



Extremely low frequency magnetic field enhances expression of a specific recombinant protein in bacterial host

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ABSTRACT

Expression of proteins in bacterial host cells, particularly *E. coli*, has gained much attention in recent years. Low expression outcome is the main technical drawback associated with this procedure, further restricting its largescale application in industry. Therefore, application of new amendments or reformations are required before further proceedings. Extremely low frequency magnetic fields (ELF-MFs) have shown to significantly affect biological processes, including gene expression, in *E. coli*. In current study, we investigated whether application of ELF-MF could result in overexpression of proteins in *E. coli* or not. Cluster of differentiation-22 (CD22), as a model protein, was expressed in *E. coli* Rosetta (DE3) under continuous exposure to ELF-MF after applying various concentrations of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.25–1.25 mM) as inducer. The strength and frequency of electromagnetic fields (EMFs) ranged between 15 and 100 mT and 2.5–20 Hz respectively. Interestingly, application of 55 mT EMFs with frequencies ranging from 2.5 to 2.8 Hz significantly enhanced the yield of expression at all studied IPTG concentrations. Contrarily, EMFs with intensities other than 55 mT meaningfully declined protein expression at IPTG concentrations equal to 1 and 1.25 mM. In conclusion, application of specific range of ELF-MFs may be exploited as a new modification for enhancing heterologous expression of proteins in *E. coli*.

1. Introduction

Recent progress and advances in different fields of proteomics and structural biology have made membranous proteins the subject of numerous studies worldwide. For instance, involvement of eukaryotic membranous proteins in numerous biological processes including regulation of cell functions, transduction of cell signaling pathways and ion transportation, has made them to be the target of about half of the developed therapeutic agents and subject of several structure-function studies [1]. As a eukaryotic membrane protein, Cluster of Differentiation 22 (CD22), is an inhibitory co-receptor of B-cell receptor (BCR) which in response to cues, modulates the antigen-receptor signals. This is crucial for ensuring initiation of a proper and prompt response against different pathogens while preventing from self-antigen reactivity and development of autoimmunity [2–4]. Although CD22 overexpression is

mostly restricted to specific group of matured B-cells, abundantly presented on malignant B-cells in Non-Hodgkin lymphoma (NHL) and acute lymphoblastic leukemia (ALL), turning it to an attractive target for cancer immunotherapy strategies [5–8].

For these unique characteristics, CD22 has become the subject of several structure-function relationship studies for both better understanding the underlying signaling pathways and developing more effective therapeutic agents in treatment of autoimmune disorders and malignancies [9]. Unfortunately, currently existing techniques for structural function studying of proteins require large amounts of homogenous proteins, mostly considered as the bottleneck or the limiting step in performing these techniques. Furthermore, purifying membrane proteins from their natural sources is not affordable because of their low abundance in cells. Besides, their high hydrophobicity makes them to be handled in vitro with difficulties [10,11]. Consequently many

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researchers have suggested alternative heterologous protein expressing hosts for overcoming these restrictions [10,12].

So far, *Escherichia coli* is the most frequently applied host for over-expression of different membrane proteins [13]. In this manner, different strands, culture media and expression requirements are thoroughly screened for developing the optimum overexpression method [14]. Even in these conditions, still the yields, particularly in the case of Eukaryotic expressing hosts, are also low and in most cases do not result in better outcomes [15]. For this reason, many investigators have focused on enhancing expression yields by applying different methods for engineering or choosing specific strains of *E. coli* with enhanced membrane proteins with retained functionality being expressed better and finally applying *E. coli*-based cell free systems [13].

While the exact mechanisms have not been clarified yet, it has been demonstrated that exposure to non-thermal extremely low frequency electromagnetic fields (ELF-EMF) significantly alters gene expression profile in *E. coli* [16,17] which may in turn, influences protein synthesis profile. Consistent with this theory, in a recent study performed by our group, it was successfully demonstrated that exposure to ELF-EMF could significantly enhance expression of Glucose oxidase as a heterologous protein in *Pichia pastoris* [18]. Furthermore, Tessaro et al. and Gaafar et al. have shown that ELF-MF can be successfully apply for enhancing growth rate of *E. coli* [19,20]. Based on these encouraging studies, in this study we investigated whether exposure to ELF-EMF could increase expression of CD22 recombinant protein, as a eukaryotic membrane protein in *E. coli* or not.

