Preparation of An scFv-Based Immunoliposome Specific towards Transferrin Receptor

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Abstract

An ideal therapeutic for cancer would be one that selectively targets to tumor cells, is nontoxic to normal cells, and that could be systemically delivered, thereby reaching metastases as well as primary tumor. Immunoliposomes directed by monoclonal antibody or its fragments are promising vehicles for tumor targeted drug delivery. Transferrin receptors (TfR) levels are elevated in various types of cancer cells and considered to correlate with the aggressive or proliferative ability of tumor cells. Therefore, TfR levels can be elaborated as a prognostic tumor marker, and TfR is a potential target for drug delivery in the therapy of malignant cells. Here, we report the preparation of an anti-TfR single-chain antibody variable (scFv) immunoliposome for tumor-targeted delivery vehicle. The cDNA encoding the variable heavy and light chain domains of the anti-TfRscFv antibody fragment was derived from the murine monoclonal antibody Clone E6, which is specific towards transferrin receptor. The gene encoding the anti-TfR scFv fragment was codon optimized for expression in *Escherichia coli*, subsequently synthesized, and cloned into the expression vector pJexpress404. The His6-tagged anti-TfR scFv fragment was expressed in *E. coli* and purified by means of immobilized metal-ion affinity chromatography on TALON™ matrix. SDS-PAGE revealed that the scFv fragment had the size of approximately 27 kDa, which corresponded with the predicted size of the protein based on its amino acid sequence. Liposome containing 5% MPB-DOPE were prepared by ethanol injection method. Afterwards, the anti-TfR scFv fragments were covalently conjugated to the liposome to produce the anti-TfR scFv immunoliposome with the size of around 200 to 300 nm.

Keywords: antibody fragment, codon optimization, immunoliposome, scFv, transferrin receptor

Introduction

Direct targeting of cancer cells with therapeutic agents has the potential to treat cancer on the basis of its molecular characteristics. Although such cancer treatments have substantial promise, many practical obstacles need to be overcome before they can fulfill their goals in the clinic (McCormick, 2001). Among the important issues associated with macromolecular treatments of cancer is the efficient delivery of the molecular therapeutics to the site(s) in the body where they are needed (Harrington *et al.*, 2001). Among the important issues associated with macromolecular treatments of cancer is the efficient delivery of the molecular therapeutics to the site(s) in the body where they are needed (Harrington *et al.*, 2001). An ideal delivery vehicle would be one that could be systemically (as opposed to locally) administered and that would selectively target tumor cells. Recent gene therapy approaches employ either viral or non-viral vector systems (Ledley, 1995). Progress has been made toward developing non-viral, pharmaceutical formulations of genes for *in vivo* human therapy, particularly cationic liposome-mediated gene transfer systems (Felgner *et al.*, 1995; Xuet *et al.*, 2001). One disadvantage of cationic liposome is that they lack tumor specificity and have relatively low transfection efficiencies as compared to viral vectors. However, this can be dramatically improved when the liposome bears a ligand, such as antibody and antibody fragments that is able to recognize a cell surface receptor (Bendas, 2001).

The use of antibody molecules to target defined cell types was actually proposed 100 years ago. It took the successful development of hybridoma technology (Köhler & Milstein, 1976) with the resulting ability to produce monoclonal antibodies (mAbs) to make this approach a reality. However, the large size of
the intact mAbs (approximately 155 kDa) limits their ability to diffuse from vasculature into a tumor due to the high hydrostatic pressure resulting from the disordered blood vessels and the lack of draining lymphatic (Jain & Baxter, 1988). Furthermore, interactions with the Fc receptors (FeR) located on normal tissue can alter the distribution of the mAbs, potentially endangering the patient when toxic payloads are attached, e.g., radioisotopes or catalytic toxins. Clearly, these characteristics limit their utility as vehicles for the treatment of solid tumors.

In order to address the limitations of large IgG molecules, smaller engineered mAb-based molecules were developed. The 25-kDa single-chain Fv(scFv) molecule is a much smaller antibody fragment developed to date with potential clinical applications. The antibody scFv fragment composed of a variable region of the light chain (V_L) and a variable region of the heavy chain (V_H) joined via a short peptide spacer sequence. It has been demonstrated that the protein could be produced in significant amounts in the cells of Escherichia coli.

