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Preliminary screening and immunogenicity analysis of antigenic epitopes of spring viremia of carp virus

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ABSTRACT

Glycoprotein (*G*) is the most common gene used in SVCV vaccine constructions. To identify the major immunogenicity determinant region of SVCV *G* gene, herein we truncated *G* gene to 4 parts (*G-1*, *G-2*, *G-3* and *G-4*). Bioinformatics and the enzyme linked immunosorbent assay (ELISA) were used to identify the antigenicity of these 4 truncated G proteins. Immunological assays (serum antibody production, enzyme activity, immune genes expression and challenge test) were carried out to further identify the immunogenicity of the screened G protein in common carp. Moreover, to further verify the immune response of the screened G protein-based subunit vaccine, its protective effects on common carp against SVCV infection using single-walled carbon nanotubes (SWCNTs) as a carrier were evaluated. Results showed that G-3 protein could induce higher antibody titer than other truncated G proteins. Furthermore, carps vaccinated with G-3 and G (positive control) showed significant enhancement of immune response (serum antibody production, enzyme activity and immune related genes expression) when compared with control groups. Meanwhile, as a promising vaccine carrier, SWCNTs could significantly enhance the immune effect of naked subunit vaccine (G-3 and G). Notably, after SVCV challenge, there was no significant difference in immune protection between G-3 and G, nor between SWCNTs-G-3 and SWCNTs-G. These results so far suggest G-3 might be the potential antigen epitope of SVCV. This study lays a foundation for developing vaccine and immunodiagnostic techniques.

1. Introduction

Spring viremia of carp virus (SVCV) is widespread throughout the world with huge economical losses to cyprinid fish farming industry [1,2]. Once SVCV outbreaks, the mortality rates can reach up to 90% in juvenile carps. Strict hygienic measures and extermination are the basic strategies to control SVCV, furthermore, no effective and safe approaches are currently available [3,4]. Vaccine is widely accepted as an effective control against viral diseases [5]. So far, protection against SVCV infection by vaccinated with DNA vaccine encoding a major antigenic viral protein have been verified, with the protection rate range from 44% to 100% [2,6,7]. SVCV encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L) [2]. Among five structural proteins of SVCV, G protein is commonly considered as the most important viral antigenic protein of SVCV. However, up to now, the major immunogenicity determinant region of SVCV *G* gene remains unclear.

Vaccination has probably become a vital way to resolve aquatic viral infection problems [8,9]. However, the immune response induced by many natural antigens cannot meet the needs of preventing infection or morbidity, and it is necessary to make a choice on the epitope level for improving the protection of protein antigen, in order to get more ideal vaccine molecules [10]. Study of dominant antigenic epitope has become a new approach of artificial vaccine design [11,12]. Through genetic engineering means, the intercepted target dominant antigenic epitope region is smaller in prokaryotic expression system, and soluble expression of target protein can be significantly improved via corresponding processing.

To identify the major immunogenicity determinant region of SVCV *G* gene, we truncated G protein to 4 sequences (G-1, G-2, G-3 and G-4) and evaluated the antibody between these 4 G sequences with G (positive control), furthermore, we analyzed the immune responses induced by G-3 and G in vaccinated fish.

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2. Material and methods

2.1. Experimental fish

Common carps (*C.carpio*) weighing 1.0 ± 0.2 g were purchased from a local SVCV-free farm in Yangling (shannxi, China). Carps were bred in laboratory for 28 days prior to vaccination. The water temperature for common carps were maintained at 20–23 °C. Commercial dry feed pellets (Hellow Fish Dry Pellets; CVM Products, Beijing, China) were used to fed carps twice daily. All of the experimental animals were handled according to the guidelines of the Animal Experiment Committee, Northwest A&F University.