2. Materials and methods

2.1. ELF-MF exposure equipment

The exposure equipment (Parto Farazan Farda, Iran) consists of two pairs of square coils in a U-shape solenoid like configuration, with an iron core for enhanced homogeneity. Some characteristics of the apparatus are summarized in Table 1. Utilizing a wave form generator (GW Instek SFG-1000 series, South Korea) attached to the coils, provided a variety of magnetic fields in range of 1–100 mT in intensity and 1–60 Hz in frequency. By performing a point-by-point calibration, a central 8×8 cm square was determined as the exposure area where the field alteration was less than 2%. The possible effects of ELF-MF exposure on CD22 expression profile in the presence of different concentrations of Iso-propyl β -D-1-thiogalactopyranoside (IPTG) was examined at three different intensities (15, 55, and 100 mT) each at different frequencies (2.5, 2.8, 10, and 20 Hz). Field homogeneity model and the calibration method has been previously described in our publication (Madjid Ansari et al., 2017).

Table 1

The intensity and frequency variables of ELF exposure set up. An intensity ranges of 15 mT–100 mT and frequency range of 2.5 Hz–20 Hz were applied.

Frequency	Intensity
2.5Hz	15 mT
10Hz	15 mT
20Hz	15 mT
2.5Hz	55 mT
2.8Hz	55 mT
10Hz	55 mT
20Hz	55 mT
2.5Hz	100 mT
10Hz	100 mT
20Hz	100 mT

2.2. CD22 gene cloning

Codon optimized coding sequence of human CD22 gene (NCBI accession No. NM-001771.3) was synthesized.

Afterwards, the *Nco*I and *Bam*HI restriction sites were included at the 5' and 3' sequence endings, respectively and a 6xHis tag was included in the C-terminus sequence right before the TAA stop codon (Genecust, Luxemburg). After digestion with *Nco*I and *Bam*HI restriction enzymes (Thermo Fisher Scientific, USA), the desired fragment was gel-extracted with GenElute Gel extraction kit (Sigma, Germany) and ligated into *Nco*I/*Bam*HI double-digested pET-15b prokaryotic expression vector (Merck-Millipore, USA) applying T4 DNA ligase (Thermo Fisher Scientific, USA). The cloning was further confirmed by enzymatic digestion applying.

*Eco*RI restriction enzyme and colony PCR with TAA-TACGACTCACTATAGGG, and.

GCTAGTTATTGCTCAGCGG forward and reverse primers, respectively.

2.3. Recombinant CD22 (rCD22) expression and extremely low frequency (ELF) exposure protocol

Calcium chloride (CaCl₂) transformation technique was utilized for transforming recombinant vector into *E. coli* Rosetta (DE3) competent cells (Merck-Millipore, USA). After inoculation of a single *E. coli* colony into a 200 mL terrific broth (TB) medium comprising 100 μ g/ml ampicillin and further culturing in a 37 °C incubator at 200 rpm until attaining an optical density equal to 0.6 at 600 nm, expression of the CD22 protein was induced utilizing IPTG (AppliChem, Germany). The possible effects of ELF-MF exposure on CD22 expression profile in the presence of different concentrations of IPTG (0.25, 0.5, 1, and 1.25 mM) was examined at three different intensities (15, 55, and 100 mT) each at 3 to 4 different frequencies (2.5, 2.8, 10, or 20 Hz).

At this point, culture media were placed in the predefined exposure area, located in a free room among the two coils distance of ELF exposure equipment and incubation was continued for 3 h at 37 °C in the presence of electromagnetic fields with different intensities and frequencies. As an ELF negative control, part of the primary culture media was located in the incubated room where the magnetic field was equal to the background level. Schematic shape of exposure and expression flask has been shown in Fig. 1.

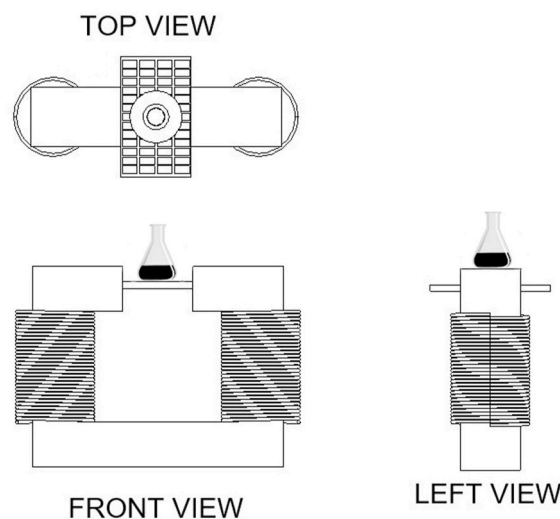


Fig. 1. Schematic shape of exposure system from 3 point of view and expression media at the exposure cite.

