Potential of Nanoparticles Chitosan for Delivery pcDNA3.1-SB3-HBcAg

Lalu Unsunnidhal[1,4], *Raudatul* Jannah[2], *Abdul* Haris[3], *Agus* Supinganto[3], *Asmarani* Kusumawati[4*]

[1]Biomedical Field, Nursing Study Program, STIKES Yarsi Mataram, West Nusa Tenggara, 83361, Indonesia
[2]Midwifery Study Program, STIKES Yarsi Mataram, West Nusa Tenggara, 83361, Indonesia
[3]Nursing Study Program, STIKES Yarsi Mataram, West Nusa Tenggara, 83361, Indonesia
[4]Department of Reproduction and Obstetrics, Faculty of Veterinary Medicine, University Gadjah Mada, Yogyakarta, 55281,Indonesia

Abstract. Hepatitis B virus (HBV) is a DNA virus that causes hepatitis in humans. This study aims to prepare a Hepatitis DNA vaccine. he optimized base sequence of the SB3-HBcAg gene was derived from the nucleotide base sequence of the Hepatitis B core antigen B3 HBcAg subgenotype, and then Cloning of the pcDNA3.1-SB3-HBcAg has been successfully performed on E. coli DH5α and confirmed by PCR, restriction analysis and sequencing. The propagated plasmids were prepared as DNA-chitosan complex and physiochemical characterized using Particle Size Analyzer. Complex with a 4:1 (wt/wt) ratio of DNA with 0.04% concentration and chitosan have a mean diameter of 231.7 nm and zeta potential +12.3 mV and the value of Cytotoxicity Assay 80-90% as compared to the untreated cells that used as negative control, so it can be concluded that nanoparticles chitosan has good potential as a carrier agent for pcDNA3.1-SB3-HBcAg. **Keywords:** A codon optimization, Chitosan nanoparticles, HBcAg, Hepatitis B virus, Delivery System

1 Introduction

Hepatitis B virus (HBV) is a DNA virus that causes hepatitis in humans. It is estimated that as many as 2 billion people have been infected, 350 million of which have developed chronic infections causing 600,000 deaths each year. Indonesia is one of the countries with the highest prevalence of HBsAg, ranging from 2.5% to 10% [1], with the highest rates reported in North Sulawesi at 33.0%, Papua at 12.8% and the lowest in Jakarta 4.0% [2].

Therapy for chronic hepatitis B can be treated with interferon and antiviral compounds (Tenofovir and entecavir), but these drugs cannot degrade all forms of viral DNA, such as cccDNA. In addition, long-term treatment with antiviral drugs can lead to viral toxicity and resistance [3]. Therefore, the most effective approach to control and prevent the spread of HBV is immunization using a competent vaccine. One type of vaccine which is a new breakthrough in vaccine technology is DNA vaccine. DNA vaccines are made by inserting DNA or genes encoding immunogenic proteins into eukaryotic expression vectors [2]. One of the structural genes of HBV used as a source of DNA vaccines is the HBcAg gene (HBV/B subgenotype B3). HBcAg was chosen as a vaccine candidate because it is more specific for cytotoxic T lymphocytes (CTL) in the liver. CTL secretes the cytokines TNF- and interferon, both of which will affect infected hepatocytes, so that HBV replication in these cells can be inhibited [2].

DNA vaccines require delivery systems to work optimally. DNA vaccines have the disadvantage of low effectiveness in triggering the immune system in organisms due to limitations in the delivery system [4]. On the other hand, the delivery system using nanoparticle chitosan is widely used as a delivery system in DNA in gene therapy; This occurs because of the positive charge of nanoparticle chitosan which can be DNA condensation reagents [5], [6]. In addition, chitosan nanoparticles are also known to have strong electrostatic interactions with negative DNA charges [5], [6]. This causes chitosan nanoparticles to be good candidates for DNA delivery systems [5], [6]. Based on the above description, this study aims to evaluate the in vitro potential of nanoparticle chitosan as a carrier system for recombinant DNA (SB3-HBcAg) as a model carrier system for the DNA vaccine.