Various reports revealed that transferrin receptors levels are elevated in various types of cancer cells, including breast cancer, cervical cancer and prostate cancer cells, and correlate with the aggressive or proliferative ability of tumor cells (Elliott et al., 1993; Qian et al., 2002). Therefore, TfR is a potential target for drug delivery in the therapy of malignant cell growth (Thorstensen & Romslo, 1993; Ryschich et al., 2004). Various laboratories have made use of the transferrin receptor to facilitate uptake of antibody-toxin conjugates (Batraet al., 1991) or to direct cationic liposomes to receptor-bearing cells (Allen et al., 1995).

In this work, we took advantage of proven successes in codon engineering for the expression of proteins in E. coli. We designed a synthetic gene encoding the anti-TfR antibody scFv fragment using the E. coli preferred codon usage. The codon optimized scFv variant was designed to be primarily expressed as a soluble protein in the periplasmic space of the bacterium. Previously, various anti-TfR antibody scFv fragments have been generated, but mostly were expressed in the E. coli cytoplasm as inclusion bodies (Xu et al., 2001; Yang et al., 2005; Ye et al., 2012). The purified scFv fragment was subsequently conjugated to a liposome by a covalent linkage between a cysteine residue at the carboxyl-terminal of the protein and a maleimide group on the liposome. In the future, the so constructed anti-TfR immunoliposome can be verified further for its potential in the targeted delivery of drugs or other therapeutic agents for the treatment of tumors expressing transferrin receptor.

Materials and Methods

Plasmid and chemicals. The expression vector pExpress404 (T5 Promotor, AmpR, pUC origin) was obtained from DNA2.0. The natural lipids HydroSoy PC (L-α-phosphatidylcholine, hydrogenated), the cationic lipids DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)-propane, DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), plant cholesterol and the fusogenic neutral helper lipid MPB-DOPE (1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine-N-{4-(p-maleimidophenyl)butyramide}) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). All other chemicals were purchased from Merck or Sigma-Aldrich except otherwise mentioned.

Construction of synthetic gene. The amino acid sequences of the heavy-chain (V_H) and the light-chain domain (V_L) of the antibody were derived from the anti-transferrin receptor monoclonal antibody Clone E6 (Hoogenboom et al., 1990). The amino acid sequences for V_H and V_L domains were obtained from GenBank (http://www.ncbi.nlm.nih.gov) with the accession number M29533 for V_H and M29534 for V_L domain, respectively. Between the C-terminal of the V_L domain and the N-terminal of the V_H domain a peptide linker (Gly5Ser)3 was inserted to covalently link both domains. At the N-terminal of the gene, a sequence encoding the signal peptide of OmpA was added for localization of the protein into bacterial periplasm during expression, whereas at the C-terminal the sequence encoding six histidines and a cysteine (His6Cys) was incorporated. The His6-tag is commonly used to facilitate protein purification, and the cysteine residue can be
used to covalently link the scFv fragment with liposome particles.

The constructed amino acid sequence for the anti-TfR-scFv antibody fragments was used as blueprint to design the synthetic gene encoding anti-TfR-scFv by using the software Gene Designer and DNA2.0 algorithms (http://www.dna20.com). The synthetic gene was designed to use the codon preference for *E. coli* as the host for gene expression. The codon frequency table for *E. coli* was deduced from Codon Usage Database (http://www.kazusa.or.jp/codon/). The gene encoding anti-TfR-scFv antibody fragment was hereinafter referred to as synAnti-TfR-scFv, subsequently synthesized by DNA2.0 (Menlo Park, CA), and cloned into the expression vector pJEexpress404. The expression vector harboring the gene synAnti-TfR-scFv was designated pJE-CompA.