2.4. rCD22 purification

Culture harvesting was carried out by centrifugation at 4500g for 15min at 4 °C. Cell pellets were then resuspended in 7 ml lysis buffer (300 mM NaCl, 10 mM imidazole, 50 mM NaH₂PO₄). After sonication (10 s on/10 s off; total on: 10 min) and centrifugation (6000 g for 30 min at 4 °C), cell lysates were harvested. Clarified cell lysates were further diluted with 6 ml lysis buffer and cell lysate was prepared by sonication. The lysed preparation harvested by centrifugation at 8000g for 20min in 4 °C and the clarified cell lysate was diluted by PBS, 50/50. The diluted lysate was applied to 1 ml HisPur Ni-NTA resin (Thermo Scientific, USA) packed within a 2 mL-Pierce Centrifuge Column (Thermo Scientific, USA). Preventing from possible non-specific bindings, column washing was performed with 10 ml washing buffer (25 mM imidazole in PBS) in room temperature after a 10 min-short time of incubation. The rCD22 was eluted by 1 mL elution buffer (250 mM imidazole in PBS).

2.5. SDS-PAGE and Western blot analysis

SDS-PAGE was carried out for analyzing recombinant CD22 protein expressions. Different prepared protein samples including cell lysate, flow through, final wash and eluted CD22 samples were electrophoresed on a 15% polyacrylamide gel that was further stained with Coomassie Brilliant Blue stain. Proteins were then transferred on a nitrocellulose membrane blocked with blocking buffer (1% casein in PBS) and incubated with 2 µg/ml Rabbit anti-Human CD22 polyclonal antibody (Sigma, Germany) in PBS and 1:1500 diluted anti-mouse HRP conjugate (Sigma, Germany). Finally, the specific band was detected utilizing 3,3'-diamino benzidine (DAB) solution (Sigma, Germany).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The antigen-antibody interaction was studied through an ELISA experiment using the anti-CD22 monoclonal antibody (eBioscience, USA). Briefly, an ELISA plate was coated with 100 µl of the expressed recombinant CD22 protein (2 µg/mL). The plate was washed with PBST (PBS with 0.05% Tween 20), then blocked with 4% skim milk in PBS for 1 h at 37 °C. After three washes with PBST, 100 µl anti-CD22 antibody (1 µg/mL in PBS) was added to each well and incubated for 1 h at 37 °C. Then, after washing, 100 µl of anti-mouse HRP conjugate, 1:4000 in PBS, was added to each well after 1 h incubation at 37 °C and were washed for three times. Finally, 75 µl of 3,3', 5,5'-tetra methyl benzidine (TMB) substrate (Sigma, Germany) was added into the wells, and incubated in 37 °C for 5 min in the dark. The reaction was stopped by adding 75 µl of stopping buffer (H₂SO₄ 0.5 N) to wells. The absorbance at 450 nm was determined by ELISA reader. Antibody dilution buffer (PBS) was used as negative control and experiment was performed in triplicate.

2.7. Protein concentration direct assay

The Absorbance measurement at 280 nm (A₂₈₀) for a protein (Protein A₂₈₀) is the most popular quantification method for protein concentration measurement in range of 0.1–100 µg/ml because it is fast and simple, requires no reagents, and consumes very little volume of sample [21].

In this study, a NanoDrop™ UV-Vis spectrophotometer (Thermo Scientific, USA) was utilized for analyzing protein samples quantification (total protein) with applications for direct protein A₂₈₀, A₂₀₅ and colorimetric assays in according to manufacturer's instruction. The total protein content accurately measured using direct absorbance at 280 nm.

2.8. Statistical analysis

SPSS software version 16.5 (SPSS Inc., Chicago, USA) was utilized for analyzing data and the results have been represented as mean ± standard deviation (SD) among replicates. Experiments were carried out in

triplicate for each analysis and Student's - *t*-test was performed for evaluating statistical significance. A probability of less than 0.05 (*P* < 0.05) was considered as significant.