2 Material and methods

2.1 Construction of SB3-HBcAg expression cassettes

In this study, a synthetic DNA vaccine was designed based on the nucleotide base sequence of hepatitis B gene core antigen B3 subgenotype HBcAg [2]. The consensus sequence was optimized using codon optimization with target host Bos taurus using the services of Gene Universal Inc. Several motif

* Corresponding author: uma_vet@ugm.ac.id

sequences that potentially produce lower expression such as Chi site (GCTGGTGG), ter site core (GTTGTAAC), polyA sites (AATAAA or ATTAAA), consensus eukaryotic promoter core (TATA), immunosuppressive telomeric motif (TTAGGG), DNA uptake sequences (GCCGTCTGAA, AAGTGCGGT or ACAAGCGGTC), consensus splice donor (AGGT), consensus splice acceptor (CAGG), polyA binding proteins consensus (AAAAA), polyT binding proteins consensus (TTTTT) and DnaA binding site (TTATCCACA) were substituted using codon optimization process [7]. The expression cassette was constructed by adding a start codon and a double-codon stop. The gene was synthesized using the Custom Gen Synthesis service by Gene Universal Inc. and cloned to one of the available standard vectors, pcDNA 3.1 to produce pcDNA3.1-SB3-HBcAg gene.

2.2 Plasmid preparation and confirmation

The cloned plasmid from Gene Universal Inc. would be transformed into the host bacteria (E. coli DH5α) for the production of DNA vaccine on a larger scale. The preparation of the chemically competent bacterial cells based on the calcium chloride method. At first, Escherichia coli DH5α was propagated in a shaker incubator at 37°C, 150 rpm for 16 hours and subcultured into a new medium with the same conditions until the optimal density value reaches 0.4. A total of 1.5 ml of bacterial culture was transferred into a 1.5 ml tube (Biologix), incubated on ice for 30 minutes, precipitated using centrifugation (4000 rpm, 10 min, 4°C). The pellets were resuspended with 1.5 ml of cold sterile CaCl2 100 mM (Sigma), incubated on ice for 30 minutes with an occasional shaking, and precipitated using centrifugation again (4000 rpm, 10 min, 4°C). The pellet was resuspended with 30 µl of cold sterile 100 mM CaCl2 and incubated again on ice for 2 hours.

An amount of 10 ng DNA plasmid pcDNA3.1-SB3-HBcAg was added to the competent cell and incubated on ice for 30 minutes. The cells were subjected to heating at 42°C for 45 seconds. The cells were incubated on ice quickly for 2 minutes. A total of 0.9 ml of Luria Bertani Broth at 37oC was added after heat shock treatment and then the cells were incubated at 37oC for 1.5 hours with shaking. A total of 50 µl cell cultures were grown on Luria Bertani agar medium containing 100 µg/ml of Ampicillin (Sigma). In addition, competent cells transformed with water were used as negative controls.

Two colonies in Ampicillin selection medium were picked and suspended into the mastermix using sterile white tip (Axigen) (10 µl) for first confirmation using PCR. The master mix contained 5 µl GoTaq Master Mix 2X (Promega), 3.4 µl nuclease-free water (Qiagen), 0.8 µl primer forward SB3-HBcAg (5'-ACGTGAATATGGGCCTGAAG- 3') 10 µM (IDT) and 0.8 µl Primer reverse SB3-HBcAg (5'-CTTCTAGGGCTCTGGCTCCT- 3') 10 µM (IDT).

These primers were designed based on the consensus sequence using primer3 software from https://primer3plus.com/cgi-bin/dev/primer3plus.cgi to amplify the specific fragment of the SB3-HBcAg gene with the product size of 250 bp. The PCR was performed under the conditions of pre-denaturation at 95oC for 5 minutes, denaturation at 95oC for 30 seconds, annealing at 53.5oC for 30 seconds, elongation 72oC for 30 seconds, post-elongation at 72oC for 10 minutes. All of the above processes were conducted for 25 cycles. The PCR products were electrophoresed in 1.5% agarose gel (Lonza) contained SYBR Safe DNA Gel Stain (Invitrogen) and visualized under the UV illuminator.