**Expression and purification of scFv fragment.** The expression vector pJE-CompA was transformed into the expression host cells *E. coli* JM109 by heat-shock at 42°C for 45 sec. *E. coli* JM109 containing pJE-CompA were grown on Luria-Bertani (LB) plates containing 100 μg/ml ampicillin. Single colonies were picked and grown overnight in 10 ml LB containing 100 μg/ml ampicillin at 37°C. This culture was then diluted 1:50 with LB-medium containing 100 μg/ml ampicillin, grown at 37°C and 200 rpm in 1-L shaking flask to an OD600 of 0.8-1.0, after which IPTG (Fermentas) was added to a final concentration of 0.1 μg/ml and growth was continued for 4 h. The cells were harvested by centrifugation (5000xg, 15 min, 4°C) and then resuspended in 50 mM PBS containing 300 mM NaCl. After addition of lysozyme to a final concentration of 0.5 mg/ml and incubation at room temperature for 20 min, the suspension was sonicated carefully to isolate the scFv fragments, which were expressed in the periplasmic space of *E. coli*.

Purification of the anti-TfR scFv-fragments was performed by immobilized metal-ion affinity chromatography (IMAC) on TALON™ (Clontech) chromatography matrix (Kusharyoto et al., 2002). Two milliliters of the matrix were loaded into a PD-10 column (GE Bioscience) and equilibrated with 50 mM PBS pH 7.2 containing 300 mM NaCl. 10 ml periplasmic extract from 0.4 litre culture were applied to the column. After washing with 40 ml of the same buffer the scFv fragments were eluted from the column with 250 mM imidazole in 50 mM PBS pH 7.2 containing 300 mM NaCl. Removal of the imidazole was performed by gel-filtration on Sephadex G-25 (GE Bioscience) in PD-10 column. Periplasmic extracts from cell extract and fractions from purification by IMAC were resolved on 12% reducing SDS–PAGE. Protein concentrations were estimated by bicinchoninic acid (BCA) protein assays (Pierce, USA). Different dilutions of BSA were used to draw a standard curve, which was used to estimate the total protein content of a sample.

**Conjugation of scFv fragment to liposome.** The purified scFv containing a cysteine residue at the C-terminus, scFv-cys, was first reduced by DTT (Sigma-Aldrich) to obtain the monomer scFv-SH. One molar DTT was added to the scFv in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) to a final concentration of 1-50 mM. After stirring at room temperature for 5–10 min, the protein was desalted on a 10-DG column (Bio-Rad). Neutral liposomes containing 5% molar MPB:DOPE of total lipids in lipidosome formulations of Lip 1 (DOTAP:DOPE, 1:1 molar ratio) or Lip 2 (HydroSoy PC:cholesterol, 1:1 molar ratio) were prepared by the ethanol injection method as described (Campbell, 1995) with minor modification. Briefly, lipids (each 2x10^{-5} mol) in 100 μl ethanol were injected quickly into 800 μl water at 55°C in a test tube while vigorously stirring. The test tube was vortexed for an additional 20 min while cooling to room temperature. The hydrated lipid suspension was extruded through a polycarbonate membrane with the pore diameter of 100 nm according to user’s manual to produce the MPB-liposome particles. Since the maleimide group is not stable in aqueous solution with a pH 7.0, the liposomes were prepared in water with a pH between 5.0 and 6.5 (Xu et al., 2002).

For conjugation, the reduced scFv-SH was added to the MPB-liposome at a protein:lipsids ratio of 1:10–1:40 (weight ratio). The solution was mixed by gentle stirring for 30 min at room temperature to produce scFv-Lip. To evaluate the efficiency of conjugation and the examine the presence of any unconjugated scFv in the scFv-liposome solution, a
discontinuous native (non-SDS) PAGE (Ornstein, 1964) was employed. Measurements of particle size distribution were performed by PT. Nanotech Indonesia (Serpong, Tangerang, Indonesia).

Results

In order to develop robust expression of a recombinant antibody scFv fragment specific towards transferrin receptor (TfR) in the E. coli periplasm, we synthesized a single chain antibody gene by using codons optimized to reflect abundantly translated E. coli mRNAs. To enable expression of the scFv fragment in the periplasm, a signal sequence OmpA, which has been used for effective translocation of various recombinant proteins in E. coli (Pines & Inouye, 1999), was added at the N-terminal of the protein.

