnight. Aggregates and precipitated SDS were removed by centrifugation at 20000×g for 20 min at 4°C. Analysis of the supernatant and the pellet after this centrifugation by SDS-PAGE showed refolding efficiency of only 20%. This means that 20% Cx32 was still present in the supernatant, while the rest 80% were found in the pellet. Similar result was reported for BR refolding in A8-35 [166], so we decided to try another approach.

In case of amphipol mediated refolding of GPCR and BR much better results were obtained with PDS precipitation form protein/SDS/A8-35 solution by addition of potassium chloride. Refolding of Cx32 form SDS in amphipol was done by stepwise addition of 1 M KCl stock solution to the protein suspension. The same procedure as described above was used. Only instead of dialysis after short incubation at room temperature the sample was kept on ice and KCl was added by small portions to a final concentration of 100 mM. Precipitated PDS was removed by centrifugation at 20000×g for 20 min at 4°C. Analysis of the supernatant and the pellet by SDS-PAGE showed that all Cx32 remained in the supernatant. Further the supernatant was analyzed by size exclusion chromatography. For this it was applied to Superose6HR 24 ml column pre-equilibrated with equilibration buffer without any detergent. Resulting chromatogram is shown on Figure 3.34 in red. Initial Cx32 solubilized in SDS before detergent precipitation is depicted with blue. Cx32 refolding to A8-35 caused significant peak narrowing in comparison to Cx32/SDS sample. The width of this peak was the same as reported previously for Cx32 solubilized from gap junctional plaques. Therefore amphipol helped us to assemble Cx32 hemichannel and to produce homogeneous in terms of oligomerization sample, suitable for further crystallization.
Figure 3.34. Size-exclusion chromatography of Cx32 solubilized in 0.5% SDS (blue) and then refolded in amphipol A8-35 (red) by SDS precipitation as its potassium salt. Cx32 in 0.5% SDS after purification by metal affinity chromatography was loaded onto pre-equilibrated with equilibration buffer (with the same SDS concentration) Superose6HR 24 ml column and eluted in the same buffer with 0.1 ml/min flow speed. For refolded in A8-35 connexin 32 equilibration buffer without any detergent was used. Grey lines correspond to calibration with globular proteins. With green elution profile of Cx32 overexpressed in Sf-9 insect cells is shown (not hemichannels but gap junctions) [16].

3.3. *E.coli* expression of optimized connexin 32 gene

In parallel to the experiments on CF connexin 32 synthesis we carried out test expression of optimized Cx32 gene in *E.coli*. First, optimized Cx32 gene with N-terminal polyhistidine tag was cloned from pivex2.3d_HxFxaM_1GoCx32 using *Xba*I and *Xho*I DNA restriction sites into empty pSCodon1.2 vector. Resulting plasmid was transformed into SE1 *E.coli* strain and test expression was carried out same as it was described in section 3.1.1.2. Results indicated that in case of optimized Cx32 gene expression level was significantly higher in comparison to non-optimized pSCodon1.2_hCx32FXaH8 construct. In order to understand whether Cx32 gene optimization or N-terminal polyhistidine tag helped to enhance expression we decided to prepare construct with optimized Cx32 gene and C-terminal affinity tag. Same procedure as described above was used. Briefly, optimized Cx32 gene with C-terminal polyhistidine tag was cloned from pivex2.3d_ohCx32FXaH10 using *Xba*I and *Xho*I DNA restriction sites into empty pSCodon1.2 vector. Resulting plasmid was
transformed into SE1 *E.coli* strain and again test expression was carried out. Cx32 expression yield was the same as for construct with N-terminal affinity tag, which indicated that gene optimization played crucial role in expression enhancement. Observed Cx32 yield was 0.5 mg of protein per 1 L of culture (before purification) and since *E.coli* expression is much cheaper than CFPS we decided to shift our attention to it.