The positives clones were picked up again from media selection and cultured on a 10 ml liquid medium containing 100 µg/ml of Ampicillin with shaking 150 rpm at 37°C for 16 hours. Subsequently, the culture was inoculated into a new 140 ml liquid medium containing 100 µg/ml Ampicillin and incubated with the same conditions before. The plasmid isolation from the bacterial cells was performed by using Plasmid DNA Extraction Maxi Kit (Favorgen Biotech Corp). Furthermore, the concentration of the obtained plasmid was measured using Nanodrop (Maestro Gen). The plasmid was visualized in 1.5% agarose gel (Lonza) with SYBR Safe DNA Gel Stain (Invitrogen). Before loading to the gel, the plasmid was mixed with a 5X dye DNA Pilot Gel (Qiagen), a loading buffer. The further confirmation was performed by restriction analysis using MluI and XhoI. Amount of 1 µg plasmid was mixed with 1 µL MluI (10 U/µl, Invitrogen), 1 µL enzyme XhoI (20 U/µL, Invitrogen), 2 µL buffer restriction enzyme (Invitrogen) and nuclease-free water (Qiagen) to the final volume of 20 µL. The mixture was incubated at 37°C overnight. The restriction enzymes were inactivated by heating at 85oC for 20 minutes. The result was electrophoresed in 1% agarose gel. The expected size of the plasmid backbone is 4600 bp, while the expected size of the Jtat fragment is 1300 bp.

The last confirmation was the sequencing of the inserted gene in the plasmid. The inserted gene was amplified using PCR. The master mix contained 30 µl GoTaq Master Mixes 2X (Promega), 4.8 µl Primer CMV-F (5'-TAATACGACTCACTATAGG-3') 10 µM (IDT), 4.8 µl Primer BGH-R (5'-TAGAAGGCACAGTCGAGG-3') 10 µM (IDT), 17.4 µl nuclease-free water (Qiagen) and 3 µl of plasmid as a template. The primer pair used was the Universal primer pair for the sequencing primers on the pcDNA3.1(+) vector. The PCR was conducted under the conditions of predenaturation at 95oC for 5 minutes, denaturation at 95oC for 30 seconds, annealing at 51oC for 30 seconds, elongation 72oC for 1.5 minutes, and post-elongation at 72oC for 10 minutes. All processes were conducted for 25 cycles. The PCR products were sent for sequencing service (First Base, Singapore) and then sequenced data were analyzed using the ClustalW online program at www.genome.jp/tools-bin/clustalw.

2.3 Preparation of chitosan-DNA plasmidcomplex

Chitosan-pcDNA3.1-SB3-HBcAg were prepared by the complex coaservation method (unsun) described as follows. A total of 0.1 g of medium chain chitosan

was dissolved in 10 mL of 1% acetic acid solution thenthe pH of the solution was adjusted using NaOH solution to reach a pH of 5.0. The solution was filtered using a 0.2 m sterile filter and stored in a sterile conickel tube and made several series of concentrations including0.02%, 0.04% and 0.06%.

A total of 1 g of plasmid and several solutions with different ratios of chitosan were heated separately at 50oC for 10 minutes. The plasmid DNA solution and the chitosan solution were mixed by vortexing at 3000 rpm for 30 seconds. The ratio of the mass ratio of chitosan and DNA used was 0.5:1, 1:1, 2:1, 3:1, 4:1 and 5:1 witha final total volume of 30 L.

2.4 Characterization: particle size, potential zeta, and cytotoxicity assay

The measurement of particle size was calculated by SZ-100 (HORIBA Scientific) at 24.9oC and scattering angle 90o, while the measurement of zeta potential was calculated by SZ-100 (HORIBA Scientific) at 24.8oC and electrode voltage 3.3 Volt.

