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Lipid–protein nanodiscs for cell-free production of integral membrane proteins in a soluble and folded state: Comparison with detergent micelles, bicelles and liposomes

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ABSTRACT

Production of integral membrane proteins (IMPs) in a folded state is a key prerequisite for their functional and structural studies. In cell-free (CF) expression systems membrane mimicking components could be added to the reaction mixture that promotes IMP production in a soluble form. Here lipid–protein nanodiscs (LPNs) of different lipid compositions (DMPC, DMPG, POPC, POPC/DOPG) have been compared with classical membrane mimicking media such as detergent micelles, lipid/detergent bicelles and liposomes by their ability to support CF synthesis of IMPs in a folded and soluble state. Three model membrane proteins of different topology were used: homodimeric transmembrane (TM) domain of human receptor tyrosine kinase ErbB3 (TM-ErbB3, 1TM); voltage-sensing domain of K^+ channel KvAP (VSD, 4TM); and bacteriorhodopsin from *Exiguobacterium sibiricum* (ESR, 7TM). Structural and/or functional properties of the synthesized proteins were analyzed. LPNs significantly enhanced synthesis of the IMPs in a soluble form regardless of the lipid composition. A partial disintegration of LPNs composed of unsaturated lipids was observed upon co-translational IMP incorporation. Contrary to detergents the nanodiscs resulted in the synthesis of ~80% active ESR and promoted correct folding of the TM-ErbB3. None of the tested membrane mimetics supported CF synthesis of correctly folded VSD, and the protocol of the domain refolding was developed. The use of LPNs appears to be the most promising approach to CF production of IMPs in a folded state. NMR analysis of ^{15}N-Ile-TM-ErbB3 co-translationally incorporated into LPNs shows the great prospects of this membrane mimetics for structural studies of IMPs produced by CF systems.

1. Introduction

Integral membrane proteins (IMPs) are responsible for a variety of essential biological functions in living cells and multicellular organisms. These proteins play a crucial role in the cell communication with the environment, intercellular recognition, signal transduction, cell energetics, and transport of various substances through the membrane [1]. According to genomic data the IMPs represent over 25% of the proteins encoded in the genome of higher animals [2]. Nevertheless, functional and structural IMP studies are complicated as compared with the situation observed for water-soluble globular proteins. These difficulties are caused by the requirement for a membrane-like environment to stabilize a correct spatial structure and to support a functional activity of IMPs in solution [1]. One of the main problems hindering IMP investigations is a large-scale production of proteins in a functional state [3]. Traditional cell-based expression systems have serious shortcomings when applied to the membrane protein production [1,3]. The efficient refolding protocol is usually needed to endow recombinant IMPs with functionality [4].

Abbreviations: Brij-35, dodecylpolyoxyethylene 35; Brij-58, hexadecylpolyoxyethylene 58; Brij-78, octadecylpolyoxyethylene 78; Brij-98, octadecylpolyoxyethylene 98; CF, cell-free; DC7PC, 1,2-diheptanoyl-sn-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; DOPG, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol; DPC, dodecylphosphocholine, FOS-12; DTSP, 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester); ESR, bacteriorhodopsin from *Exiguobacterium sibiricum*; FM, feeding mixture; FOS-14, tetradecylphosphocholine; HSQC, heteronuclear single quantum correlation; IMP, integral membrane protein; LDAO, n-dodecyl-N,N-dimethylamine-N-oxide; LPN, lipid–protein nanodisc; LS, lauryl sarcosine; MSP, membrane scaffold protein; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; RM, reaction mixture; R_{St}, hydrodynamic radius of a particle, Stokes radius; shTM-ErbB3, shortened transmembrane domain of human receptor tyrosine kinase ErbB3; SDS, sodium dodecyl sulfate; TM, transmembrane; TM-ErbB3, transmembrane domain of human receptor tyrosine kinase ErbB3; TROSY, transverse relaxation-optimized spectroscopy; Triton X-100, polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether; Tween-20, polyoxyethylene(20)sorbitan monolaurate; VSD, voltage-sensing domain of KvAP channel from *Aeropyrum pernix*

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In recent years, cell-free (CF) systems [5] have attracted much attention as alternative tools for the recombinant production of membrane proteins [6]. As compared to the cell-based expression, these systems display several advantages: they allow synthesis of toxic proteins [7,8], significantly simplify synthesis of the selectively labeled proteins for their structural and biochemical studies [9–13] and permit a direct addition of the specialized agents or co-factors to the reaction mixture (RM), which promote the formation of the native structure of the synthesized protein and enhance its stability in solution [6]. The components of the classical membrane mimicking media such as detergent micelles [14,15], lipid/detergent mixtures (bicelles) [16], liposomes [17], as well as synthetic surfactants like amphipols [18], fluorinated surfactants [19], and fructose-based polymers (NVoy) [20] can be added to the RM to facilitate membrane protein production in a soluble form. At the same time the question on the functional properties of the synthesized soluble IMPs still remains open.

The lipid–protein nanodiscs (LPNs) represent the recently introduced membrane mimicking medium consisting of discoid patches of the lipid bilayer (~10×4 nm, ~150 lipids) stabilized in solution by two copies of either apolipoprotein or special membrane scaffold protein (MSP) [21]. It was suggested that the LPN membrane supported the native state of the incorporated membrane proteins [22] providing a suitable environment for functional and structural studies [23–27]. Several reports demonstrated a successful application of nanodiscs for CF synthesis of soluble IMPs [28–30]. Two methods were described: an addition of preformed nanodiscs in the RM [28,29] and cell-free co-expression of the MSP with membrane proteins in the presence of phospholipids [30].

The properties of the lipids used for the LPN formation (charge, saturation, etc.) could be important for folding and functioning of the synthesized proteins. In the present study the preformed LPNs of different lipid compositions (DMPC, DMPG, POPC, or POPC/DOPG 3:1 mixture) were compared with the detergent micelles, lipid/detergent bicelles, and lipid vesicles by their ability to promote the cell-free synthesis of IMPs in soluble and folded forms. We also investigated the refolding of the precipitated IMPs synthesized without membrane mimetics. There were investigated three model membrane proteins with the different number of transmembrane helices: the homodimeric transmembrane domain of human receptor tyrosine kinase ErbB3 (TM-ErbB3, residues 632–675, 1TM), the voltage-sensing domain of K^+ channel KvAP from *Aeropyrum pernix* (VSD, residues 1–148, 4TM), and bacteriorhodopsin from *Exiguobacterium sibiricum* (ESR, 7TM). The structural and functional (if possible) properties of the obtained protein preparations were analyzed to determine the functional relevance of their structural states.

Recent NMR study of the CF expressed TM-ErbB3 in the environment of DPC (FOS-12) micelles revealed the formation of the specific parallel homodimers of the domain [31]. The homodimeric structure of the TM-ErbB3 probably corresponds to the native state of this domain in the biological membrane [32]. In the present work the spatial structure of the TM-ErbB3 synthesized in the presence of different membrane mimetics was tested using chemical cross-linking and NMR spectroscopy. It was assumed that only a correctly folded domain was able to form homodimers providing 2D $^{1}H,^{15}N$-correlation spectra (HSQC or TROSY) similar to the previously reported ones [31]. The spatial structure of the CF synthesized VSD was analyzed by $^{1}H,^{15}N$ NMR spectroscopy using the published spectra of the natively folded domain extracted from the bacterial membrane [23,33]. The functional activity of the CF synthesized ESR preparations was monitored by UV–vis spectroscopy, assuming that the functionally active protein produced in the presence of the *all-trans*-retinal should demonstrate the absorbance at 534 nm and a purple color, similar to the protein expressed within the *Escherichia coli* membrane [34].