2.2. Virus and cell lines

SVCV (strain 0504) kindly provided by Professor Qiang Li (Dalian Ocean University, Dalian, China), was propagated in epithelioma papulosum cyprini (EPC) cell line (Bioleaf, China) at 20 °C in minimum essential medium (MEM), pH 7.0 (Life Technologies, USA) plus 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Virus titers for challenge were determined as the established protocols [6], with a modified incubation temperature of 20 °C.

Epithelioma papulosum cyprinid (EPC) cells (kindly provided by Prof. Ling-bing Zeng in Yangtze River Fisheries Research Institute, Wuhan, Hubei, China) were cultured at 25 ± 0.5 °C in humidified atmosphere with 5% CO_2, and maintained in Medium 199 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; ZETA LIFE, USA).

2.3. Purified G protein

The purified G protein (freeze-dried powder) were manufactured and stored in our Lab.

2.4. Raw SWCNTs

The raw SWCNTs (95% purity black powder, 1–2 nm in outside diameter, 0.4–3 μm in length) were purchased from Chengdu Organic Chemicals Co., Ltd. Chinese Academy of Science (Chengdu, China).

2.5. Prediction of the transmembrane and antigenic domain of SVCV G protein

To analysis the transmembrane domain of SVCV G protein, protein transmembrane region analysis tools including SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui), DNAStar 6.0 (Prorean), DAS (M. Cserzo et al., 1997): http://www.sbc.su.se/~miklos/DAS, TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM-2.0) and TMpred (Holfmann, Stoffel 1993): http://www.ch.embnet.org/software/TMPRED_form .html. For antigenic domain prediction of G protein, tools including SignalP 3.0 (Jannick Dyrlov Bendtsen et al., 2004): http://www.cbs.dtu.dk/services/SignalP/, InterProScan: http://www.ebi.ac.uk/InterProScan/and BepiPred 2.0 (http://www.cbs.dtu.dk/services/BepiPred/) were used.

2.6. Recombinant G plasmid constructions

The pET32a (+) (Novagen, Madision, USA) was used as the original plasmid. QIAGEN Viral RNA Mini Kit (Qiagen, Hiden, Germany) was used to extract the viral genomic RNA of SVCV, and RNA PCR Kit (AMW) Ver.3.0 (Takara, Shiga, Japan) was used to converted the extracted RNA into cDNA. Specific primers used to clone SVCV *G-1*, *G-2*, *G-3* and *G-4* genes were designed based on the reference sequence published in GenBank which accession number is NC_002803. Primers can be found in Table 1 (GGATCC shows *BamH*I site, CTCGAG indicates *Xho*I site). The purified PCR products of *G-1*, *G-2*, *G-3* and *G-4* genes were digested with *BamH*I or *Xho*I and inserted into pET32a (+) plasmid to generate recombinant pET32a-G-1, pET32a-G-2, pET32a-G-3 and pET32a-G4 plasmids, respectively. PCR amplification, restriction enzyme digestion and DNA sequencing were used to verify the construction of these 4 recombinant G plasmids.

2.7. Expression and purification of the recombinant G proteins

Isopropyl-β-D-thiogalacto-pyranoside (IPTG; Sigma-Aldrich Trading Co., Ltd, Shanghai, China) was used to induce the expression of recombinant G-1, G-2, G-3 and G-4 protein. The empty plasmid pET32a (+) was the control fusion protein. The fusion protein was analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamid gel electrophoresis) following the previously method [13]. The recombinant proteins were assessed by western blotting analysis. The antibodies were used the diluted anti-His-tag monoclonal antibody (abcom, Cambridge, MA, USA) (1:1500) and HRP-conjugated goat anti-mouse IgG (1:1000) (Beijing CoWin Biotech Corp., Beijing, China). DAB horseradish peroxidase color development Kit (Qiagen, Hilden, Germany) were used to visualize the result. The recombinant *G* protein with the hexa-histiding tag at the N-terminus was purified by using Ni-NTA agarose (Qiagen, Shanghai, China) and then determined by Micro BCA Protein Assay Kit (Beijing CoWin Biotech Corp., Beijing, China).