The V\textsubscript{H} and V\textsubscript{L} domains of the constructed scFv fragment was derived from the anti-TfR monoclonal antibody Clone E6 produced by mammalian cell lines (Hoogenboom et al., 1990). Although an E. coli cell has a great capacity to produce large quantities of protein, there are limits when the composition of the mRNA or protein is not typical. Within E. coli, a clear bias exists among the amino acid codons found within the population of mRNA molecules and the translation rate is slowed when the target protein codon usage differs significantly from the average codon usage of the host organism (Angov et al., 2008).

When we analyzed the codon usage in the murine anti-TfR-scFv gene, almost 19% of codons showed rare occurrence in E. coli genes. Majority of them were arginine (7 of total 8), glycine (13 of 17), proline (6 of 10), serine (12 of 27), isoleucine (2 of 11), leucine (2 of 16) and threonine (2 of 19) (Figure 1). Rare codons and poor codon bias in the native scFv gene may be a possible cause of the inefficient translation and scFv production in E. coli. Such rare codons are not only strongly associated with low levels of protein expression due to ribosome stalling and failed translation (Hayes et al., 2002), but also implicated in frame shift and amino acid miss-incorporation (McNulty et al., 2003). To overcome this limitation, the murine anti-TfR-scFv gene was codon optimized for increased soluble expression in E. coli. The codon usage was adapted to the codon bias of E. coli genes with the help of Gene Designer software (Villalobos et al., 2006). All the rare codons and less preferred codons of the natural scFv gene were replaced with synonymous codons of high-frequency in the synthetic scFv gene.

Several reports and web-based programs proposed algorithms for gene optimization on the basis of different calculation methods (Puigbò et al., 2007). Codon usage preference in a gene is often measured by codon adaptation index (CAI) and the codon bias of a gene toward common codons is reflected in CAI (Sharp & Li, 1987). It is useful for predicting the level of expression of a gene and for making comparisons of codon usage in different organisms. The genes designed to match host bias or obtained by maximizing CAI value have been expressed successfully in different studies (Gustafsson et al., 2004).

We have calculated the CAI by using online tool available in the website https://www.genscript.com/cgi-bin/tools/rare_codon_analysis. The CAI of the native scFv gene was 0.65, whereas the design of the gene resulted in an increase of CAI to 0.83. However, such optimization approach might have some disadvantages like congestion of tRNAs due to repeatedly choosing the same codon for the frequently used amino acids, and an increase possibility of the occurrence of stable secondary structures such as hairpins in the vicinity of the 5' and 3' encoding regions of the corresponding mRNA sequence (Kurland & Gallant, 1996). To avoid these potential difficulties, we considered to use second-preferred codons alternatively with the most preferred codons during optimization.

The designed gene encoding the anti-TfR scFv was subsequently synthesized and cloned into the expression vector pJE-CompA (Figure 2). Verification by enzymatic digestion with NdeI and HindIII restriction sites, resulted in the expression vector pJE-CompA (Figure 2). Verification by enzymatic digestion with NdeI and HindIII as well as sequencing revealed that the gene synAnti-TfRscFv was successfully inserted into with the expression vector pJE-CompA (Figure 3). Following expression in E. coli JM109, periplasmic scFv extracts having His6-tag were purified by immobilized metal chelate affinity chromatography (IMAC) on TALONTM protein purification resin. Upon verification by SDS-PAGE, the anti-TfRscFv antibody fragments appeared as a single band at 27 kDa with relatively high purity, which corresponded to the calculated molecular
weight of scFv fragment with its C-terminal His-tag and an additional cysteine residue (Figure 4). Under appropriate experimental conditions, we observed from three independent experiments a yield of approximately 2 mg of purified scFv from the periplasmic extract of 1-liter *E. coli* culture.

Digestion with either *Nde*I or *Hind*III resulted in a linearized vector with the size of approximately 4,800 bps. Double digestion with both *Nde*I and *Hind*III yielded a DNA band with the size of around 800 bps for the synAnti-TfR-scFv gene.

Two different formulations of liposomes were prepared by ethanol-injection method (Campbell, 1995). Neutral liposome was prepared from a mixture of HydroSoy PC and cholesterol with a molar ratio 1:1. Such neutral liposome could especially be used for the delivery of anti-cancer drug molecules (Lee et al., 2002). Cationic liposome, which is particularly suitable as delivery vehicle for DNA or RNA molecules, was prepared from a mixture of DOTAP and DOPE with a molar ratio 1:1 (Pirollo et al., 2006). In each liposome formulation, MPB was included to facilitate the covalent linking of the scFv-fragment. Nanoliposomes were obtained by extrusion method using membrane with the pore size of 100 nm.