The effect of the transfecting agents on the cells after transfection were investigated using the MTT assay. The HeLa cells were seeded in a 96 well plate with the initial density of 5x103 cells per well in 200 µL supplementedDMEM medium. After 24 h, the cells were treated with plasmid, chitosan, and chitosan-DNA complex for 4 h. The medium was replaced with fresh medium and incubated for 24 h. Then, the medium was replaced by 100 µL of MTT stock solution and incubated again for 3 h at 37°C in a CO2 incubator. The stopper reagent contained SDS 10% was added into the well. The untreated HeLa cells were used as a negative control. The absorbance of the dye in the solution was measuredat the wavelength of 500 nm with ELISA plate reader.

3 Results

3.1 Construction of SB3-HBcAg expression cassettes

Codon optimization is a gene engineering method that changes existing codons with synonymous codons to increase the production of the protein they encode. Codon optimization is carried out using the services of Gene Universal Corp. The optimized base sequence of the SB3HBcAg gene is derived from the nucleotide base sequence of the Hepatitis B core antigen B3 HBcAg subgenotype [2]. The results of the optimization of the codon of the SB3-HBcAg gene are shown in Figure 1A.The expression cassettes were constructed by adding BamHI and EcoRI restriction sites for cloning sites, ATG codon as a start codon, TAATAA codons as a double-codon stop. The gene was synthesized using the Custom Gen Synthesis service by Gene Universal Inc. and cloned to one of the available standard vectors, pcDNA3.1 (+) to form the recombinant plasmid pcDNA3.1-SB3-HBcAg gene.

A

Optimization Report
Gene Universal Corp.

Before Codon Adjustment
CAI: 0,68

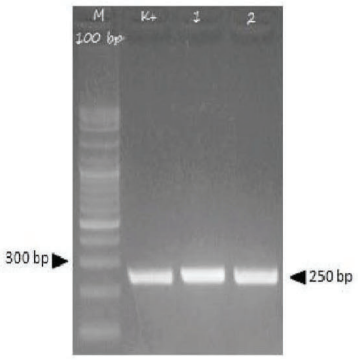
Original Codon:
ATGGACATTGACCCGTATAAAGAATTTGGAGCTTCTGTGGAGTTACTCTCTTTTTT
GCCTTCTGACTTCTTTCCTTCTATTCGAGATCTTCTCGACACCGCCTCTGCTCTGTA
TCGGGAGGCCTTAGAGTCTCCGGAACATTGTTCACCTCACCATACGGCACTCAGG
CAAGCTATTTTGTGTTGGGGTGAGTTGATGAATCTAGCCACCTGGGTGGGAAGTA
ATTTGGAAGACCCTGCATCCCGGGAATTAGTAGTCAGTTATGTCAATGTTAATAT
GGGCCTAAAAATCAGACAACTATTGTGGTTTCACATTTCCTGTCTTACTTTTGGAA
GAGAAACTGTTCTTGAATATTTGGTGTCTTTTGGAGTGTGGATTCGCACACCTCCT
GCATATAGACCACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTG
TTAGACGACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCACCTCGCAGACGAA
GGTCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAG

Original Protein:
MDIDPYKEFGASVELLSFLPSDFFPSIRDLLDTASALYREALESPEHCSPHHTALRQAI
LCWGELMNLATWVGSNLEDPASRELVVSYVNVNMGLKIRQLLWFHISCLTFGRETV
LEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRR
RSQSRESQC*

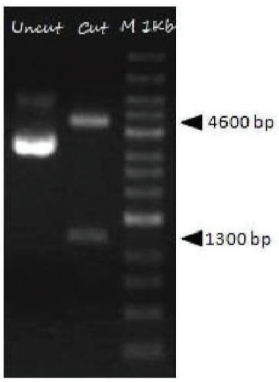
After Codon Adjustment
CAI: 0,97

Optimized Codon: **Seluen Gen SB3-HBcAg (Telah Dioptimasi) dirahasiakan oleh peneliti untuk kepentingan Paten**
Optimized Protein:
MDIDPYKEFGASVELLSFLPSDFFPSIRDLLDTASALYREALESPEHCSPHHTALRQAI
LCWGELMNLATWVGSNLEDPASRELVVSYVNVNMGLKIRQLLWFHISCLTFGRETV
LEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRR
RSQSRESQC*