“Preparative scale” expression was carried out as described in section 2.2.3 of materials and methods with small deviations. Briefly, one colony of freshly transformed bacteria was grown overnight at 37°C in 50 ml of LB broth with 1% glucose and 100 mg/l ampicillin in 500 ml flask. Next morning 0.5 L of AIM without 5052 but with 1% glucose and antibiotic was inoculated with this overnight culture and was grown at 37°C for approximately 9 h in 2 L flask. When glucose in pre-culture was depleted 6 L of AIM with 100 mg/l ampicillin were inoculated with this pre-culture at starting OD$_{600}$ around 0.2. The bacteria were cultured in 2 L flasks at 37°C with vigorous shaking (120 rpm). Glucose level in media was controlled by commercial glucose tests (Merckoquant®). When glucose was depleted (around 3-4 h after inoculation), culture was cooled to 20°C for overnight incubation. The cells were harvested by centrifugation at 5000×g for 30 min at 4°C and then either immediately used for protein purification or frozen in liquid nitrogen and stored at -80°C.

3.3.1. Purification in NLS/SDS and further Cx32 refolding

Bacterial pellets (around 30 g) were resuspended in 150 ml of ice-cold 10% glycerol solution. Deoxyribonuclease I from bovine pancreas was added to cleave DNA in the cell suspension after lysis (0.6 mg of DNAse per 30 g of cells). First, the cells were disrupted by passing through the microfluidizer precooled at 4°C with one pass. Next, NaOH was added to mixture to final concentration 20 mM. The solution was incubated for 30 min on ice with vigorous mixing. Then, it was passed through the precooled microfluidizer two additional times. The suspension was centrifuged at 120000×g for 1 h at 4°C (Beckman Ti70 rotor) to separate *E.coli* total membranes. The supernatant was discarded; the pellet was resuspended in 150 ml of ice-cold homogenization buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.5). To wash out membrane associated proteins and some lipids we used 1% Triton X-100. It was added to the membrane suspension, which were next incubated for 1 h at 4°C with vigorous mixing. The washed membranes were collected by centrifugation at 120000×g for 1 h at 4°C and again resuspended in 150 ml of the same homogenization buffer. For Cx32 solubilization 1.5 g of NLS was added to the suspension (1%) and mixture was incubated overnight at 4°C with vigorous mixing.
Next morning the suspension was centrifuged at 120000×g for 1 h at 4°C to remove insoluble material. The supernatant containing solubilized proteins was first diluted with solubilization buffer to reduce NLS concentration to 0.5%, 20 mM Imidazole was added to increase binding specificity. Then this mixture was added to 3 ml of pre-equilibrated Ni²⁺-NTA beads (with solubilization buffer containing 0.5% NLS) and incubated at 4°C for 5 h under gentle rotation. The resin with bound protein was loaded onto chromatographic column and further washed with 5×CV of washing buffer (20 mM Tris-HCl, 100 mM NaCl, 20 mM Imidazole, 0.5% NLS, pH 7.5) using AKTA purifier chromatographic system. Next washing step was carried out to exchange NLS for SDS. For this purpose the column was washed additionally with 5×CV of washing buffer, containing 0.2% SDS. Finally, bound proteins were eluted in 2×CV of elution buffer (20 mM Tris-HCl, 100 mM NaCl, 400 mM Imidazole, 0.2% SDS, pH 7.5). The purification on chromatographic system was done at room temperature (since SDS precipitated under lower temperatures).

### 3.3.1.1. Refolding of Cx32 from SDS

First, we decided to screen several detergents for their ability to stabilize proper Cx32 refolding and its oligomerization. 9 detergents with 5×CMC concentrations (0.05% DDM, 0.05% DDM/CHS, 1.24% Cymal-5, 0.26% Fos12, 0.23% LDAO, 0.28% F6TAC8.5, 0.13% F6DiGluM, 0.09% HTAC and 0.15% A8-35) were added to the eluates of Cx32 in 0.2% SDS after affinity chromatography. As before, after 1h incubation of these mixtures at room temperature they were cooled down on ice. Refolding of Cx32 from SDS was done by stepwise addition of 1 M KCl stock solution to the protein solution. Small portions of KCl were added to final concentration of 100 mM. Precipitated potassium dodecylsulphate was removed by centrifugation at 20000×g for 20 min at 4°C. Analysis of the supernatants and the pellets by SDS-PAGE showed that in case of 5 detergents (DDM, DDM/CHS, Fos12, F6DiGluM, A8-35) all Cx32 remained in the supernatant. For the rest – connexin precipitated together with PDS and no protein was found in the supernatants after centrifugation.