**Figure 1.** Codon optimization of the gene encoding the anti-TfR scFv-fragment for expression in *E. coli*. Sequence alignment of native or wild-type (Wt) and codon-optimized anti-TfRscFv gene (Opt) is shown. The rare codons and rare codon clusters are marked in red. The shaded horizontal boxes indicate the sequences for OmpA and the linker (G₄S), respectively. Around 19% of codons showed rare occurrence in *E. coli* genes. AA: the amino acid sequence
Figure 2. Schematic presentation of the vector pJE-CompA harbouring the synAnti-TfRscFv gene for the expression scFv-fragment in *E. coli*. The signal sequence OmpA was used to direct the protein translocation into the bacterial periplasm. His6 was used to facilitate protein purification, while the residue Cys enables covalent linkage with liposome containing maleimide group.

Figure 3. Verification of the presence of synAnti-TfR-scFv gene in the expression vector pJE-CompA by enzyme digestion.

For the conjugation of the anti-TfRscFv to MPB-liposome, the scFv sequence was constructed to include a cysteine residue at the carboxy-terminal. This carboxyl-terminal cysteine contains a free sulfhydryl group, which facilitated the conjugation of the scFv to MPB-liposome. Since it is important to have mainly monomer scFv fragments for conjugation, the scFv-cys was first reduced with DTT to obtain the monomer, scFv-SH. Our trials revealed that between 1 and 10 mM DTT was appropriate to reduce the scFv-cys (data not shown), which gave rise to approximately one free sulfhydryl per scFv molecule.

Figure 4. SDS–PAGE analysis of codon-optimized anti-TfRscFv protein from periplasmic extract. Lane M: protein marker; lane CE: periplasmic extract of *E. coli* JM109 transformed with codon-optimized synAnti-TfRscFv gene construct; lane FT: flow through of column containing Talon™ resin; lane W: fraction from washing steps; lane E1–E4: fractions from elution steps of purified anti-TfRscFv. The protein anti-TfR scFv was obtained mainly in second elution fraction.

Figure 5. Verification of the conjugation between the anti-TfRscFv and liposome particles by means of a 4–20% gradient natural (non-SDS) PAGE. scFv: unconjugated anti-TfRscFv; scFv-Lip 1: anti-TfRscFv conjugated with cationic liposome; scFv-Lip 2: anti-TfRscFv conjugated with neutral liposome.

The scFv-SH was subsequently conjugated to the maleimide group of MPB-liposome for the generation of the anti-TfRscFv-liposome.
In order to verify the conjugation, a discontinuous native (non-SDS) PAGE (Ornstein, 1964) was used, which showed that only small amount of free scFv-cys was detected in two different preparations of scFv-liposome at a scFv:l lipid weight ratio range of 1:20 to 1:40 (Figure 5), indicating that the sulphydryl-maleimide covalent conjugation between scFv-cys and MPB-liposome was quite efficient. Once conjugated to the liposome, the scFv protein will not be able to enter the PAGE gel. Therefore, only the unconjugated free scFv will be detected. It is difficult, however, to determine accurately the position of the free scFv-cys monomer under non-denaturing (non-SDS) conditions. Nevertheless, we suggest that the band indicated by the arrow in Figure 5 represents any unconjugated scFv-cys protein. The generated anti-TfR immunoliposome showed a particle size distribution of mainly between 200–300 nm (Figure 6).

Figure 6. Representative diagram of particle size distribution of the anti-TfR immunoliposome generated by conjugation of anti-TfR scFv fragment and HydroSoy PC/cholesterol neutral liposome (results of two independent preparations).

Discussion

It has been well accepted that bacterially expressed antibody fragments (Fv, scFv or Fab) provide a simple and cost-effective alternative to the traditional production of diagnostic monoclonal antibodies by animal cell tissue culture. Numerous recombinant antibody fragments have been successfully produced through periplasmic expression using E. coli. The scFv format is advantageous in prokaryotic expression because of its single polypeptide structure.