3. Results

3.1. rCD22 cloning and expression

CD22 coding sequence was inserted between *Nco*I and *Bam*HI restriction sites in pET15b expression vector. The Initial verification of pET15-CD22 sequence was carried out by double digesting of the purified plasmid, utilizing.

*Nco*I and *Bam*HI restriction enzymes. A band in size of 2544 bp was observed on the agarose gel which was predictable based on the length of CD22 gene, applied primers and their specific binding sites. In addition, bands resulted from enzymatic digestion with *Bam*HI and *Nco*I restricting enzymes were observed. Furthermore, SDS-PAGE analysis demonstrated a prominent 70 kDa protein band, identified by applying the anti-CD22 antibody through western blotting which was in agreement with the expected molecular mass (Fig. 2).

3.2. In vitro binding assay

ELISA was carried out in order to evaluate the interaction between rCD22 and the anti-CD22 corresponding antibody. The reaction between the rCD22 and anti-CD22 antibody resulted a significant positive response at 450 nm (OD_{450 nm} 2.63) compared to the control (Creative BioMart, USA) (OD_{450 nm} 0.1) (Fig. 3). Second ELISA also demonstrated a significant immune response in immunized mouse serum samples. No significant immune response was observed with normal serum (Fig. 3).

3.3. Effects of ELF-EMF exposure on expression profile of rCD22

Effects of ELF-EMF exposures on expression profile of rCD22 was evaluated by comparing OD values of exposed samples and densitometry of protein bands in electrophoresis gels. In whole experiments, exposure was performed continuously, magnetic field intensity varied between 15 and 100 mT, the frequency ranged between 2.5 and 20 Hz and IPTG, as inducer of experiment, altered between 0.25 and 1.25 mM. As depicted in Fig. 4, the most significant increase in expression was obtained upon exposing 0.25 mM IPTG induced medium to 55 mT, 2.8 Hz EMF. Similarly, exposure to the EMF with 55 mT intensity and 2.5 Hz frequency could significantly enhance protein expression in next place compared to non-exposed media.

Utilizing IPTG concentration of 0.5 mM, a significant increase in expression profile of CD22 was observed following exposure to magnetic field intensity of 55 mT and frequencies of 2.5 and 2.8 Hz. No statistically significant differences were observed between protein expression profiles of these two frequencies. Upon raising IPTG concentration to more than 1 mM, significant increase in protein expression profile was observed only when system was exposed to 55 mT EMF with 2.8 Hz frequency. Therefore, by increasing IPTG concentrations from 0.25 mM–1.25 mM, significant increase in protein expression restricted to 55 mT magnetic field intensity and 2.8 Hz frequency. Contrarily, using IPTG concentration of 1.25 mM, exposure to 15 and 100 mT EMFs significantly decreased protein expression profile compared to non-exposed media.

Densitometric Analysis of protein bands in electrophoresis gels using ImageJ software also demonstrated comparable patterns to those obtained from spectrophotometric results. As depicted in Fig. 5, increase in protein expression was gradually decreased by rising IPTG concentrations from 0.25 to 1.25 and the most significant fold change in protein expression occurred with 55 mT EMF and 2.8 Hz frequency. Overall, the most significant increase in protein expression was obtained at lower IPTG inducing concentrations and upon application of ELF-EMF with