Further the supernatants with Cx32 were analyzed by size-exclusion chromatography using Superose6HR 24 ml column pre-equilibrated with equilibration buffer (20 mM Tris-HCl, 100 mM NaCl, 1mM EDTA, 1 mM EGTA, pH 7.5) containing 1×CMC of these 5 detergents. Resulting chromatograms indicated that in all cases except A8-35 Cx32 aggregated and migrated through the column either in the void volume or as big particles of size over then 720 kDa (with peak elution volumes between 6 and 10 ml). In case of amphipol
peak was left shifted and a little wider comparing to initial sample in SDS (Figure 3.35 blue line).

Figure 3.35. Size-exclusion chromatography of Cx32 solubilized in 0.5% SDS (red) and then refolded in amphipol A8-35 (blue) by SDS precipitation as its potassium salt. With yellow elution profile of Cx32 transferred into DDM after amphipol refolding is shown. Cx32 in 0.5% SDS after purification by metal affinity chromatography was loaded onto pre-equilibrated with equilibration buffer (with the same SDS concentration) Superose6HR 24 ml column and eluted in the same buffer with 0.1 ml/min flow speed. For refolded in A8-35 connexin 32 equilibration buffer without any detergent was used. For Cx32/DDM 0.2% DDM solution in the same buffer was used for column equilibration. With green elution profile of A8-35 is shown. Grey lines correspond to calibration with globular proteins.

Since connexin was expressed in E.coli some lipids might be still associated with protein even after purification. This may explain observed peak widening in comparison to Cx32/A8-35 from CFPS. In CFPS most E.coli lipids are removed from lysate on preparation stage so much less of them appear in the expression mixture. Cx32/A8-35 peak shift in comparison with Cx32/SDS peak may indicate that in case of amphipol less detergent molecules are involved in hemichannel stabilization and that protein is less unfolded. For further in meso crystallization experiments it was important to remove excessive amphipol, not-associated with the hemichannel. With this size-exclusion chromatography also helped, since amphipol without protein migrated significantly faster (Figure 3.35 green line) and could be eliminated in such a manner from the protein sample.

Since Cx26 solubilized in DDM [10] was successfully crystalized we decided to try replacing A8-35 with DDM. For this purpose to Cx32/A8-35 eluate after size-exclusion chromatography we added 1% DDM. The sample was incubated for 1 h at 4°C with gentle
stirring. Then it was applied onto Superose6HR 24 ml column pre-equilibrated with a buffer containing 0.2% DDM. Elusion profile is shown on Figure 3.35 with yellow line. Significant peak widening indicates that Cx32 is destabilized when transferred to DDM and may be also partially unfolded. For this reason we decided to decline this procedure. For further crystallization Cx32/A8-35 complex was chosen.

3.3.1.2. DLS with Cx32 in amphipol

To study refolded into amphipol connexin monodispersity dynamic light scattering was used. The middle of the Cx32/A8-35 peak after size-exclusion chromatography was analyzed. Results are shown on Figure 3.36.

![Figure 3.36](image)

Figure 3.36. DLS measurements of Cx32/A8-35 complex monodispersity. The middle of Cx32/A8-35 peak after size-exclusion chromatography was analyzed. Measurements were carried out at 20 °C.

Main fraction of particles in the sample had hydrodynamic radius of 5.8 nm that is in a good agreement with the previously reported data for Cx26 hemichannel [190]. The deviations of 0.4 nm can be explained by method error and lipids depletion during purification process.