2.8. Titers analysis of 4 truncated recombinant G protein

The titers of 4 truncated G proteins (G-1, G-2, G-3 and G-4) were measured by ELISA (Enzyme-linked immunosorbent assay) as described elsewhere [14]. Common carp (30 fish per treatment group) were intraperitoneally injected with purified G protein and 4 truncated G proteins, respectively. After 21 days, serum samples were collected from the tail veins of 6 fish per treatment group, as previously reported [15]. Briefly, the blood collected from the caudal vein of common carp was placed overnight at 4 °C and then centrifugated at 5000 × *g* for 15 min. The supernatant was collected and stored at −20 °C until use. Purified recombinant G, G-1, G-2, G-3 and G-4 protein were used as antigen. Anti-6X His tag antibody [HIS.H8] ab18184 (1:1500, Abcam, Cambridge, MA, USA) was used as primary antibody. HRP-conjugated goat anti-mouse IgG (1:2000, Beijing CoWin Biotech Corp., Beijing, China) was used as secondary antibody, followed by color development using tetramethylbenzidine, TMB (Tiangen Biotech, Beijing, China) was used as colorimetric substrate. The plate was read at 450 nm by using a precision microplate reader (Molecular Devices Corp., Palo Alto, CA). $\triangle\mathbf{A} = (A_{test} - A_{blank})/(A_{negative} - A_{blank})$, If $\triangle\mathbf{A}$ value > 2.1, then the results could be considered as positive.

2.9. Preparation of SWCNTs based subunit vaccine

The preparation of SWCNTs based subunit vaccine was according to our previous study [13]. Briefly, the SWCNTs were dispersed in mixture of concentrated H_2SO_4 and concentrated HNO_3 (3:1 v/v) under reflux with stirring at room temperature for 36 h to generate the carboxylate SWCNTs (SWCNTs-COOH). The protein combined with the SWCNTs-COOH were carried out through the amidation reactions, briefly, 5 g SWCNTs-COOH, 1-Ethyl-3-(3-dimenthylaminopropyl) carbodiimide hydrochloride (9 g) and N-hydro-succinimide (12.3 g) were dispersed in 3000 mL 2-(N-morpholino) ethanesulfonic acid buffer solution (0.1 M,pH = 5.6) for 3 h. Meanwhile, after the vacuum freeze-dried G protein (5.0 g) sonicated in the 500 mL PBS solution (phosphate buffered saline, pH = 7.4) for 3 h, the mixture was stirred for other 48 h. Then the treated SWCNTs-COOH and protein were mixed and stirred for 36 h (in shade). Subsequently, after centrifugation and freeze-drying the SWCNTs-protein powder was obtained.

2.10. Vaccination

During the vaccination, the rearing water temperature were kept at

Table 1
Primers used for the analysis of mRNA expression by qRT-PCR.