The anti-TfRmAb Clone 6 was shown to bind to a specific leukemia cancer cell expressing TIR, but it had only been expressed by hybridoma cell culture (Hoogenboom et al., 1990). We used the information of the amino acid sequence of the anti-TfRmAb Clone 6 to design a gene encoding the variable fragment of the mAb, connected the VL domain and the VH domain by a short linker sequence and added a sequence of Hys6Cys at the 3’-end of the gene. Upon analyzing the gene sequence of murine anti-TfR variable fragment, poor codon bias in its coding gene sequence was found with almost 19% of codons showing less than 10% frequency (below threshold value) of occurrence in E. coli genes. Besides strong association with low levels of protein expression due to ribosome stalling and failure in translation, such rare codons might have implication in frame shift and amino acid misincorporation (McNulty et al., 2003). Therefore, the gene was codon-optimized to adapt to the codon bias of E. coli genes in order to increase the expression of soluble protein in E. coli. The optimized codon usage was indicated by the increase in the CAI from 0.65 to 0.83.

We used the strategy of expressing the antibody fragment in the periplasm of E. coli in order to obtain soluble proteins. In contrast to the reducing conditions in cytoplasm, the oxidizing environment of the periplasmic space of E. coli, which is rich in proteins important for folding and catalyzing disulfide bond formation (Goemans et al., 2013) or...
chaperones such as SKp (Fernandez, 2004) is most suitable for proper folding of scFv fragment, which has two intramolecular disulfide bonds. Antibody fragments expressed in the periplasmic space have been shown to be correctly folded with excellent yield (Kusharyoto et al., 2002; Chen et al., 2004). Additionally, extraction of proteins expressed in the periplasmic space can easily be performed by a simple osmotic shock procedure or addition of lysozyme, and purification of antibody fragments from periplasmic extracts is more straightforward than purification from total cell lysates.

The expression of the antibody fragment in the periplasm of E. coli, however, is influenced by the choice of the signal sequence and the amino acid sequence of the antibody fragment itself. We obtained 2 mg protein per liter culture, which represents only a moderate level of protein expression in the bacterial periplasm under the control of lac operator/promoter system. In order to increase the expression level, future works will also be directed to consider utilizing other signal sequences such as pelB, phoA or DsbA (Thie et al., 2008), and other bacterial transcription control mechanism such as rhamnose regulon (Giocalone et al., 2006).

The size of the generated anti-TfR immunoliposome were mostly between 200-300 nm, which is in the range of nanoliposome size suitable for in vivo target delivery (Mokhtarieh et al., 2013). It is well known that the particle size plays an important role on the alternation of pharmacokinetics by affecting the tissue distribution and clearance. Vehicles with small particle are known to increase the accumulation of drug in the tumor via enhanced permeability and retention (EPR) effect, although this behavior is dependant on the tumor type (Xu et al., 2005). On the other hand, liposomes of large size (>500 nm) are rapidly cleared from blood circulation (Immordino et al., 2006). We used DOTAP as cationic lipid as it is the most widely used lipid for lipofection (Bringles et al., 2012). HydroSoy PC, DOPE, and cholesterol were used as neutral (helper) lipids. DOPE is known to be fusogenic and promotes transfection efficiency in vitro (Fletcher et al., 2006), whereas cholesterol decreases fluidity of the bilayer, increases stability, and is considered to be more efficient in vivo (Templeton et al., 1997).

Further experiments are still necessary in order to verify the binding affinity of the resulted anti-TfR immunoliposome towards transferrin receptor expressed on different cancer cell lines as well as its potency in internalizing therapeutic drugs and killing of cancer cells expressing transferrin receptor.

**Conclusion**

We constructed an anti-TfR immunoliposome comprised of an anti-TfRscFv antibody fragment that was covalently linked to the liposome via a cysteine residue at the carboxyl-terminal of the protein and a maleimide group on the liposome. Such immunoliposome has the potency to be used as a delivery vehicle for DNA or drugs to target cancer cells, which still needs to be verified further. In contrast to the whole antibody or transferrin molecule itself, the scFv fragment has a much smaller size for better penetration into solid tumors, and is a recombinant protein rather than a blood product. Therefore, large-scale production and strict quality control is feasible.

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