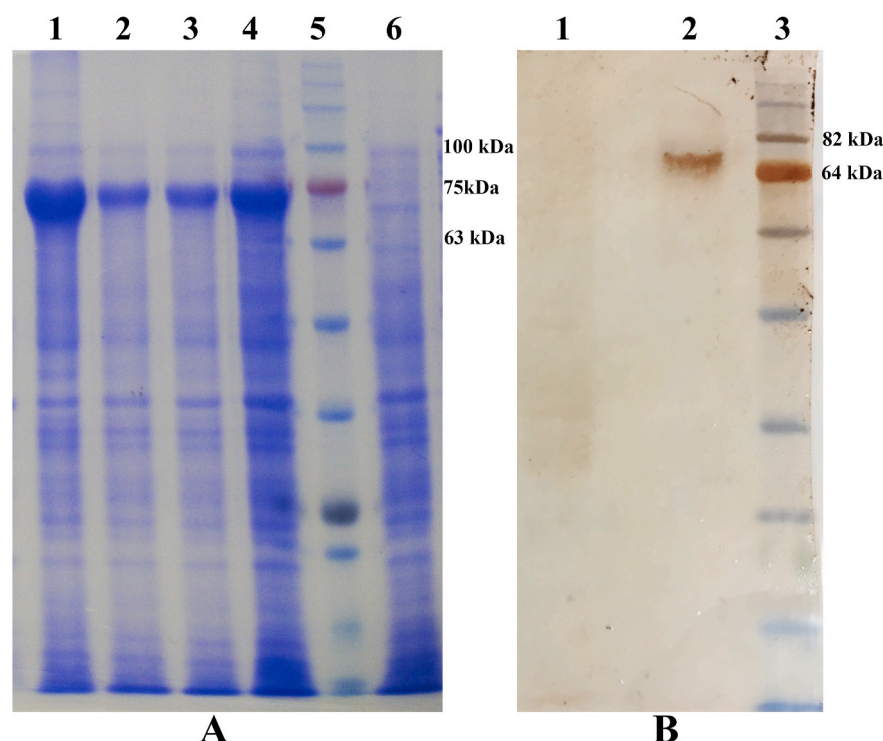


Fig. 2. Characterization of expressed recombinant CD22 by SDS-PAGE (A) and Western blotting (B). From left to right: lane A1: 3-hour expression, lane A2: 2-hour expression, lane A3: 1-hour expression, lane A4: overnight expression, lane A5: BLUelf Pre-stained Protein Ladder (GeneDireX, Taiwan), lane A6: Non-induced cell lysate, lane B1: Non-induced cell lysate, lane B2: purified recombinant CD22, and lane B3: Bench Mark Prestained Protein Ladder (Thermo Fisher Scientific, USA). The recombinant CD22 was expressed as a single band corresponding to about 70 kDa. Expression was performed at a concentration of 1 mM IPTG at 37 °C in a shaker incubator and also samples were in sham exposure conditions.

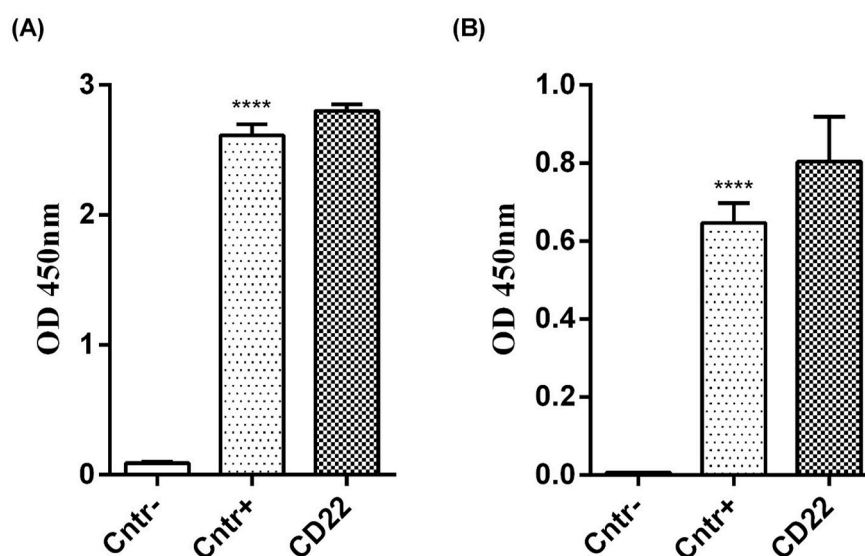


Fig. 3. (A) The reaction between the rCD22 and anti-CD22 monoclonal antibody resulted a significant positive response at 450 nm (OD450 nm 2.63) compared to the control (Creative BioMart, USA) (OD450 nm 0.1) (B) In the second ELISA, a strong immune response to the CD22 was detected in sample of immunized mouse serum (OD450 nm 1.3) in comparison with normal serum (OD450 nm 0.2).

intensity of 55 mT and frequencies of 2.5 and 2.8 Hz in first place and 10 Hz in next.

4. Discussion

Despite the existing controversies, different studies have confirmed the extremely low frequency magnetic field effects on biological systems. Multiple studies have clearly demonstrated the effects of ELF-MF influence on protein synthesis, DNA-binding complexes and biochemical reactions in different multi-cellular eukaryote and prokaryote cells [22–24]. Alongside, changes in rate of cell division, protein synthesis and genome conformation state of.