Genes	Accession no.		Primer sequences (from 5′ to 3′)	Product size
β-actin	M24113	Forward	GCTATGTGGCTCTTGACTTCG	85 bp
		Reverse	CCGTCAGGCAGCTCATAGCT	
N	KJ513477.1	Forward	ATGAGTGTCATTCGGATCAAAACA	417 bp
		Reverse	AAGACCCAGCAAGAAGAGAGGCAGCC	
G-1	AY527273.1	Forward	GGATCCATGTCTATCATCAGCTAC	408 bp
		Reverse	CTCGAGTTAACTTTGAGGGGGAAACCC	
G-2	AY527273.1	Forward	GGATCCGGGTTTCCCCCTCAAAGT	408 bp
		Reverse	CTCGAGTTATTTTTCTACCCAGTCTCC	
G-3	AY527273.1	Forward	GGATCCGGAGACTGGGTAGAAAAA	408 bp
		Reverse	CTCGAGTTAATCTAACTCCCAGTCGTC	
G-4	AY527273.1	Forward	GGATCCGACGACTGGGAGTTAGATGGC	417 bp
		Reverse	CTCGAGTCAAACGAAGGACCGCATTTC	
TNF-α	AJ311800.2	Forward	TGTGCCGCCGCTGTCTGCTTCACGCT	291 bp
		Reverse	GATGAGGAAAGACACCTGGCTGTAGA	
IL-10	JX524550.1	Forward	GTCATCCTTTCTGCTCTGGTT	91 bp
		Reverse	CCACAAATGAGCAACAGTCA	
Cxca	AJ421443	Forward	CTGGGATTCCTGACCATTGGT	88 bp
		Reverse	GTTGGCTCTCTGTTTCAATGCA	
IgM	AB004105	Forward	CACAAGGCGGGAAATGAAGA	145 bp
		Reverse	CTGATAAAGCTTTGCACTTCAGCA	
CD4	DQ400124.1	Forward	AGTGGGATCCAAAGGGCGAA	214 bp
		Reverse	ATTCCAGAGACAGAGAGT	
MHC-II	S62611.1	Forward	TGCAGTGCCTATGACTTC	191 bp
		Reverse	GAGCTGGCGTGCTCCA	
IFNg2b	JX181980.1	Forward	GCTCAAGAAGTATGCAGAAACTC	151 bp
		Reverse	TCTGGCTTGTCGTCTCCT	
I-IFN	AB376666.1	Forward	CAGAGTCAATGCTCCGCTT	297 bp
		Reverse	CTCAGATGACTGCCGTTGC	

20 ± 0.5 °C. Disease-free common carp were randomly separated into nine groups (120 fish per serving), including three control groups (PBS group, pET32a group, SWCNTs group) and 8 vaccinate groups (G-3 groups containing three dose (1 μg, 5 μg and 10 μg), G groups containing three dose (1 μg, 5 μg and 10 μg), SWCNTs-G-3 (10 μg) group and SWCNTs-G (10 μg) group. Before intramuscular injection, fish were anaesthetized in 0.01% benzoncaine, the injection dosages were 30 μL per fish and the intramuscular injection position were in the right, dorsal, epaxial muscle in front of the dorsal fin [16]. Subsequently, the vaccinated fish were transferred to different tanks and monitored daily.

2.11. Measurement of antibody level

The presence of specific, neutralizing antibodies was determined by ELISA according to our previous studies [14]. Vaccinated and control fish (6 fish per group) were randomly sampled to prepare sera weekly until 6 weeks for antibody determination. Sera serially diluted with PBS containing 3% skimmed milk were used as primary antibodies. Purified G protein and G-3 protein were used as antigen. Anti-Common carp (*Cyprinus carpio carpio*)/Koi carp (*Cyprinus carpio koi*) IgM monoclonal antibody labeled with horseradish peroxidase (Aquatic Diagostics Ltd., England) was diluted with PBS containing 3% skimmed milk at the ratio of 1:1000 before use, followed by color development using tetramethylbenzidine, TMB (Tiangen Biotech, Beijing, China) was used as colorimetric substrate. A precision microplate reader (Molecular Devices Corp., Palo Alto, CA) was used to analyze the plate at 450 nm.

2.12. RNA isolation, cDNA synthesis and qPCR assays

To analyze the immune genes expression, total RNAs were obtained from the kidney tissues in each group (3 fish per group) at 1, 3, 7, 14 and 21 days after vaccination with TRIzol reagent. HiScript Q Select RT SuperMix for aPCR (+gDNA wiper) (Vazyme, China) was performed to reverse transcribed the purified RNA into cDNA.