3.3.1.3. Electron microscopy with Cx32 in amphipol and DDM

For electron microscopy analysis fractions with connexin 32 refolded in amphipol after size-exclusion chromatography corresponding to hexamer (200 kDa) by size were supplied. Resulting photographs are shown on Figure 3.37 (A and B). Observed particles had the size that was in a good agreement with the previously reported for connexin 32 solubilized from gap junctional plaques [16]. The size distribution and presence of small aggregates were caused by amphipol, since the same effects were previously reported in case of bacteriorhodopsin trapped with amphipol [191]. Absence of doughnut-shaped connexons could be explained by pore blockage with side-chain groups of amphipol or with C-terminus of connexin 32 (also polyhistidine tag might make a contribution).
Figure 3.37. Solubilized connexin 32 negatively stained with uranyl acetate. (A) and (B) Cx32 was solubilized from total E.coli membranes in 1% NLS, purified by affinity chromatography in 0.2% SDS and then refolded in amphipol A8-35 by SDS precipitation as its potassium salt. Prior to EM analysis Cx32 was additionally purified by size-exclusion chromatography, excessive amounts of amphipol not associated with connexin were removed. (C) and (D) Cx32 transferred to DDM after amphipol assisted refolding from SDS. Before EM measurements the sample was applied to Superose6HR 24 ml column, pre-equilibrated with 0.2% DDM to remove amphipol. Fractions corresponding to dodecamer by size were further studied by EM. (A), (B) and (C) bars 20 nm, (D) bar – 100 nm.

Transfer of Cx32 from amphipol to DDM resulted in protein aggregation (C and D) that corresponded to observed SEC peak widening (Figure 3.35 yellow line). Connexons were probably destabilized with A8-35 removal, which resulted in their aggregation.

3.3.1.4. Crystallization of Cx32/amphipol in meso

Fractions corresponding by size to hemichannel after size-exclusion chromatography of Cx32 refolded into amphipol were concentrated to final protein concentration 30 mg/ml using centrifugal filter with membrane cut-off 30 kDa. Protein concentration was controlled by NanoDrop spectrophotometer.

Detergents (OG, DDM, FOS12) were added to the concentrated protein solution till the aimed concentration is achieved (12% OG, 24-30% DDM, 30% Fos12). Detergent
concentration was checked by IR spectroscopy. The protein solution was added to monooleoyl in a ratio 1:1 (vol:vol) and several passes through interconnected syringes for cubic phase preparation were performed. Cubic phase was incubated overnight at room temperature and then used for crystallization screenings.

Crystallization was carried out using robot “NT 8 Crystallography” for in meso membrane protein crystallization. Crystallization probes were visualized and systematically scanned by automated system for imaging crystallization probes.

Till now (two months) no crystals have been observed in the probes. But since in case of bacteriorhodopsin under certain crystallization conditions first crystals were found only after 6 months we continue crystallization experiments with Cx32/A8-35 varying different parameters.

3.3.2. Purification in NLS/DDM

To obtain Cx32 solubilized in “mild” DDM or Fos12 first we tried to replace NLS on Ni\(^{2+}\)-NTA column. For this the same procedure as described above was used only SDS in all buffers was replaced with either 0.2% DMM or 0.2% Fos12. Unfortunately, no connexin 32 was observed in the eluates, seemed that it stuck on the resin.

Then we decided to try “two-detergent” approach, previously used for purification of Cx32 in fusion to Mistic. Isolation and washing of E.coli total membranes was done following the same procedure as described above. On solubilization stage combination of two detergents was added – either 1% NLS and 1% DDM or 1% NLS and 1% Fos12. First, more “mild” detergent was added to the membranes suspension and the mixture was incubated for 1 h at 4°C with vigorous mixing. Next, NLS was added and solubilization was carried out overnight.

The supernatants after removal of insoluble fragments by centrifugation were diluted with solubilization buffer to reduce NLS concentration to 0.5%, 20 mM Imidazole was added to increase binding specificity. The mixtures were applied to Ni\(^{2+}\)-NTA columns pre-equilibrated with solubilization buffer (containing 0.5% NLS/0.5% DDM or 0.5% NLS/0.5% Fos12). Columns were washed with 5×CV of washing buffer with 0.5% of both detergents. Then, washing with 5×CV of washing buffer, containing only “mild” detergents (0.2% DDM or 0.2% Fos12) was carried out. Finally, bound proteins were eluted in 2×CV of elution buffer containing same concentrations of “mild” detergents. The purifications on chromatographic system were done at 4°C.
To study oligomeric composition of purified connexin 32 these eluates were further subjected to size-exclusion chromatography. The detergent concentrations in the elution buffer corresponded to the ones of Ni\(^{2+}\)-NTA elution buffers. Resulting chromatograms are illustrated on Figure 3.38.