B



C



D

```
CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: Optimization   564 bp
Sequence 2: Sequencing_F   764 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score:  100
Guide tree file created:   [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences:   2      Score:10716
Alignment Score 4336

CLUSTAL-Alignment file created  [clustalw.aln]
```

Figure 1. Construction of SB3-HBcAg expression cassettes and preparation of DNA plasmid. HBsAg SB3 Gene Codon Optimization Results (A). Results of Recombinant DNA Plasmid Amplification with SB3HBcAg gene detection primer. Synthetic gene SB3-HBcAg (K+) was used as a positive control, while plasmids isolated from transformant colonies (repeats 1 and 2) were tested to detect the insert gene SB3-HBcAg, marker 100 bp (M) (B). Restriction results of pcDNA 3.1-SB3HBcAg with MluI and XhoI, for confirmation (C). The results of data processing using Clustal W from the results of sequencing using Universal Primer for Sequencing the Insert gene on Plasmid pcDNA 3.1 (+), namely at the position of the CMV Promoter and bGH poly(A) signal (D).

3.2 Preparation of DNA plasmid

The synthetic gene was chemically produced and cloned into pcDNA3.1(+) vector with restriction sites of EcoRI and BamHI to build pcDNA3.1-SB3-HBcAggene. The transformation into E. coli DH5α as vector cloning was performed for larger scale production of DNA plasmid. This plasmid has a selectable marker for ampicillin resistance. Two chosen ampicillin-resistant bacteria in the selection media were confirmed by colony-PCR with primer SB3-HBcAg-For and SB3-HBcAg-Rev and showed a specific band (250 bp) (Figure 1B). The plasmid extracted from those positive clones were also checked with restriction analysis with MluI and XhoI (Figure 1C) to give two distinct bands that corresponding with the vector fragment (about 4600 bp) and target fragment (1300 bp). One of those clones was chosen to be confirmed with Sanger sequencing using CMV-F and BGH-R primers. The result of the sequencing and the initially designed transgene were aligned using online software and the results showed that the transformation process into the cloning vector did not induce any mutations in the nucleotide composition of SB3-HBcAg gene (Figure 1D).

3.3 Chitosan-DNA Complex Preparation

The chitosan-pcDNA3.1-SB3-HBcAg nanoparticle complex in this study was prepared by the complex coaservation method by varying the concentration of chitosan against DNA into several variations of concentration including 0.02%, 0.04%, and 0.06% and each concentration was made in 6 ratios (Chitosan:DNA) ie 0.5:1 to 5:1. To evaluate the formation of the chitosan-pcDNA3.1-SB3-HBcAg nanoparticle complex, entrapment observations were carried out using 1% 100V agarose gel electrophoresis for 30 minutes with the observed indicator in the form of DNA capable of being perfectly entangled in chitosan nanoparticles in the hope that no DNA was released during the visualization process. . All complexes were prepared in a total volume of 30 L (10 L DNA and 20 Lchitosan). The results of the electrophoresis of the complex formed are presented in Figure 2.

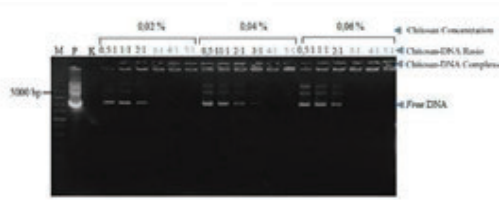


Figure 2. Visualization of the chitosan-pEGFP-C1-tat complex. M) marker 5000 bp. P) pEGFP-C1-tat plasmid. K) chitosan without the addition of DNA. 0.5:1 to 5:1) chitosan-DNA complex with 0.02%, 0.04% and 0.06% chitosan concentrations.