The obtained results revealed a marked dependence of the CF synthesis efficiency on the topology of the IMPs and properties of the membrane-mimicking system. The majority of detergents significantly enhanced the solubility of all the synthesized IMPs relative to the control experiments performed without any membrane mimetics. At the same time the detergents were able to support the quasi-native folding only of the 1TM protein (TM-ErbB3) and did not provide the correct folding of the polytopic proteins (VSD and ESR). The lipid/detergent bicelles demonstrated a diminished yield of the soluble IMPs as compared to the detergent micelles, but supported synthesis of correctly folded ESR and TM-ErbB3. An enhancement of the IMP solubility upon the CF synthesis in the presence of liposomes was observed only for the TM-ErbB3. In contrast to that the LPNs significantly enhanced the solubility of all IMPs irrespective of the lipid composition. The LPNs formed of the saturated lipids (DMPC, DMPG) appeared the most stable during the co-translational incorporation of the membrane proteins. The application of the nanodiscs resulted in the correct folding of TM-ErbB3 and ESR, but the produced VSD was misfolded. The refolding from the RM precipitate was the only approach to produce the VSD with a native-like spatial structure. NMR analysis of the ^{15}N-Ile-labeled TM-ErbB3 points to a possibility of direct structural studies of membrane proteins co-translationally incorporated into nanodiscs.

2. Materials and methods

2.1. Cell-free production of the IMPs

IMPs were produced using the continuous exchange cell-free system as a modification of the earlier described method [6]. The bacterial S30 extract was prepared from *E. coli* (strain A19). The T7 polymerase was produced as described in [35]. The RM contained the following components: 100 mM HEPES-KOH (Sigma, St. Louis, MO), pH 8.0, 8 mM (11 mM for the TM-ErbB3) $Mg(OAc)_2$ (Sigma), 130 mM (80 mM for the TM-ErbB3) KOAc (Sigma), 25 mM acetyl phosphate (Sigma), 2 mM 1,4-dithiothreitol (Sigma), 25 mM phosphoenol pyruvate (Sigma), 2.3 mM of each amino acid: Arg, Cys, Trp, Met, Asp and Glu (Sigma); 1.3 mM of every other amino acid (Sigma), 0.15 mg/ml folic acid (Sigma), 1.4 mM ATP (Sigma), 1 mM of GTP, CTP, and UTP (Sigma), 1 tablet per 10 ml of a complete protease inhibitor (Roche Diagnostics, Mannheim, Germany), 0.05% NaN3 (Sigma), 2% PEG 8000 (Sigma), 0.3 U/μl RiboLock (Fermentas, Vilnius, Lithuania), 0.04 mg/ml of pyruvate kinase (Fermentas), 0.0055 mg/ml T7 RNA polymerase, 0.5 mg/ml tRNA from *E. coli* MRE 600 (Roche Diagnostics), 0.2 mg/ml of plasmid *pET28a(+)/VSD* [33] or 0.3 mg/ml of *pET-22b(+)/TM-ErbB3* [36] or 0.3 mg/ml of *pET-32a(+)/ESR* [34], 30% of the S30 extract from *E. coli*. The concentrations of $Mg(OAc)_2$, KOAc and the plasmid were optimized for each protein. The feeding mixture (FM) contained the same components except high-molecular substances, i.e. the ferments, S30 extract, plasmid, inhibitor of ribonucleases. The FM/RM ratio was 15/1. Cell-free reactions were performed using the membrane tubing with the cut-off of 12 kDa (Sigma). The synthesis was carried out at 30 °C with a gentle mixing for 20 h. A shortened variant of TM-ErbB3 (residues 639–670, shTM-ErbB3) was synthesized using the same protocol as for the TM-ErbB3. For the ESR synthesis the RM and FM were supplemented with 0.1 mM of *all-trans*-retinal (Sigma). The retinal addition did not influence the yield of the synthesized protein. The ^{15}N-Ile-labeled TM-ErbB3 and ^{15}N-Ala-labeled VSD were produced by replacement of Ile and Ala in the RM and FM with ^{15}N-Ile and ^{15}N-Ala (CIL, Andover, MA), respectively.

The detergents (Brij-35, Brij-58, Brij-78, Brij-98, LS, SDS, Triton X-100, Tween-20) were the product of Sigma and (CHAPS, cholate, DPC, DDM, FOS-14, LDAO) were charged from Anatrace Inc. (Maumee, OH). The detergents (DHPC, DC7PC) and the lipids used (DMPC, DMPG, POPC, DOPG) were the product of Avanti Polar Lipids (Alabaster, AL). The LPNs were prepared using the membrane binding domain (44–243 fragment, 26 kDa) of the human apolipoprotein A-I containing *N*-terminal His_6-tag (MSP) by the protocol described in [24,37]. The

LPN concentration was calculated from the UV absorbance at 280 nm provided that each nanodisc contains two copies of MSP ($E_{280,\ MSP}$ = 26,930). DMPC small unilamellar vesicles were prepared using the Avanti Mini-Extruder and 100 nm polycarbonate membranes. The formation of DMPC/DHPC and DMPC/CHAPS (1:2 = q = 0.5) bicelles was optimized by several freezing/thawing cycles.

The detergents were added in various concentrations to the RM and FM, if needed. The membrane mimicking components such as LPNs, liposomes, and bicelles were added only to the RM. In the case of the DMPC/DHPC and DMPC/CHAPS bicelles the equivalent concentrations of DHPC and CHAPS were added to the FM to prevent detergent dilution. An optimal concentration of the detergents in all cases (except DC7PC, and FOS-14/CHAPS mixture) was 1%, use of a lower detergent concentration reduced the quantities of the synthesized soluble proteins. An increase in the detergent concentration up to 2% didn't enhance the yield of soluble IMPs. The concentrations of the DC7PC (0.2%) and FOS-14/CHAPS mixture (2 mM/3 mM) were taken as in [38,39] and were not further optimized. The unilamellar DMPC vesicles were used at the 30 mM lipid concentration. The optimal concentration of DMPC/DHPC bicelles was found to be ~15/30 mM (that corresponds to 1.3% of DHPC), and of the LPNs — 0.05 mM.

2.2. Quantification and purification of the IMPs

Soluble and insoluble fractions of the RM after CF synthesis were separated by centrifugation for 15 min, 14,000 rpm. The obtained fractions were analyzed by SDS-PAGE. All the studied IMPs contained the *C*-terminal His_6-tag. The quantities of the proteins produced in soluble and insoluble forms in the presence of detergent micelles, bicelles, liposomes and LPNs were estimated by Western blot using His-tag monoclonal antibodies (Merck, Darmstadt, Germany). For this purpose each SDS-PAGE gel was supplemented with a 'reference' lane(s) containing the IMP/detergent complex(es) of the known concentration. The TM-ErbB3, VSD, and ESR samples synthesized and purified in the presence of Brij-58, Brij-35, and Brij-98, respectively, were used as the references. The blots were analyzed by densitometry using the OptiQuant 3.00 software (Packard Instrument Company). The total amount of the synthesized protein was calculated as a sum of the protein amounts in soluble and insoluble fractions of the RM.

IMP/detergent complexes were purified from the RM soluble fraction on the Ni^{2+}-Sepharose 6 Fast Flow column (GE Healthcare, Sweden) by elution with the buffer A (20 mM Tris–HCl, 250 mM NaCl, 1 mM NaN3, pH 8.0) containing 100 mM (VSD, ESR), or 300 mM (TM-ErbB3) of imidazole and 0.2% of an appropriate detergent. The concentration of the purified IMPs was quantified according to the molar extinction coefficients ($E_{280,\ shErbB3}$ = 6990, $E_{280,\ ErbB3}$ = 6990, $E_{280,\ VSD}$ = 18,910, $E_{280,\ ESR}$ = 46,870) by measuring the UV absorbance at 280 nm. Molecular masses of the proteins were 4693 Da, 5972 Da, 17,328 Da, and 28,848 Da for the shTM-ErbB3, TM-ErbB3, VSD, and ESR, respectively. The concentration of the active ESR was measured from the absorbance at 534 nm assuming the molar extinction coefficient of 43,000. IMP/LPN complexes were purified from the RM soluble fraction on the Ni^{2+}-column by elution with the buffer A containing 300 mM imidazole.