E. coli during exposure to several frequency windows have also been reported by many investigators [25]. Explaining these effects, different mechanisms and models have been proposed so far [26–28]. These mechanisms are mostly based on the assumption that alternating magnetic fields just like static fields can modulate different cells naturally high frequency oscillations [29]. For instance, it has been showed previously that low intensity millimeter waves (MMWs) can significantly affect *E. coli* GCS resonance [30]. Furthermore, since these effects are strongly depended on the exposed cells concentrations, it has been demonstrated that these resistance effects are mostly in cooperative manner. So far, the most accepted explanation for these observations is that the exposed cells themselves possess natural high frequency

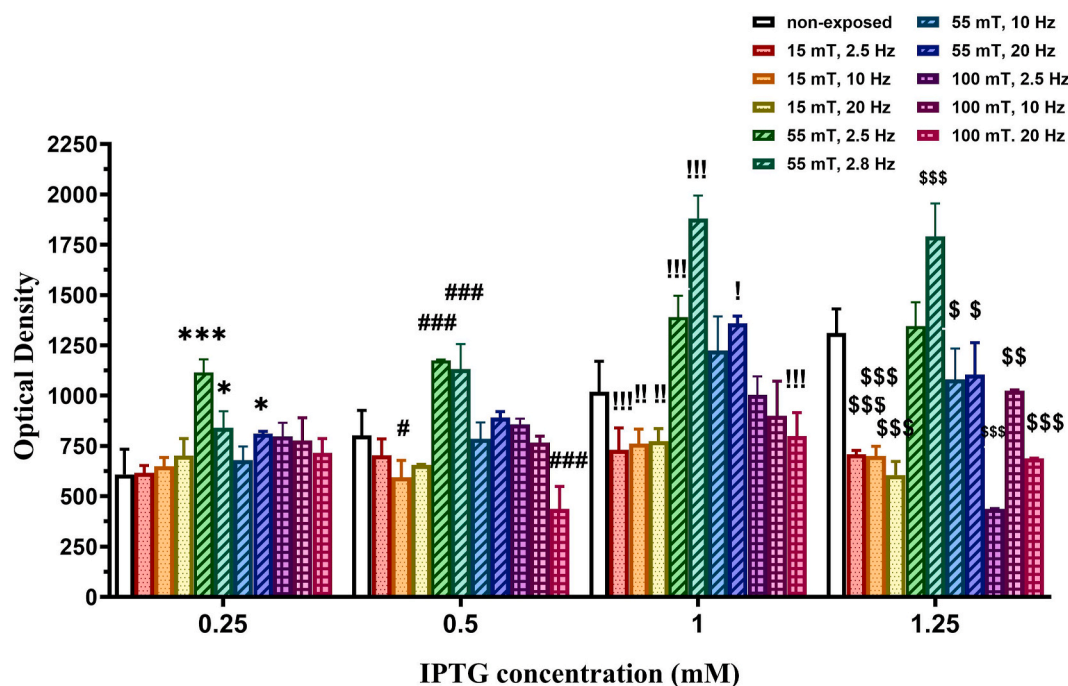


Fig. 4. Protein direct OD values of exposed samples to the different settings of ELF-EMF and at the presence of different concentration of IPTG (0.25–1.25 mM). Each exposure setting has been shown in a specific colored column. White column is representative of sham exposed values. Significances are indicated by marker characters (*, #, !, \$).

radiations [31]. If ELF-MFs could interact with these naturally high frequency oscillations, certain variations would be expectable to take place in these cells natural radiations. Results of these cells changes can further affect other cells and explain the dependencies of ELF effects on cells concentrations. This assumption has been clearly demonstrated in a study performed by Belyaev et al. [30]. Although the precise mechanism of interaction between ELF-EEMF and prokaryote cells is not clear, it has been proposed that elevation of reaction oxygen species (ROS) levels, as well as prolongation of their lifetime could be the most probable event underlying observed biological effects [32,33], (Tiwari R, Lakshmi N, Bhargava S, Ahuja Y, 2014), (Tiwari R, Lakshmi N, Bhargava S, Ahuja Y, 2015). In this context, it has been shown that ROS elevation is associated with an alteration in expression of numerous genes and consequently, related proteins, as well as several enzymes activity in bacteria [34–36]. This may happen mostly due to the activation of numerous bacterial pathways which cause resistance against environment-induced inactivation factors [37]. Similarly, it is probable that during the process of recombinant protein expression in bacteria, overexpression of ROS caused by ELF-EMF may induce certain alterations in enzymes or genes involved in expression of recombinant proteins. Clearly, different amplitudes and frequencies will cause different extends of increase in development of ROS and hence, alteration in activity of enzymes and gene expression will be different. Overall, differences in frequencies and intensities of ELF-EMF will cause different amounts of ROS expression and hence, different expression of recombinant proteins in bacteria. Similarly, an enhanced IPTG amount may cause an enhancement in production of ROS which may become enhanced upon interaction with those produced by ELF-EMF exposure.