3.4 Characterization of chitosan-DNA plasmid

The physicochemical characterizations were performed to investigate the further properties of the chitosan-DNA complex. These properties were measured by using dynamic light scattering (DLS) method by examining the random changes in the scattering light intensity detected in the solution. The average size and surface charge of the complex prepared from unmodified chitosan are shown in Figure 3. The mean particle size for chitosan-DNA complex was 231.7 nm, while the zeta potential of this complex was found to be +12.3 mV.

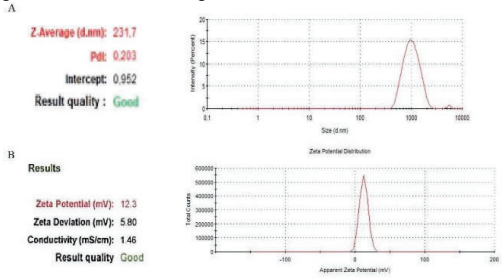


Figure 3. Characterization analyses of chitosan-DNA plasmid complex: particle size distribution (A) and zeta potential value measurement (B) using particle size analyser.

3.5 Cytotoxicity assay of chitosan-DNA plasmid

The cytotoxicity assay in this study was utilized to evaluate the impact of the chitosan-DNA plasmid complex on cellular viability. MTT assay will measure the decrease in mitochondrial activity in the cells in thepresence of our complex at the defined contact time. This assay for the chitosan-DNA complex formed at pH
5.0 showed that in the exposure time as long as 4 hours,this chitosan complex was not harmful to the cells. Theviability of the HeLa cells was about 80-90% as compared to the untreated cells that used as negative control.

4 Discussion

Plasmid pcDNA3.1(+) has been used previously as a DNA vaccine vector against various infectious disease such as Brucella abortus [8], grass carp reovirus [9] or

human immunodeficiency virus-1 [10]. This study will use the pcDNA3.1(+) vector to encode a synthetic gene constructed from a consensus sequence of one gene from JDV. This strategy that designs a novel vaccine based on the consensus of amino acid sequences has been previously implemented to develop a vaccine against rabies virus [11], avian influenza [12] or hepatitis B [13]. Moreover, this study will also test the success of this vaccine delivery on in vitro level by formulating it in the form of chitosan complex. Chitosan is broadly employed in the pharmaceutical sector as a carrier agent that is able to release molecules in a controlled manners, such as DNA, vaccines, peptides, or antibiotics. This kind of delivery system has been employed in different vaccine candidate cases, such as intranasal chitosan-DNA vaccine for influenza virus [14], the oral chitosan-DNA vaccine against nodavirus [15] or vaccine for Trueperella pyogenes infection [16]. This study will evaluate chitosan complex as DNA vaccine delivery system at in vitro level to deliver the DNA plasmid encoding a gene of interest from JDV. In this case, the HeLa cell line would be used as a model to test the delivery of DNA vaccine across the eukaryotic membrane cells.

The main stage for the designing of this DNA vaccine is the construction of a codon-optimized nucleotide sequence which encodes SB3-HBcAg antigen. Based on the codon optimization report by Gene Universal Corp. above, the Codon Adaptation Index (CAI) value increased from 0.68 to 0.97 (Figure 1). CAI itself calculates the frequency of use of each codon and calculates the geometric mean of the frequency of use in each protein in the target Expression System [17]. So that the increase in CAI can optimize the expression of the SB3-HBcAg gene that encodes the Hepatitis B core antigen B3 subgenotype protein. Additionally, some motif sequences were suggested to be excluded to avoid the lower plasmid production in host cloning or lower translation of target protein in the host expression [7]. Prior to the preparation of plasmid in a higher yield, the transformed plasmid in the host cloning had to be confirmed to make sure there is no mutation take place in the host cloning. The confirmations such as PCR, restriction analysis and sequencing were performed to ensure the expression cassette could produce the desired amino acid sequence. In this study, the mean diameter of chitosan-DNA complex 231.7 nm with a polydispersion index value of 0.203 (Figure 3). This size falls in the range around 100-250 nm so it showed that this complex has a normal size for a complex that produced by using coarsevation complex method [18]. The particle size is thought to be the main factor that affects particle uptake and gene delivery into the cells [19]. A particle with a size higher than 100 nm is proven to enter the cells through endocytosis or pinocytosis [20]. Another physiochemical character observed was zeta potential that shows the charged of the complex. The complex produced in this study has a zeta potential value of +12.3 mV. A positively charged complex was likely to be easily attached to the cell membrane that has a negative charge and be internalized into the cells through endocytosis [19].