2.3. Refolding of the VSD from the RM precipitate

The RM containing the VSD synthesized without membrane mimetic components was centrifuged for 15 min, 14,000 rpm. The precipitate was disintegrated in the buffer A (20 ml of buffer per 1 mg of the VSD) by sonication (Branson Digital Sonifier, Danbury, CT) at the output power 50 W and at 4 °C for 1 min. Then 8 M urea and 1% SDS were added to the protein suspension. Denatured VSD was loaded on the Ni^{2+}-column and refolded by a gradual replacement of the urea and SDS with DPC. For this aim, the Ni^{2+}-Sepharose 6 Fast Flow column (10 ml of resin per 1 mg of the VSD) was washed with 10 column volumes of 1% DPC at the flow rate 0.3 ml/min. Refolded VSD was eluted with the buffer A containing 100 mM imidazole and 0.2% DPC.

2.4. Size-exclusion chromatography

The IMP/LPN complexes were analyzed by size-exclusion chromatography performed on the Superdex-200, Tricorn 5/200 column (GE Healthcare) loaded into 20 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 1 mM NaN3, pH 7.4. Dextran 2000, thiroglobulin (MW 669 kDa, hydrodynamic Stokes radius (R_{St}) 85 Å), ferritin (MW 440 kDa, R_{St} 61 Å), catalase (MW 232 kDa, R_{St} 52.2 Å), aldolase (MW 158 kDa, R_{St} 48.1 Å), BSA (MW 67 kDa, R_{St} 35.5 Å), and ovalbumin (MW 43 kDa, R_{St} 30.5 Å) from high and low weight calibration kits (GE Healthcare) were used for a calibration. The elution rate was 0.3 ml/min, the wave length of detection was 280 nm. The measured elution volumes were converted to the R_{St} values via the linear calibration graph (elution volume versus $Log(R_{St})$).

2.5. NMR spectroscopy

2D sensitivity enhanced ^{1}H,^{15}N-HSQC or TROSY spectra were measured on the Bruker AVANCE 700 spectrometer equipped with a cryoprobe. Unless otherwise stated, the spectra were acquired at 43 °C and pH 5.0 (samples in detergent micelles and lipid/detergent bicelles) or at pH 7.0 (samples in LPNs). The measurement time of the HSQC/TROSY spectra was 2 h for the samples in detergent micelles or lipid/detergent bicelles and 12 h for the samples in LPNs. In the last case a slow tumbling of IMP/LPN complexes in solution led to a significant increase in transverse nuclear relaxation and loss of sensitivity, thus requiring prolonged measurements. The buffer composition of the samples after purification on Ni^{2+} column was changed to 10 mM Tris/Ac (pH 7.0) or to 10 mM NaAc (pH 5.0) by repeated dilution/concentration cycles using the Ultrafiltration Cell (NMWL 10,000, Millipore, Billerica, MA).

3. Results

3.1. CF synthesis of IMPs in the presence of detergent micelles

To test the ability of the detergents to support the CF synthesis of the TM-ErbB3, VSD and ESR in a soluble form, a set of the most frequently used non-ionic detergents (DDM, Brij-35, Brij-58, Brij-78, Brij-98, Triton X-100, and Tween-20) was evaluated. The zwitterionic detergent DPC (FOS-12) was also analyzed as it could maintain close to native spatial structure of the TM-ErbB3 [31] and the VSD [33]. In each case the amounts of the IMPs synthesized in soluble and insoluble forms were determined (Fig. 1A). An addition of the DPC micelles to the RM almost completely inhibited synthesis of the model IMPs (Fig. 1A). The application of DDM and Triton X-100 also suppressed the CF production of the VSD and ESR, but resulted in high-yield synthesis of the soluble TM-ErbB3 (Fig. 1A). Tween-20 added to the RM did not suppress the CF system, but supported the synthesis in a soluble form of only TM-ErbB3. On the contrary, the Brij detergents provided the environment for high-yield synthesis (1–2 mg/ml of RM) of all three IMPs in a soluble form (Fig. 1A). Interestingly, distinct detergents from the Brij family were optimal for the production of the different proteins (Fig. 1A). These findings are in good agreement with the results of Klammt et al. [14], indicating the ability of the Brij detergents to support the CF synthesis of soluble IMPs. Noteworthy is the fact that a CF extract contains some fraction of the *E. coli* lipids [6]. This could explain the minor synthesis of the soluble IMPs observed in the control experiments without any membrane mimicking components (Fig. 1A).

To analyze an oligomeric state of the TM-ErbB3 synthesized in the presence of the Brij-58 micelles (this environment provides the

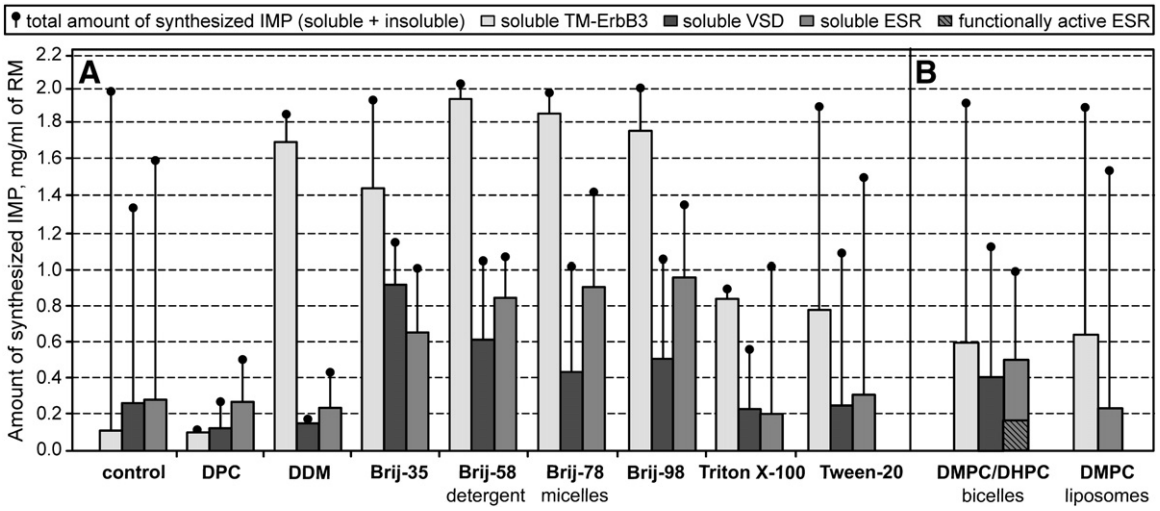

Fig. 1. CF synthesis of the TM-ErbB3, VSD, and ESR in the presence of detergent micelles, bicelles and liposomes. (A) Synthesis in the presence of the detergent micelles (1% w/v). (B) Synthesis in the presence of the DMPC/DHPC (15/30 mM) bicelles and DMPC liposomes (30 mM). The amounts of the IMPs synthesized in a soluble form (bars) were compared with the total amounts of the synthesized IMPs (dots), including a fraction of insoluble proteins. The amounts of the IMPs (mg/ml of RM) were estimated by Western blot as compared to those of the TM-ErbB3, VSD, and ESR synthesized and purified in the presence of Brij-58, Brij-35, and Brij-98, respectively (see Materials and methods). The amount of the functionally active ESR within soluble fraction of RM (hatched bars) was determined by UV–vis spectroscopy. Each value represents the average of the three experiments. A systematic error doesn't exceed 15%. The RM and FM in the control experiments didn't contain any membrane mimicking components.

highest yield of the soluble protein), chemical cross-linking was used. The TM-ErbB3 contains two amine groups (NH_3^+) at Lys639 and Lys674, and a significant extent of cross-linking was expected providing that the domain formed parallel helical homodimers. Indeed, SDS-PAGE analysis of the TM-ErbB3/Brij-58 sample treated by DTSP revealed some quantity of protein homodimers (Fig. 2F, lane 3). The TM-ErbB3 structure in Brij-58 was analyzed by NMR spectroscopy using the selectively ^{15}N-Ile-labeled sample of the domain. The obtained HSQC spectrum (Fig. 2A) did not match well with the HSQC spectrum of the homodimeric ^{15}N-Ile-labeled TM-ErbB3 in the DPC micelles (Fig. 2B) pointing to the differences of the domain spatial structure in these environments. Nevertheless, the observed cross-linking pattern indicated that the domain with the distorted conformation was still capable of forming homodimers. A subsequent exchange of Brij-58 for DPC on Ni^{2+}-column resulted in the properly folded TM-ErbB3 that was confirmed by NMR analysis.