![Figure 3.38](image)

**Figure 3.38.** Size-exclusion chromatography of Cx32 solubilized in 0.2% Fos12 (blue) or 0.2% DDM (red). Cx32 in 0.2% Fos12 or 0.2% DDM after purification by metal affinity chromatography was loaded onto pre-equilibrated with equilibration buffers (with the same Fos12 and DDM concentrations) Superose6HR 24 ml column and eluted in the same buffers with 0.1 ml/min flow speed. Grey lines correspond to calibration with globular proteins. With green elution profile of Cx32 refolded in amphipol after SDS precipitation is depicted.

In Fos12 (blue line) several oligomeric states were present, which indicated that it did not stabilize desirable hexamers properly and could not be used for its purification. In DDM we also observed polydispersity (red line). But particles with 200 kDa size dominated and could be separated from others. This allows preparing monodisperse sample, suitable for further crystallization by vapor diffusion method.

3.3.2.1. EM with Cx32 purified in DDM

Again for electron microscopy analysis fractions with connexin 32 solubilized and purified in DDM and Fos12 after SEC corresponding to hexamer (200 kDa) by size were supplied. Resulting photographs are shown on Figure 3.39.

In case of DDM usage for solubilization and purification (Figure 3.39A) observed particles had similar geometry and size distribution as previously received for Cx32 refolded...
in amphipol (Figure 3.37A). This time we also did not observe doughnut-shaped connexons and again blamed detergent and C-terminus.

**Figure 3.39.** Solubilized connexin 32 negatively stained with uranyl acetate. (A) Cx32 was solubilized from total *E. coli* membranes in 1% NLS and 1% DDM, purified by affinity chromatography in 0.2% DDM. Prior to EM analysis Cx32 was additionally purified by size-exclusion chromatography, the peak corresponding by size to hexamer was further supplied for EM. (B) Cx32 was solubilized from total *E. coli* membranes in 1% NLS and 1% Fos12, purified by affinity chromatography in 0.2% Fos12. As for DDM the peak corresponding by size to hexamer after size-exclusion chromatography was further supplied for EM. Bars, 20 nm.

Again for electron microscopy analysis fractions with connexin 32 solubilized and purified in DDM and Fos12 after SEC corresponding to hexamer (200 kDa) by size were supplied. Resulting photographs are shown on **Figure 3.39**.

In case of DDM usage for solubilization and purification (Figure 3.39A) observed particles had similar geometry and size distribution as previously received for Cx32 refolded in amphipol (Figure 3.37A). This time we also did not observe doughnut-shaped connexons and again blamed detergent and C-terminus.

In case of Fos12 observed by SEC sample inhomogeneity was visualized also by EM. Some filament-like aggregates were found in the sample. Similar particles were previously reported when purified Cx32 was incubated with polyethylene glycol 2000 [16].
References


[139] A.L. Harris, Connexin channel permeability to cytoplasmic molecules., Progress in Biophysics and Molecular Biology. 94 (n.d.) 120–43.


Genes of proteins used in this work for generating Cx32 constructs are listed below.