Quantitative real-time PCR (qRT-PCR) was performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using AceQ® qPCR SYBR® Green Master Mix (Vazyme, China) with the following procedure: 95 °C for 5 min and 40 cycles at 95 °C denaturation for 15 s, followed by 60 °C annealing for 60 s. The extracted DNA were used as template for RT-PCR amplification with specific primers SM-F/R. The *β-actin* was used as an internal control (Table 1). All qRT-PCR reactions were performed for three biological replicates and repeated with two independent samples. Relative mRNA expression was calculated using $2^{-\triangle\triangle Ct}$ method with the formula, $F = 2^{-\triangle\triangle Ct}$, $\triangle\triangle C_t = (C_{t,\ \text{target gene}} - C_{t,\ \text{reference gene}}) - (C_{t,\ \text{target gene}} - C_{t,\ \text{reference gene}})_{\ \text{control}}$ [17].

2.13. Virus challenge

Before virus challenge, the water temperature was gradually lowered from 20 °C to 15 °C at a rate of 1–2 °C/day. At the time point of 21 days post-vaccination, fish in vaccinated and control groups (each group, n = 52) were transferred to new tanks and challenged by intraperitoneal injection with 50 μL 6.0×10^4 $TCID_{50}$ mL^{-1} of live SVCV in saline buffer, and the relative percentage survival (RPS) were recorded daily for 22 days after viral challenge. Dead fish were collected daily, recorded, and examined for clinical signs of SVCV. Moreover, PCR assay was used to confirm SVCV infection in challenged fish, primers (N) used for viral detection can be found in Table 1.

Relative percentage survival (RPS) = 1- [% mortality rate (vaccinated fish)/% mortality rate (control fish)] × 100.

2.14. Statistical analysis

All data were analyzed using SPSS 22 Software (IBM, USA) and expressed as means ± standard division. Serum antibody production were analyzed with two-tailed student's t-test (**$P < 0.01$; *$P < 0.05$). For immune parameters and immune-related genes expression were analyzed with one-way ANOVA (analysis of variance) followed by Duncan multiple range tests, values with different letters are significant ($P < 0.01$).