SDS-PAGE analysis of the VSD sample synthesized in Brij-35 revealed the partial protein aggregation (Fig. 3F, lane 2). The VSD structure in this environment was analyzed by NMR spectroscopy. Significantly different spectral patterns were revealed for the ^{15}N-Ala-labeled VSD in the Brij-35 micelles (Fig. 3B) and the uniformly ^{15}N-labeled domain extracted from the *E. coli* membranes in the native-like conformation and solubilized in the DPC/LDAO micelles [23,33] (Fig. 3A). Notably, no characteristic up- and downfield shifts of the Ala74, Ala96, Ala100 signals (Fig. 3A, encircled) originated from intra- and interhelical interactions within the tertiary structure of the VSD [33] were observed in Brij-35. It is known that the Brij detergents do not provide a suitable environment for NMR spectroscopy, however the lack of the characteristic shifts of the Ala resonances pointed to the absence of the native-like tertiary domain structure in the Brij-35 micelles. The attempts to exchange Brij-35 for DPC by Ni^{2+}-chromatography, unlike the TM-ErbB3 situation, resulted in a complete VSD aggregation on the column, that also pointed to incorrect folding of the domain upon synthesis.

Recently DC7PC and FOS-14/CHAPS mixtures were successfully applied for the CF synthesis in the functional form of the 7TM proteins: the proteorhodopsins from the marine proteobacteria [38] and bacteriorhodopsin [39], respectively. These detergents were evaluated for CF production of the ESR in addition to the set of the detergents used for the VSD and TM-ErbB3. The functional activity of the ESR preparations was monitored by UV–vis spectroscopy. The soluble fraction of the RM remained colorless in all the detergents tested pointing to incorrect folding of the ESR. At the same time, the formation of the colored RM precipitate was observed in Tween-20 indicating that this detergent is able to support synthesis of the correctly folded protein, but unable to stabilize it in a soluble state.

3.2. CF synthesis of IMPs in the presence of bicelles and liposomes

The ability of the DMPC/CHAPS and DMPC/DHPC bicelles and DMPC liposomes to support the CF synthesis of the soluble IMPs was investigated. The use of the DMPC/CHAPS (1:2) bicelles almost completely suppressed the IMP synthesis, probably due to the inhibitory activity of CHAPS [39]. In contrast to that, the DMPC/DHPC (1:2) bicelles increased the solubility of the synthesized TM-ErbB3, VSD and ESR, but with a lower efficiency compared with the detergent micelles (Fig. 1B).

For NMR analysis the RM soluble fraction containing the synthesized ^{15}N-Ile-labeled TM-ErbB3 in bicelles was concentrated twice, heated at 45 °C (pH 5.0) for 1 h, and then centrifuged to additionally purify the protein/bicelle complexes from the CF system components. The 2D ^{1}H,^{15}N-TROSY spectrum measured for the supernatant (Fig. 2C) revealed the very similar spectral pattern to that observed for the homodimeric domain in the DPC micelles (Fig. 2B). That pointed to the formation of the native-like spatial structure of the TM-ErbB3 during the co-translational incorporation into the DMPC/DHPC bicelles. It should be noted that the components of the bicelles (DMPC and DHPC) could be hydrolyzed under acidic conditions. The stability of the TM-ErbB3/DMPC/DHPC sample was analyzed by 1D ^{1}H NMR spectroscopy. The obtained spectra revealed no lipid hydrolysis products, but detected a considerable amount of low-molecular weight components of the CF system in the sample (data not shown).

The VSD/DMPC/DHPC preparation was unstable and precipitated upon concentration, thus hindering further NMR analysis. The sample instability indicated incorrect folding of the VSD co-translationally incorporated into the bicelles. Notably, according to the published data, the DMPC/DHPC bicelles can support the native-like structure of the VSD extracted from the *E. coli* membranes [23]. UV–vis and Western blot analysis revealed that only 30% of the soluble ESR synthesized in the presence of the DMPC/DHPC bicelles was functional (Fig. 1B).

Application of unilamellar DMPC vesicles did not inhibit the CF system and similar to the bicelles enhanced the TM-ErbB3 solubility