Another important assay that had to be performed before transfection study is the cytotoxicity assay. In this study, the MTT assay was conducted to evaluate the effect of the chitosan complex to the viability of the cells during exposure time with the cells. The positive charge of the polycationic charge like chitosan complex can interact strongly with plasma membrane protein through an electrostatic bond, destabilize it and ultimately rupture the membrane structure of the cell [19]. Under 4 h of transfection, chitosan solution and chitosan-DNA complex showed the relative viability with the value higher than 90%. The similar chitosan complexes that result to a relative viability ranging from 80-90% was still reviewed to have no high toxicity potential [5], [6]. This test indicates that chitosan complex does not exhibit a high toxicity potential for the cell during exposure time for transfection study.

References

1. H. Gunardi, F. Zaimi, L, R. Soedjatmiko, A, and H. Muljono, D, “Current prevalence of hepatitis B infection among parturient women in Jakarta, Indonesia,” Acta Med Indones, vol. 46, pp. 3–9, 2014.
2. R. Waty, Z. Mustopa, Apon, Suharsono, A. Ningrum, Ratih, and M. Hidayah, “Soluble expression and purification of hepatitis B core antigen (HBcAg) subgenotype B3 in Escherichia coli using thioredoxin fusion tag,” Asian Pacific J. Trop. Dis., vol. 7, no. 8, pp. 496–501, 2017.
3. F. Akbar, S, M, M. Al-Mahtab, H. Uddin, M, and I. Khan, S, “HBsAg, HBcAg, and combined HBsAg/HBcAg-based therapeutic vaccines in treating chronic hepatitis B virus infection,” Hepatobiliary Pancreat Dis Int, vol. 12, no. 4, pp. 363–369, 2013.
4. G. Wang, L. Pan, and Y. Zhang, “Approaches to Improved Targeting of DNA Vaccines,” Hum Vaccines., vol. 7, pp. 1271–1281, 2011, doi: DOI:10.4161hv.7.12.17983.
5. L. Unsunnidhal, J. Ishak, and A. Kusumawati, “Expression of gag-CA Gene of Jembrana Disease Virus with Cationic Liposomes and Chitosan Nanoparticle Delivery Systems as DNA Vaccine Candidates,” Trop. Life Sci. Res., vol. 30, no. 3, pp. 15–36, 2019, doi: https://doi.org/10.21315/tlsr2019.30.3.2.
6. J. Ishak, L. Unsunnidhal, R. Martien, and A. Kusumawati, “In vitro evaluation of chitosan-DNA plasmid complex encoding Jembrana disease virus Env-TM protein as a vaccine candidate,” J. Vet. Res., vol. 63, no. 1, pp. 7– 16, 2019, doi: https://doi.org/10.2478/jvetres- 2019-0018.
7. J. Williams, “Vector Design for Improved DNA Vaccine Efficacy, Safety and Production,” Vaccines, vol. 1, no. 3, pp. 225– 249, 2013, doi: 10.3390/vaccines1030225.
8. D. Luo et al., “Protective Immunity Elicited by A Divalent DNA Vaccine Encoding Both The L7/L12 and Omp16 Genes of Brucella abortus in

BALB/c Mice," Infect Immun, vol. 74, no. 5, pp. 2734–2741, 2006, doi: DOI:10.1128/IAI.74.5.2734-2741.2006.