**Mistic (MstX) from Bacillus subtilis:**

```
atgttttgtacatatttttggaaaacatccggaagctgggttactgttagagataaagcagcgggttactgggttagctgtataatctatctggac
aggtgaggaagagaagacgtgacacagcagcaaatctgaccgcacgtgcactgtcgagctgtgattaatatttgagacgctgcagctggtgaatg
```

**Wild type human connexin 32:**

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agtgaactgagacaggttgttacattcctgctgatgctgtctgacatcaggccgagtggtctgtctgtgtttctttcttctgtctgttgtgctgttgatggt
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Abstract

The genome sequencing projects reveal that integral membrane proteins represent up to one third of the predicted proteins of all organisms examined so far. Furthermore, membrane proteins are targets of the large majority of the drugs that are currently in use. About 80% of all cellular responses are thought to occur through the proteins linked to the cell membrane. An ultimate understanding of their function depends on detailed structural data for each class of membrane proteins, such as transporters, receptors, channels and pumps. The structural information could also greatly improve the efficiency of drug discovery. To date, the 3D structures of only a tiny fraction of membrane proteins (around 218) are known in atomic details, though structures of around 50000 soluble proteins have already been solved.

Difficulties to determine 3D structures of integral membrane proteins are related to their low natural abundance, hydrophobic nature, and inhomogeneity due to post-translational modifications. They are expressed at rather low levels and usually constitute less than 30% of a total cellular protein. This requires developing of an efficient overexpression system to produce a sufficient amount of protein for crystallization experiments.

*E.coli* is a convenient host for heterologous protein expression. Its advantages include well-established protocols, fast growth, high level of expression and scalability of experiments and low cost. Also it is important to mention that 80% of all protein structures deposited in the protein data bank were overexpressed in *E.coli*. However, heterologous gene expression in *E.coli* can lead to the production of misfolded, aggregated, and/or nonfunctional target proteins. This is often due to the absence of cofactors or lack of post-translational modifications required for function, stability and folding.

Taken together these features make the development of methods for the study of membrane proteins lag far behind those of soluble proteins. Finally, one has to point out that large amounts of pure and homogeneous protein are absolute prerequisites to begin with crystallization experiments.

Connexins are the class of membrane proteins. In living organisms connexins are the principal protein component of gap junctions – specialized cell-cell junctions that directly link the cytoplasm of adjacent cells. Recent discoveries provide the direct evidence that gap junctional intercellular communication is essential for tissue functions and organs development, and that its dysfunction causes diseases. At least nine human maladies are currently associated with mutations in genes encoding connexins. Also there are strong evidences that connexins function as malignant tumor suppressors.

Despite connexins relevance and the fact that connexin studies have been started long time ago atomic resolution structure of gap junction formed by human connexin 26 is only available. This is mostly explained by high complexity of overexpression and purification of connexins in sufficient for crystallization amounts in mammalian or insect cells, which are commonly used for functional expression of mammalian proteins. The second problem is that connexins are subjected to post-translational modifications, which need to be removed to proceed with crystallization. And each additional purification step is crucial for final protein yield. On the other hand expression of recombinant proteins in *E.coli* eliminates most of post-translational modifications and can solve the problem.
Human connexin 32 among other members of connexin family it is one of the best studied. A lot of data concerning its conductance and permeability properties are available. Furthermore, connexin 32 was previously successfully produced and purified from mammalian cells, \textit{in vitro}, and from baculovirus/insect cells. That is why there exists a good possibility to compare functional properties of this connexin overexpressed in different systems. However, previous attempts at expression and purification of full-length connexin 32 from \textit{E.coli} were not successful. In this work, we show that this is indeed possible. We developed the protocol for expression and purification of functional protein, able to form hemichannels. It is important to stress that there is no experimental atomic resolution structure of connexin 32 that can elucidate its mechanism of function. Understanding the great demand for atomic structure two models based on cryo-electron microscopy maps were proposed. But still high-resolution X-Ray structure of connexin 32 is to be determined.

To summarize, in this work we overexpressed connexin 32 using membrane-targeting expression tag. Membrane-integrating protein MstX from \textit{Bacillus subtilis} was attached to N-terminus of Cx32 and helped to increase tremendously expression yield. Since addition of N-terminal extensions suppresses connexins functions all excessive amino acids must be removed. We demonstrated that MstX is resistant to various cleavage conditions including different proteases usage and variations of linker in between. Also we expressed Cx32 without any expression tag \textit{in vitro} using \textit{E.coli} S30 extract and refolded purified connexins into hemichannels. Then, since expression yield was still not high enough, we did broad screening of cell free reaction conditions and examined influence of short peptides addition on N-terminus. Next, we managed to express and purify connexin 32 from \textit{E.coli} using certain gene sequence optimization. The refolding procedure was developed and Cx32 functionality was confirmed by assembling it into hemichannels. Further this protein was used for crystallization experiments.
Zusammenfassung