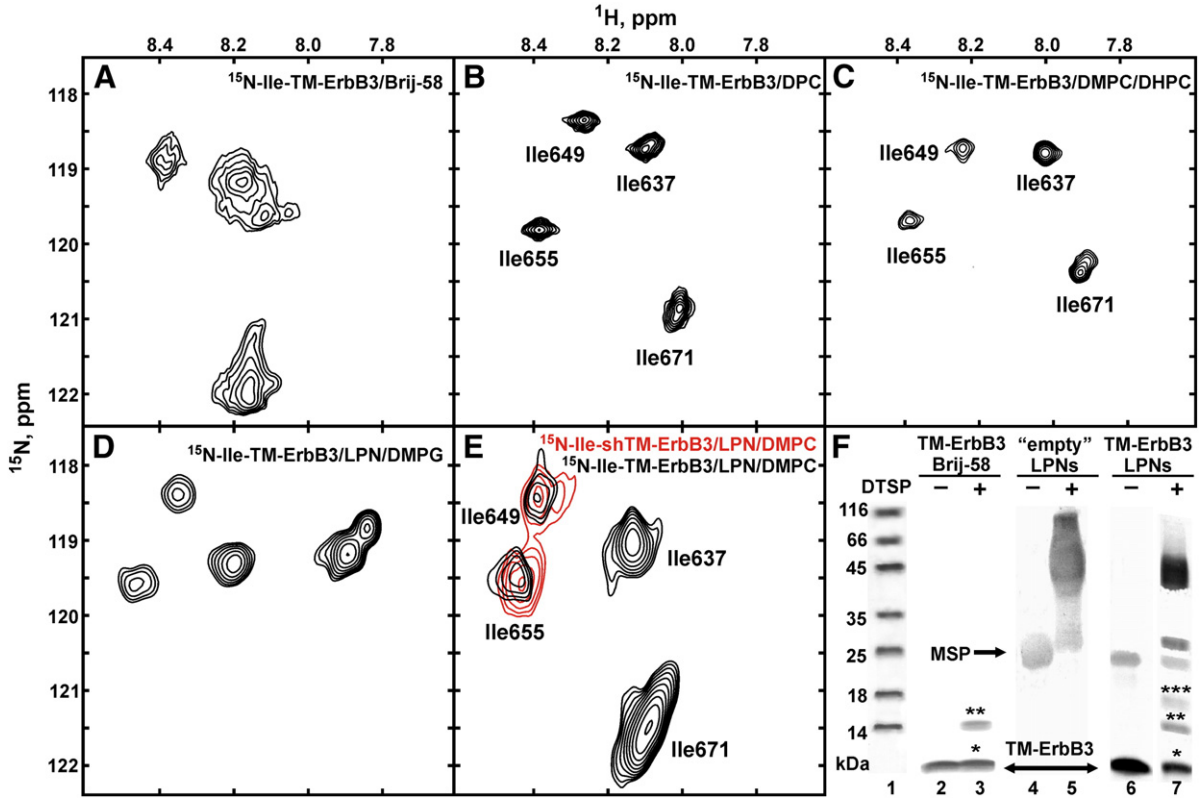

Fig. 2. 2D ^{1}H,^{15}N NMR and SDS-PAGE analysis of the TM-ErbB3 preparations. (A) HSQC spectrum of the ^{15}N-Ile-TM-ErbB3 synthesized in the presence of Brij-58 (pH 5.0). (B) HSQC spectrum of the ^{15}N-Ile-TM-ErbB3 synthesized in the presence of Brij-58 and transferred to DPC. (C) TROSY spectrum of the RM containing the ^{15}-Ile-TM-ErbB3 synthesized in the presence of the DMPC/DHPC (1:2) bicelles. (D) TROSY spectrum of the ^{15}N-Ile-TM-ErbB3 synthesized in the presence of the LPN/DMPG. (E) Superposition of the TROSY spectra of the ^{15}N-Ile-TM-ErbB3 (black) and ^{15}N-Ile-shTM-ErbB3 (red) synthesized in the presence of LPN/DMPC. In each case the concentration of the TM-ErbB3 was about 0.2 mM and the temperature was 43 °C. In (A) and (B) the detergent concentrations were ~10 mM, respectively. The concentrations of DMPC/DHPC in (C) were ~30/60 mM. The concentration of the LPNs in (D, E) was about 0.03 mM. In (A–C) and (D and E) pH values were about 5.0 and 7.0, respectively. (F) SDS-PAGE analysis of the TM-ErbB3 preparations after purification on the Ni^{2+}-column. 1 — Molecular mass markers; 2 — the TM-ErbB3 synthesized and purified in the presence of Brij-58; 3 — the TM-ErbB3/Brij-58 sample treated by DTSP as described in [40]; 4 — the LPN/DMPC sample; 5 — the LPN/DPMC sample treated by DTSP; 6 — the TM-ErbB3/LPN/DMPC sample; and 7 — the TM-ErbB3/LPN/DMPC sample treated by DTSP. The bands corresponding to the monomeric MSP and the TM-ErbB3 are marked by the arrows. The bands corresponding to the monomer, dimer and trimer of the TM-ErbB3 are marked by one, two and three asterisks, respectively.

to ~30% of the totally produced protein (Fig. 1B). At the same time, the ESR synthesized in the presence of the DMPC vesicles formed the purple colored precipitate. Thus, the zwitterionic lipid DMPC in bicelles or liposomes can support only limited synthesis of the TM-ErbB3 and ESR with the native-like conformation, and demonstrates a lower ability to stabilize the synthesized proteins in solution as compared to the detergents micelles.

3.3. Cell-free synthesis of IMPs in the presence of lipid–protein nanodiscs

To investigate the influence of the LPN lipid composition on the CF synthesis of IMPs, the nanodiscs formed from the lipids with a different charge and degree of saturation (DMPC, DMPG, POPC or POPC/DOPG 3:1) were used. SDS-PAGE and Western blot analysis demonstrated that the efficiency of the CF synthesis and the yield of the soluble proteins did not strongly depend on the nanodisk lipid composition. In each case more than 60% of the synthesized IMPs accumulated in a soluble fraction of the RM (Fig. 4A). An optimal concentration of the LPNs was ~0.05 mM. The use of a lower nanodisc concentration diminished the yield of synthesized soluble proteins. An increase in the LPN concentration up to 0.2 mM didn't significantly enhance the production of soluble IMPs (data not shown).

Unlike the conventional membrane mimetics, LPNs involve restricted fragments of a lipid bilayer and the movement of an encapsulated IMP between different nanodiscs is probably energetically forbidden. In this case the molar ratio of a synthesized IMP to LPNs in the RM could be an important factor controlling CF synthesis efficiency. The observed levels of the IMP synthesis in the presence of nanodiscs (~1.8 mg/ml of RM for the TM-ErbB3, and ~1.4 mg/ml of RM for the VSD and ESR) corresponded to the protein molar concentrations of 0.3, 0.08 and 0.05 mM, respectively. The application of 0.05 mM LPNs for the TM-ErbB3 synthesis provided a theoretical protein/nanodisc ratio of 6:1, thus enhancing a probability of the homodimer formation. On the other hand, the insertion of several copies of the VSD and ESR into the same LPN could potentially lead to non-specific aggregation of the IMPs or even to destabilization of a nanodisc structure. To avoid this, the concentration of the LPNs in the experiments with the VSD and ESR was increased up to 0.15 mM (protein/nanodisc ratio of ~1:2 and 1:3, respectively).

The IMP/LPN complexes were purified on the Ni^{2+}-column and analyzed by size-exclusion chromatography. According to the obtained data LPNs composed of the saturated lipids (DMPC or DMPG) retained the nanodisc structure (characteristic diameter~10 nm) upon the IMP incorporation (Fig. 4C–E). On the contrary, significant amounts of the large-sized (15–25 nm) and small-sized (~6 nm) particles were observed in the IMP/LPN samples containing unsaturated POPC and POPC/DOPG lipids (Fig. 4C–E). In the last case the incorporation of synthesized IMPs probably destabilized the nanodisc structure that led to a release of free MSP molecules (characteristic diameter~6 nm [37]) and a formation of large IMP/lipid complexes. Noteworthy is the fact that the minor content of the large-sized particles was also detected for the DMPC and DMPG LPNs (Fig. 4).