9. Y. Gao, C. Pei, X. Sun, C. Zhang, L. Li, and X. Kong, "Plasmid pcDNA3.1-s11 Constructed Based on The S11 Segment of Grass Carp Reovirus as DNA Vaccine Provides Immune Protection," Vaccine, vol. 36, no. 25, pp. 3613–3621, 2018, doi: 10.1016/J.VACCINE.2018.05.043.
10. M. T. Shata, M. S. Reitz Jr., A. L. DeVico, G. K. Lewis, and D. M. Hone, "Mucosal and systemic HIV-1 Env-specific CD8+ T-cells Develop after Intragastric Vaccination with A Salmonella Env DNA Vaccine Vector," Vaccine, vol. 20, no. 3–4, pp. 623–629, 2001, doi: 10.1016/S0264-410X(01)00330-9.
11. S. Starodubova, E, V. Kuzmenko, Y, A. Latanova, A, V. Preobrazhenskaya, O, and L. Karpov, V, "Creation of DNA vaccine vector based on codon-optimized gene of rabies virus glycoprotein (G protein) with consensus amino acid sequence," Mol. Biol., vol. 50, no. 2, pp. 328–331, Mar. 2016, doi: 10.1134/S0026893316020242.
12. D. J. Laddy, J. Yan, N. Corbitt, G. P. Kobinger, and D. B. Weiner, "Immunogenicity of novel consensus-based DNA vaccines against avian influenza," Vaccine, vol. 25, no. 16, pp. 2984–2989, Apr. 2007, doi: 10.1016/j.vaccine.2007.01.063.
13. R. Jannah and L. Unsunnidhal, "KONSTRUKSI DAN KLONING PLASMID PCDNA3 . 1 (+) DENGAN SUBGENOTIP B3 HEPATITIS B CORE ANTIGEN (HbcAg SEBAGAI KANDIDAT VAKSIN DNA HEPATITIS B," J. Penelit. dan Kaji. Ilm. Kesehat., vol. 5, no. 2, pp. 125–131, 2019.
14. C. Sawaengsak et al., "Intranasal Chitosan- DNA Vaccines that Protect Across Influenza Virus Subtypes," Int J App Pharm, vol. 473, no. 1–2, pp. 113–125, 2014, doi: 10.1016/j.ijpharm.2014.07.005.
15. Y. Valero et al., "An Oral Chitosan DNA Vaccine Against Nodavirus Improves Transcription of Cell-Mediated Cytotoxicity and Interferon Genes in The European Sea Bass Juveniles Gut and Survival Upon Infection," Dev Comp Immunol., vol. 65, pp. 64–72, 2016, doi: 10.1016/j.dci.2016.06.021.
16. T. Huang et al., "Chitosan - DNA Nanoparticles Enhanced The Immunogenicity of Multivalent DNA Vaccination on Mice agaInst Trueperella pyogenes Infection," J. Nanobiotechnology, vol. 16, no. 8, pp. 1–15, 2018.
17. P. M. Sharp and W. H. Li, "The Codon Adaptation Index–a Measure of Directional Synonymous Codon Usage Bias, and Its Potential Applications," Nucleic Acids Res, vol. 15, pp. 1281–1295, 1987.
18. A. E. Gregory, R. Titball, and D. Williamson, "Vaccine Delivery using Nanoparticles," Front Cell Infect Mi, vol. 3, p. 13, 2013, doi: 10.3389/fcimb.2013.00013.
19. S. Nimesh, M. M. Thibault, M. Lavertu, and M. D. Buschmann, "Enhanced Gene Delivery Mediated by Low Molecular Weight Chitosan/DNA Complexes: Effect of pH and Serum," Mol Biotechnol., vol. 46, no. 2, pp. 182–196, 2010, doi: 10.1007/s12033-010- 9286-1.
20. J. C. Venter et al., "The sequence of the human genome," Science (80-.)., vol. 291, pp. 1304–1351, 2001.