Genomsequenzierungsprojekte zeigen, dass integrale Membranproteine bei allen bisher untersuchten Organismen bis zu ein Drittel der vorhergesagten Proteine ausmachen. Darüber hinaus zielt die große Mehrzahl heute verwendeter medizinischer Wirkstoffe auf Membranproteine ab. Es wird angenommen, dass etwa 80 % aller Zellreaktionen über die mit der Zellmembran verbundenen Proteine ablaufen. Ein abschließendes Verständnis ihrer Funktion erfordert detaillierte Strukturdaten für alle Arten von Membranproteinen, z. B. Transporter, Rezeptoren, Kanäle und Pumpen. Diese Strukturinformationen könnten auch die Effizienz bei der Entdeckung neuer Wirkstoffe stark erhöhen. Obwohl die Struktur von etwa 50.000 löslichen Proteinen bereits aufgeklärt werden konnten, ist bis heute nur von einem kleinen Bruchteil der Membranproteine (ca. 218) die 3-D-Struktur auf atomarer Ebene bekannt.

Schwierigkeiten bei der Bestimmung der 3-D-Strukturen integraler Membranproteine hängen mit ihrem geringen natürlichen Vorkommen, ihrer hydrophoben Beschaffenheit und der Inhomogenität aufgrund posttranslationaler Modifikationen zusammen. Sie werden in recht kleinen Mengen exprimiert und machen in der Regel weniger als 30 % des Gesamtvorkommens eines Zellproteins aus. Es muss daher ein effizientes System für die Überexpression entwickelt werden, um eine für die Kristallisation ausreichende Proteinmenge herzustellen.


Aufgrund all dieser Faktoren liegt die Methodenentwicklung für die Untersuchung von Membranproteinen im Vergleich zu löslichen Proteinen noch weit zurück. Nicht zuletzt sind große Mengen reinen, homogenen Proteins eine Grundvoraussetzung für Kristallisationsversuche.


Trotz der Bedeutung von Connexinen und der Tatsache, dass sie bereits seit einiger Zeit untersucht werden, ist bisher nur für das menschliche Connexin 26 die atomare Struktur der Gap Junction bekannt. Dies ist vor allem auf die hohe Komplexität der Überexpression und


In dieser Arbeit wurde Connexin 32 mithilfe eines Membranexpressions-Tags überexprimiert. Das integrale Membranprotein MstX aus *Bacillus subtilis* wurde am N-Terminus von Cx32 angebaut, was zu einer enormen Verbesserung der Expressionsausbeute beitrug. Da die Zugabe N-terminaler Erweiterungen Connexinfunktionen unterdrückt, müssen alle überschüssigen Aminosäuren entfernt werden. Es konnte gezeigt werden, dass MstX gegenüber verschiedenen Spaltungsbedingungen resistent ist, darunter der Verwendung verschiedener Proteasen und der Variation des zwischengeschalteten Linkers. Außerdem wurde Cx32 ohne Expressions-Tag unter Verwendung von S30-Extrakt von *E. coli* *in vitro* exprimiert und die aufgereinigten Connexine wurden wieder in Hemikanäle gefaltet. Da die Expressionsausbeute noch immer nicht ausreichend war, wurde ein grobes Screening der Bedingungen für zellfreie Reaktionsvorgänge durchgeführt und der Einfluss einer Zugabe kurzer Peptide am N-Terminus untersucht. Daraufhin konnte Connexin 32 durch Optimierung einer bestimmten Gensequenz in *E. coli* exprimiert und aufgereinigt werden. Es wurde ein Vorgehen für die Neuufaltung entwickelt und die Funktionalität von Cx32 wurde durch die Neuufaltung des Proteins in Hemikanäle nachgewiesen. Das Protein wurde darüber hinaus auch für Kristallisationsversuche verwendet.