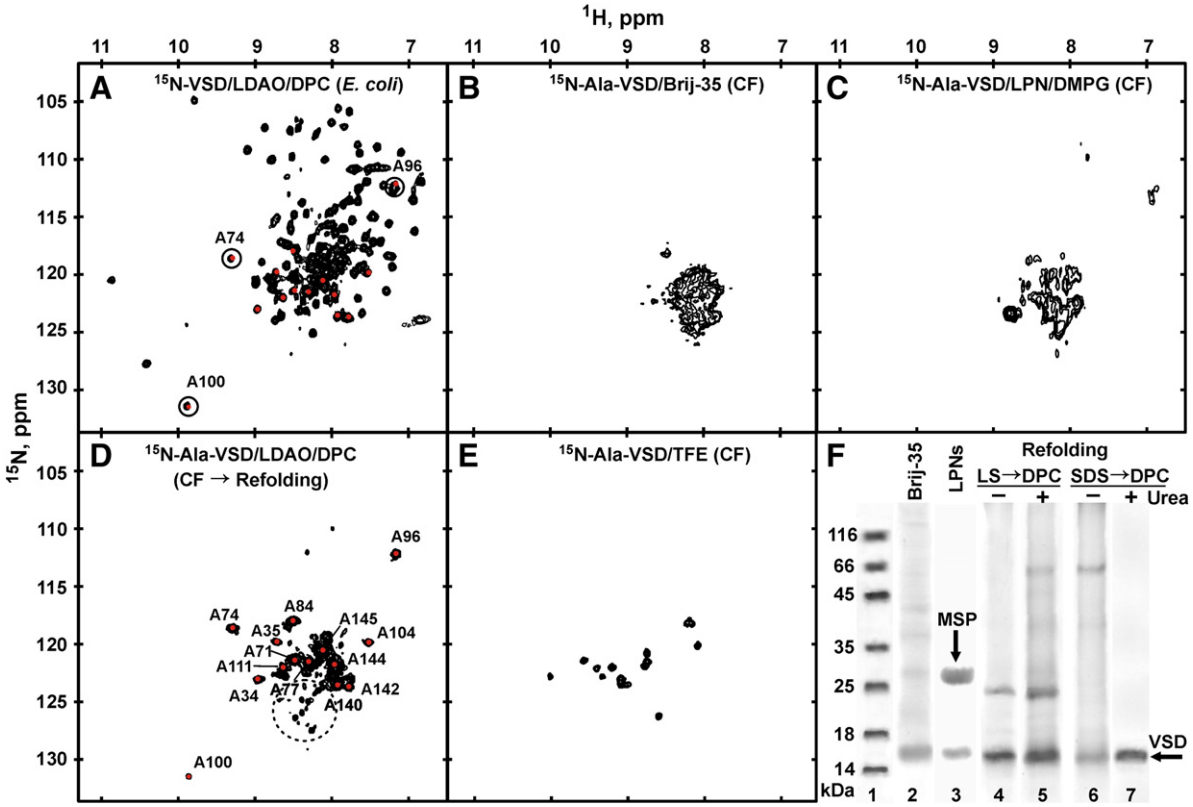

Fig. 3. 2D ^{1}H,^{15}N NMR and SDS-PAGE analysis of the VSD preparations. (A) HSQC spectrum of the totally ^{15}N-labeled VSD extracted from *E. coli* membranes in a native-like state and solubilized in the DPC/LDAO (2:1) mixture. The characteristic signals from Ala72, Ala96, and Ala100 are encircled. The positions of the signals observed for the refolded ^{15}N-Ala-VSD in DPC/LDAO (panel D) are marked by red dots. (B) HSQC spectrum of the ^{15}N-Ala-VSD synthesized and purified in the presence of Brij-35. (C) TROSY spectrum of the ^{15}N-Ala-VSD synthesized in the presence of the LPN/DMPG. (D) HSQC spectrum of the ^{15}N-Ala-VSD refolded from the RM precipitate in the presence of the DPC micelles and solubilized in the DPC/LDAO (2:1) mixture. The positions of the cross-peaks are marked by red dots. Signals originated from the misfolded domain are encircled by the dashed line. (E) HSQC spectrum of the ^{15}N-Ala-VSD refolded from the RM precipitate in the presence of the DPC micelles and re-solubilized in 100% d2-TFE. In each case the concentration of the VSD was about 0.1 mM, the concentration of the detergents in (A, B, D) was ~20 mM, and the concentration of the LPNs in (C) was about 0.2 mM. In (A–D) and (E) the temperature was 43 °C and 30 °C, respectively. In (A, B, D) and (C) the pH values were about 5.0 and 7.0, respectively. (F) SDS-PAGE analysis of the VSD preparations after purification on the Ni^{2+}-column. 1 — Molecular mass markers; 2 — the VSD synthesized and purified in the presence of Brij-35; 3 — VSD/LPN/DMPG sample; 4–7 — the VSD refolded from the RM precipitate under different conditions of the initial solubilization: (lane 4) 1% LS, (lane 5) 1% LS and 8 M urea, (lane 6) 1% SDS, and (lane 7) 1% SDS and 8 M urea. The bands corresponding to the MSP and VSD are marked by the arrows.

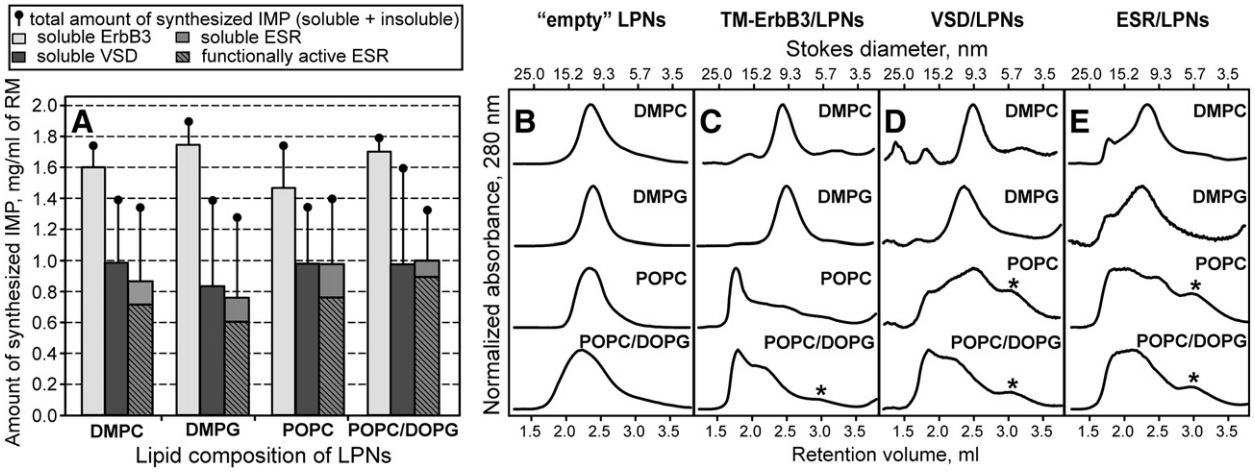

Fig. 4. CF synthesis of the TM-ErbB3, VSD, and ESR in the presence of LPNs of different lipid compositions. The LPN concentrations were 0.05 mM (TM-ErbB3) and 0.15 mM (VSD, ESR). (A) The amounts of the IMPs synthesized in a soluble form (bars) were compared with the total amounts of the synthesized IMPs (dots), including a fraction of insoluble proteins. The amounts of the synthesized IMPs (mg/ml of RM) were estimated by Western blot as compared to those of the TM-ErbB3, VSD, and ESR synthesized and purified in the presence of Brij-58, Brij-35, and Brij-98, respectively (see Materials and methods). The concentration of functionally active ESR within soluble fraction of the RM (hatched bars) was determined by UV–vis spectroscopy. Each value represents the average of the three experiments. A systematic error doesn't exceed 15%. (B–E) Size-exclusion chromatographic analysis of the LPNs of different lipid compositions before the IMP synthesis (B), and after synthesis of the TM-ErbB3 (C), VSD (D), and ESR (E). The peak corresponding to the MSP monomer is marked by the asterisk. The chromatographic profile of the pure MSP (not shown) can be found in [37].

An oligomeric state of the TM-ErbB3 incorporated into the LPN/DMPC was analyzed using chemical cross-linking. SDS-PAGE analysis of the sample treated by DTSP revealed monomers, dimers, and trimers of the TM-ErbB3 and monomers and dimers of the MSP (Fig. 2F, lane 7). The TM-ErbB3/MSP cross-linking was also observed. The results suggested that a fraction of the incorporated TM-ErbB3 could form homodimers within the LPN membrane. On the other hand, the formation of the trimeric TM-ErbB3 assemblies pointed to unspecific interactions between TM fragments within the LPN membrane. The 2D $^{1}H,^{15}N$-TROSY spectrum of the ^{15}N-Ile-labeled TM-ErbB3 incorporated into the LPN/DMPC (Fig. 2, panel E) revealed a similarity of the domain spatial structure and that in the DPC micelles (Fig. 2, panel B) [31]. To confirm the putative assignment of the ^{15}N-Ile resonances, a shortened variant of the TM-ErbB3 (shTM-ErbB3) containing merely two TM Ile residues (Ile649 and Ile655) was synthesized in the presence of the LPN/DMPC. The TROSY spectrum of the shTM-ErbB3/LPN/DMPC preparation proved the assignment of the Ile signals (Fig. 2, red lines in panel E) and confirmed that all Ile residues (including the TM ones) provide observable NMR signals in the LPN environment. Interestingly, the TROSY spectrum of the TM-ErbB3 measured in the LPNs containing anionic lipid DMPG (Fig. 2, panel D) also had some similarity to the spectra of the domain in the DPC and LPN/DMPC (Fig. 2, panels B and E). Prominent differences in the chemical shifts were observed only for the signals of residues Ile637 and Ile671, located above the interfacial region of the membrane [31]. Most probably, the electric charge of the lipid headgroups is responsible for these differences.

The ESR synthesis in the presence of LPNs resulted in the soluble and active (purple) protein regardless of the nanodisc lipid composition (Fig. 5). The yield of the functionally active ESR was 80–90% of the amount of the soluble protein and corresponded to 0.6–0.9 mg/ml of RM (Fig. 4C).

In contrast to the TM-ErbB3 and ESR, the VSD synthesized in the presence of LPNs didn't fold into the native-like conformation. The 2D $^{1}H^{15}N$-TROSY spectra of the ^{15}N-Ala-labeled VSD/LPN/DMPC and VSD/LPN/DMPG complexes (Fig. 3C) revealed the absence of the characteristic Ala signals observed for the VSD extracted from the *E. coli* membranes in the folded state (Fig. 3A, encircled). It should be noted that according to the published data, the VSD extracted from the *E. coli* membranes could be incorporated into the LPNs without a disruption of its spatial structure [23].

3.4. Refolding of IMPs from RM precipitate

Recently, a successful application of the DPC micelles for refolding of the precipitated TM-ErbB3 synthesized without membrane mimicking additives was demonstrated [31,36]. To show the possibility of CF production of the VSD with the native-like spatial structure, we used an approach that previously has been successfully applied for a refolding of membrane proteins and their domains [4,41]. The VSD precipitate was solubilized with the harsh anionic detergents SDS or LS and the chaotropic agent (urea). The solubilized VSD was loaded on the Ni^{2+} affinity resin, the refolding was initiated by a gradual replacement of the chaotropic agent and the harsh detergent with the milder detergent (DPC). SDS-PAGE analysis showed that the VSD refolded from the SDS/urea mixture was predominantly monomeric (Fig. 3F, lane 7). In all other cases (SDS without urea, LS with or without urea) the purified VSD/DPC sample contained the aggregates of the domain (Fig. 3F, lanes 4–6). The yield of the VSD after refolding was about 0.6 mg/ml of RM.