The other recently developed theory for explanation of these findings is the “quantum-mechanical mechanism” which is based on the quantum state interference of ions which are fitted inside a proper cavity. This theory mostly proposes that ion states superposition results in a specific non-uniform probability density pattern which consists from a set of more and less fragments. In the presence of a DC field, this pattern rotates with the cyclotron frequency inside the cavity. However, in the AC field with specific characteristics, revolution for the large section of this

rotation cycle is retarded and the ion escapes from the cavities becomes mostly facilitated. The main influence of ion scape is on the biochemical reactions equilibrium which can induce a biological endpoint [38,39]. Since ions are mostly associated in the structure of RNA polymerases and transcription factors of different bacteria, it is not surprising that ELF can influence protein synthesis process [25]. As an example, RNA polymerase of *E. coli* contains two Zn^{2+} ions, one in substrate binding subunit and the other in DNA template binding subunit [40]. From other side, three Mg^{2+} ions are necessary for the assembly of a transcription active complex. The DNA template binding subunit of RNA polymerase possesses a preserved motif, containing 3 aspartates which are assumed to be crucial for the formation of Mg^{2+} binding pockets [41]. Exchanging of a Zn^{2+} ion results in a conformational change in transcription factors which can impose a significant effect on activation of transcription process [42].

As the concentration of IPTG is increased, more allosteric repressors of Lac operon will be removed from the Lac operator and more gene transcription will occur which is together with involvement of more RNA polymerase enzymes, more transcription complex formation and more exposure of bound ions with enzymes to the electromagnetic field. Furthermore, these newly formed transcription complexes and dissociation of repressors from DNA can also result in more changes in GCS of *E. coli* which may in part explain the reason why we observed a shift from 2.5 Hz to 2.8 Hz as the concentration of IPTG is increased. The other important finding of our study was that in magnetic field strengths other than 55 mT and at high concentrations of IPTG decrease in protein expression was observed. Multiple transportation pathways may exist for IPTG [43]. In low concentrations, lactate permease is mostly responsible for IPTG uptake by *E. coli*, however, at high concentrations, usually in the case of inducing protein synthesis, the entrance of IPTG can be independent from the lactose permease transporters [44]. As ELF may affect distribution pattern of proteins and lipid domains and result in conformational changes in lipid-protein interactions in different bacterial cells, this may result in deficient uptake of IPTG compare to unexposed cells and consequently decrease in overexpression of heterologous proteins in exposed cells.