The comparison of the 2D $^{1}H,^{15}N$-HSQC spectra of the refolded ^{15}N-Ala-VSD in the DPC/LDAO micelles solution (Fig. 3D) and of the uniformly ^{15}N-labeled domain extracted from the *E. coli* membranes in the native-like conformation (Fig. 3A) revealed the very similar resonance positions for all the 14 Ala residues. This fact proves a successful refolding of the VSD. At the same time, some superfluous signals were detected in the spectrum of the refolded VSD (Fig. 3D, encircled). To find a source of these impurities, the refolded VSD/DPC sample was lyophilized and dissolved in the TFE solution. The HSQC spectrum measured in the low-polarity organic solvent exhibited only 14 signals originating from the Ala residues (Fig. 3E). The obtained data indicated that the excessive $^{1}H,^{15}N$ signals in the spectrum of the refolded ^{15}N-Ala-VSD belonged to another, probably misfolded, form of the domain. The comparison of the signal intensities of two VSD forms (folded and misfolded) showed the efficiency of refolding to be about 70%, providing about 0.4 mg/ml of the correctly folded domain.

The protocol developed for the VSD refolding was applied to the ESR synthesized as RM precipitate. SDS-PAGE analysis of the obtained ESR preparations revealed the aggregated protein (data not shown). UV–vis analysis of the ESR/DPC samples refolded in the presence of *all-trans*-retinal did not detect the active protein.

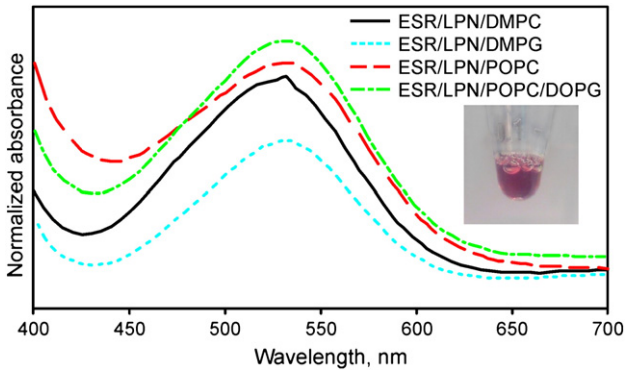

Fig. 5. UV–vis spectra of the ESR/LPN complexes of different lipid compositions. The photo of the soluble RM fraction after the ESR synthesis in the presence of the LPN/DPMC is shown in the inset.

4. Discussion

High-level production of integral membrane proteins in a folded and functionally active state is the major bottleneck in functional and structural studies of this important class of biomolecules [1,3,4,42]. The effective method of an IMP production should solve two general problems, to facilitate the folding of the produced protein into a native structural state and to ensure its stability in solution. Herein, we have analyzed several approaches to the production of membrane proteins by the CF system using three helical model IMPs of different topologies (1TM, 4TM and 7TM). The classical membrane mimicking additives: detergent micelles, lipid/detergent bicelles, and liposomes were compared with lipid–protein nanodiscs in their ability to support correctly folded IMP synthesis.

The synthesis in the presence of detergent micelles is a frequently used approach to the CF production of soluble IMPs [6,14,43]. It was earlier demonstrated that non-ionic detergents from the Brij-family, DDM and Triton X-100 could provide a suitable environment for synthesis of various helical IMPs, including multidrug transporters, ion channels, bacteriorhodopsins, proteins from the GPCR family, and others [14,15,43–49].

The present results indicate that the efficiency of the CF synthesis and structural properties of the obtained IMP preparations depend on the topological protein complexity. The dependence can be illustrated by the following points. First, all the tested detergents (except DPC) promote high-efficient synthesis of the soluble TM-ErbB3, the protein of simple 1TM topology, however not all of them support the effective synthesis of soluble polytopic IMPs (VSD and ESR). Second, according to chemical cross-linking, the TM-ErbB3 forms homodimers with the distorted spatial structure in the Brij micelles. Besides, the TM-ErbB3 can be refolded via substitution of this detergent for DPC. At the same

time, the 4TM protein VSD, which also adopts the misfolded conformation within the Brij micelles, cannot be refolded using this technique. Finally, none of the used detergents supported synthesis of the soluble and active ESR, the bacteriorhodopsin of the 7TM topology. Thus, according to our data the CF expression in the presence of detergent micelles doesn't guarantee a native folding of the polytopic VSD and ESR synthesized in a soluble form, providing the quasi-correct folding of the simple bitopic helical IMP TM-ErbB3 (Table 1). Nevertheless, several examples illustrated a successful detergent application for the CF production of polytopic IMPs in a folded state [43]. It was reported that DDM, DC7PC, FOS-14/CHAPS mixture, and detergents from the Brij family could support synthesis of the functional 7TM proteins: bacteriorhodopsins, proteorhodopsins, and human endothelin receptors [19,38,39,48,49]. It should be noted that the content of the functionally active proteins in most studies did not exceed 50% of the amount of the target IMPs synthesized in a soluble form. Despite that, none of those detergents could support the CF synthesis of the functional 7TM bacteriorhodopsin from *E. sibiricum*, indicating that the folding of the CF-produced IMPs crucially depends not only on the topology, but also on the other structural protein properties.

There are several membrane mimicking media containing fragments of the lipid bilayer and being used for CF synthesis of IMPs. For example, CF synthesis of the functional bacteriorhodopsin from *Halobacterium salinarum* directly into the liposomes was recently described [17]. Several lipid compositions were tested, but even for the best (DOPC), the yield of the functional protein incorporated into the liposomes was only 20 μg/ml of the RM [17]. In agreement with that data, we did not observe quantitative synthesis of the soluble and functional ESR in the environment of lipid vesicles, and a production level of the soluble TM-ErbB3 also decreased (Fig. 1, Table 1). The semi-rigid two-dimensional lipid matrix of a liposome, as opposed to rather loose and dynamic arrangement of a detergent micelle, presumably prevents an insertion of the synthesized protein chain into the bilayer, thus hindering the formation of the IMP tertiary structure. Most probably, the formed TM helices of the membrane protein associate with the liposomes surface resulting in the protein-mediated aggregation of the lipid vesicles and precipitation of large protein/lipid complexes. The formation of the colored CF precipitate upon synthesis of the ESR in the presence of the DMPC vesicles indicates that partial folding of the IMP takes place even under these conditions. The obtained results could be interpreted using the data from [12], where the well-formed helical elements of the secondary structure were detected within the IMP precipitate synthesized without any membrane mimicking components. The formation of the colored CF precipitate was also previously described for proteorhodopsins [38].

The in-cell machinery for membrane protein production involves a special translocon system, which promotes transpiercing of the ribosome synthesized protein chain through the lipid bilayer [50]. Probably, the RM supplement with components of translocon could facilitate a correct insertion of synthesized IMPs into lipid vesicles. A similar approach was recently utilized for production of aquaporin Z in the presence of liposomes [51]. The application of the soluble translocon components (signal recognition particle (SPR) and signal recognition particle receptor (FtsY)) moderately enhanced the solubility of the synthesized aquaporin, but did not influence the yield of the active protein [51]. This suggests that the membrane embedded translocon components are also needed for correct co-translational incorporation of the synthesized IMP chain into the lipid bilayer.

An application of the bilayer containing membrane mimicking media such as bicelles [16] and LPNs [28] could provide a way for the efficient folding of the IMPs upon CF synthesis. Structural and dynamic properties of the lipid bilayer within the bicelles [52] and LPNs [53,54] differ from those of liposomes. For example, the recent thermodynamic investigation revealed that a lipid transfer rate between LPNs and aqueous solution is 20-fold higher than that in lipid vesicles [54]. In principle, the altered lipid packing properties in the bicelles and LPNs could facilitate the co-translational TM insertion of the polypeptide chain. Data obtained for the bicelles and nanodiscs are in general agreement with this expectation. In contrast to the detergent micelles, the DMPC/DHPC (1:2) bicelles enable synthesis of the soluble and folded TM-ErbB3 and ESR, although with the diminished yield. At the same time, the bicelles did not provide a long-term stability to the synthesized VSD and, probably, did not support the correct domain folding. The application of the lipid/detergent mixtures (bicelles) for the CF synthesis of IMPs was recently described by Shimono et al. [16]. The use of the egg PC/cholate and the egg PC/CHAPS mixtures resulted in synthesis of the functional bacteriorhodopsin with the yield about 0.5 mg/ml of RM [16]. In our study we observed a significant suppression of the CF system in the presence of the DMPC/CHAPS (1:2) bicelles. This discrepancy could be explained by different experimental protocols employed. For example, in the work [16] the detergent components of the bicelles were added only to the RM resulting in the detergent dilution during the synthesis and, probably, diminishing its inhibitory effect.

Recent studies revealed that the LPNs containing the zwitterionic lipid DMPC added to the RM extremely increased the solubility of 32 CF synthesized IMPs of different origin (human, murine, and bacterial) [28]. At the same time, the functional activity of the IMP/LPN complexes was tested only for two bacterial proteins: the multidrug transporter EmrE from *E. coli* and the bacteriorhodopsin from *H. salinarum* [28]. The application of the DMPC/LPNs for CF synthesis of the functional β-adrenergic receptor/lysozyme chimera was recently reported [29].

Here for the first time we studied the influence of the LPN lipid composition on the efficiency of the CF synthesis of IMPs. Our results indicated that the yield of the soluble IMPs did not strongly depend on the charge and saturation of the lipids used. Contrary to other bilayer mimetics (bicelles and liposomes) the yield of IMPs in nanodisc based media was comparable with the highest one observed in the detergent micelles (Fig. 4). However, as opposed to the detergents, the major fraction of the soluble ESR and TM-ErbB3 synthesized in the presence of nanodiscs turned out to be folded. In other words,

Table 1
Comparison of various approaches to the CF synthesis of soluble and folded IMPs of different topologies.

Membrane mimetic RM additive/approach	TM-ErbB3 (1TM)		VSD (4 TM)		ESR (7 TM)	
	Solubility[a]	Folding[b]	Solubility	Folding	Solubility	Folding
Detergent micelles	+	±	+	−	+	−
Bicelles	±	+	±	−	±	+
Liposomes	±	n/d	n/d	n/d	−	−
LPNs	+	+	+	−	+	+

[a] The solubility values classified into the three categories. '+' means that more than 50% of the synthesized protein was soluble. '±' or '−' means that a soluble fraction of the synthesized protein was above or equal to the amount of the soluble protein synthesized without any membrane mimetics, respectively.
[b] The extent of the protein folding classified into the three categories. '+' means that the protein adopted a folded state during synthesis, '±' means that the synthesized protein demonstrated some distortions from the folded state, and '−' means that the protein did not adopt the folded state. n/d — not determined.

the nanodiscs can support a large-scale CF synthesis of the soluble and functional IMPs of different topologies (Table 1). Nevertheless, not all lipid compositions provide enough LPN stability to withstand the co-translational incorporation of IMPs. According to the obtained data (Fig. 4), only the LPNs composed of the saturated lipids (DMPC, DMPG) maintained the nanodisc structure during the process. A partial disintegration of the LPNs formed from the unsaturated lipids (POPC, POPC/DOPG) was observed. That experimental finding was rather surprising, because all the experiments were conducted under the conditions (30 °C) when saturated and unsaturated lipids were in a liquid-crystalline state. We can speculate that the enhanced dynamics and a larger area occupied by fatty acid chains in unsaturated lipids promoted the formation of transient membrane packing defects upon the IMP incorporation. These defects in turn could induce the 'fusion' of nanodiscs resulting in the formation of large protein/lipid particles and the release of free MSP molecules. Application of LPNs with different lipid properties provided the similar yield of the functionally active ESR (Fig. 4A), indicating that the nanodisc disintegration does not hinder the insertion and folding of the synthesized IMP. Most probably, the fused LPNs preserve the bilayer organization. Our previous studies revealed no disintegration/fusion of the LPNs formed from the anionic unsaturated lipid DOPG upon incorporation of small (16 a.a.) helical antimicrobial peptide antiamoebin I [24]. The difference in the topology of the protein/membrane interaction ('in-plane' in the case of the antiamoebin and TM in the case of IMPs) could be responsible for the discrepancy observed in the nanodisk stability.

The in vitro and in vivo fusion of high-density lipoprotein particles (natural prototype of nanodiscs) accompanied by a release of lipid-poor apolipoprotein molecules (so-called 'lipoprotein remodeling') was previously described [55,56]. Under physiological conditions, a spontaneous lipoprotein fusion is very slow, but denaturing conditions [55], as well as involvement of the plasma lipophilic enzymes (e.g. lecithin:cholesterol acyltransferase, LCAT) [56], could accelerate it. Interestingly, the formation of large protein/lipid particles with diameters up to 17 nm was observed in vitro during the fusion of spherical high-density lipoproteins under action of LCAT [56].

The IMP/LPN molar ratio in CF synthesis could influence the folding of the produced protein and the assembly of protein/protein complexes. The IMP molecules synthesized in the presence of nanodisc are encapsulated in small membrane patches, consisting of ~150 lipids. This feature of nanodiscs could be utilized to control intermolecular interactions. We used a relatively high TM-ErbB3/nanodisc ratio (6:1) to enhance a likelihood of a homodimer formation. Several important points should be discussed: (1) an insertion of the synthesized IMP into LPNs is the stochastic process and the TM-ErbB3/LPNs complexes probably contained a slightly different number of the incorporated protein molecules per nanodisc; (2) unspecific protein/protein interactions, e.g. the formation of TM-ErbB3 trimers (Fig. 2F, lane 7), could occur under these conditions. Nevertheless, hypothetically the LPNs could be used for CF production of IMP supramolecular complexes, either by simultaneous translation from several vectors, or via stepwise incorporation of different proteins to the same nanodisc preparation.

As mentioned above, the efficiency of the IMP production in a soluble and folded state could depend on the individual protein properties. The voltage sensing domain of the KvAP channel represents the protein for which all the tested methods, including application of the bilayer-containing mimetics (bicelles and LPNs), were inefficient. The VSD folding problems are probably stipulated by specific details of its structure. The TM helices of the VSD contain a large portion of the charged residues, which form a number of the interhelical salt-bridges located in the interior of the four-helical bundle of the folded domain [33,57]. Probably, these charged residues stabilize individual helices of the VSD on the surface of the bicelle or LPN bilayer, thus preventing their transition into the TM state. The refolding from the RM precipitate is the only method for the CF production of such "problem" proteins. Data obtained indicate that this approach could be quite efficient providing about 0.4 mg/ml of the correctly folded polytopic IMP. Nevertheless, an additional purification step (e.g. the affinity chromatography with ligands or antibodies) is required to separate the folded protein from the misfolded one. The example of unsuccessful bacteriorhodopsin refolding using detergent micelles emphasizes different structural properties of the two studied polytopic proteins. Compared to the VSD, the TM helices of the ESR do not contain a high proportion of the charged residues, and they could adopt the TM orientation in the nanodisc membrane leading to the correct protein folding. According to this hypothesis the environment of the lipid bilayer is needed for successful refolding of the ESR.

5. Conclusions

For the first time, the efficiency of the CF expression of helical IMPs in the environment of lipid–protein nanodiscs was compared with that in the presence of the classical membrane mimetics (detergents, bicelles, liposomes). Data obtained indicate that the LPNs provide better membrane mimicking properties for the CF production of IMPs in a soluble and folded state. The dependence of the CF expression on the lipid composition of nanodiscs was analyzed. Only LPNs composed of the saturated lipids were shown to preserve their structure upon co-translational incorporation of the membrane proteins. A possibility of direct NMR structural studies of the IMPs synthesized in the presence of LPNs without laborious procedures of the protein refolding and screening for the suitable detergent-based membrane mimetics was shown. The observed compatibility between two techniques (the CF production of membrane proteins and the methods for their stabilization in solution by the nanodiscs) opens new perspectives for functional and structural IMP investigations.

Acknowledgements

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