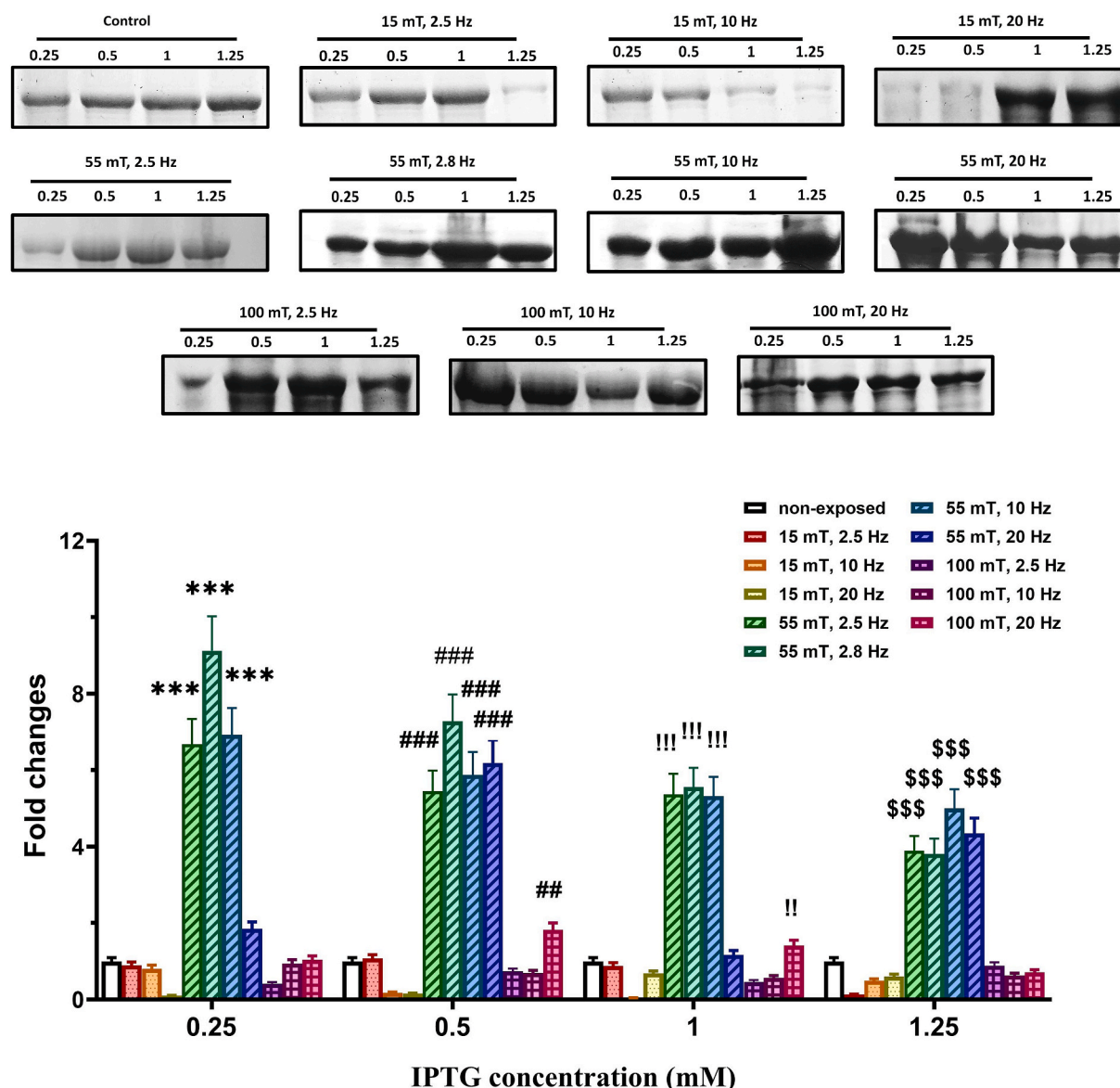


Fig. 5. Protein expression profile of rCD22 under different conditions; Effects of exposure to ELF-EMF on expression profile of CD22 were evaluated at the presence of different concentrations of IPTG (0.25–1.25 mM) as protein expression inducer.

5. Conclusion

Improving expression of Eukaryotic membrane proteins in different bacterial hosts, especially *E. coli* due to its numerous advantages is an important challenge which has been mentioned in several studies. Based on results, in current study, we demonstrated that along with other applied methods for improving protein expression yields, exposure to non-thermal ELF-MF within a specific narrow frequency window can significantly improve heterologous expression of CD22 as a Eukaryotic membrane protein in *E. coli*. As a novel non-thermal, non-expansive and comprehensive downstream interaction device, coupling an ELF-MF generator to a bio-reactor could be costless method in order to improve recombinant protein yield in bacterial host compared to the other expensive methods such as thermodynamic modulations of expression condition, co-expression of chaperones with recombinant protein and inclusion body re-solubilizing methods as well. Although in its infancy, these promising results open a new field for further studies evaluating promising effects of this approach in improving protein expression yields in different hosts. Further studies are also required for determining the effective frequency windows for different species and

also the probable underlying mechanisms of these promising results achieved by exposure to ELF-MF in order to further improving protein expression yields.

CRediT authorship contribution statement

Behnaz Rashidieh: Investigation, Writing – original draft. **Alireza Madjid Ansari:** Conceptualization, Methodology, Supervision, Project administration. **Mahdi Behdani:** Validation, Methodology. **Behrad Darvishi:** Formal analysis, Writing – original draft, Writing – review & editing. **Mahdi Habibi-Anbouhi:** Conceptualization, Methodology, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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