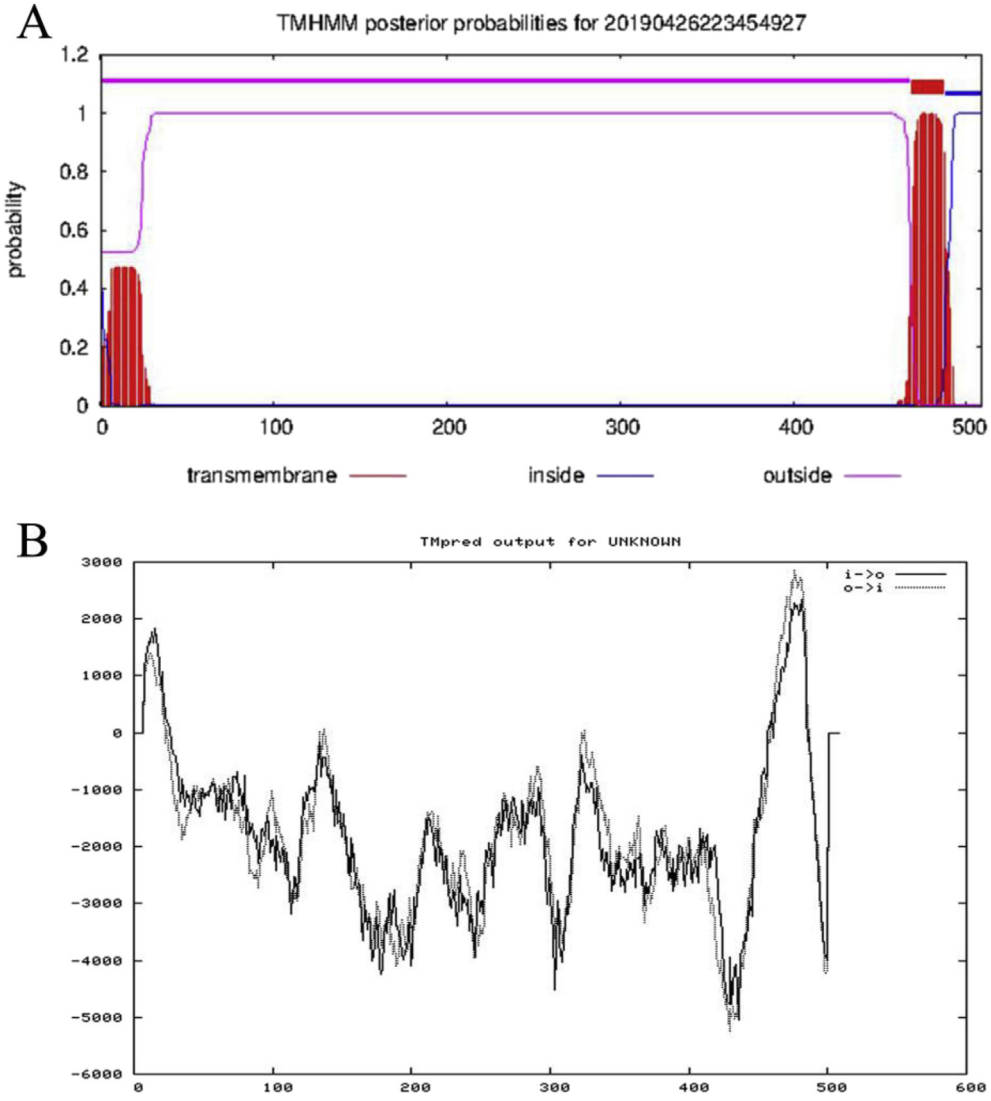

Fig. 1. Prediction of the transmembrane domain of SVCV G protein: (A) TMHMM prediction. (B) TMpred prediction.

3. Results

3.1. Bioinformation analysis of SVCV glycoprotein

As shown in Fig. 1 and Fig. 2, the transmembrane domain of G protein is approximately located between 467 amino acid (aa) to 492 aa. For antigenic region prediction, there are 10 possible regions located in 50 aa-450 aa, which is corresponding with the major hydrophilic region.

3.2. Construction of 4 truncated recombinant plasmids

The SVCV *G* gene (complement code sequence size in 1530 bp) was divided into 4 parts: *G-1* (1–393 bp), *G-2* (376–768 bp), *G-3* (751–1143 bp) and *G-4* (1126–1530 bp) (Fig. 3A). These 4 truncated genes were amplified through PCR using specific primer pairs (shown in Table 1) from the viral genome cDNA mix and the result was shown in Fig. 3B. The construction of 4 recombinant pET-G plasmids were identified by restriction enzyme digestion (Fig. 3C) and sequenced to analyze its base composition (date not shown). Above mentioned results confirmed that these 4 recombinant expression plasmids (pET32a-G-1, pET32a-G-2, pET32a-G-3 and pET32a-G-4) were successfully constructed. Fig. 3D shows that 4 appropriate-sized protein (about 30 KDa) was expressed in *Escherichia coli* when induced by IPTG, furthermore, Western blot using monoclonal mouse anti-his-tag antiserum confirmed that theses 4 recombinant protein are all 30 KDa his-tag fusion proteins, which was the molecular weight expected (Fig. 3E).

3.3. Antibody titers analysis of 4 truncated recombinant G proteins

At the time point of 21 days after vaccination, the antibody titers between 4 truncated recombinant G proteins were analyzed. Table 2 shows the antibody titers of G and 4 truncated G proteins, which indicated that with the serum dilution increase, the antibody responses of G and 4 truncated proteins were declined. Moreover, the highest antibody titers (1:6400) were observed in G-3 and G, which indicated G-3 could confer stronger antibody response than other truncated G proteins.

3.4. Serum antibody production

The antibody level of sera samples obtained from vaccinated fish during 1–6 weeks were evaluated. As shown in Fig. 4, there were significant enhancement of antibody levels in all vaccinated fish. In addition, the antibody level reached a peak titer at about 3-week post immunization and subsequently they were gradually attenuated. At the same vaccine dose and time point, G-3 groups shown similar serum antibody level with G groups. Moreover, the antibody SWCNTs-protein

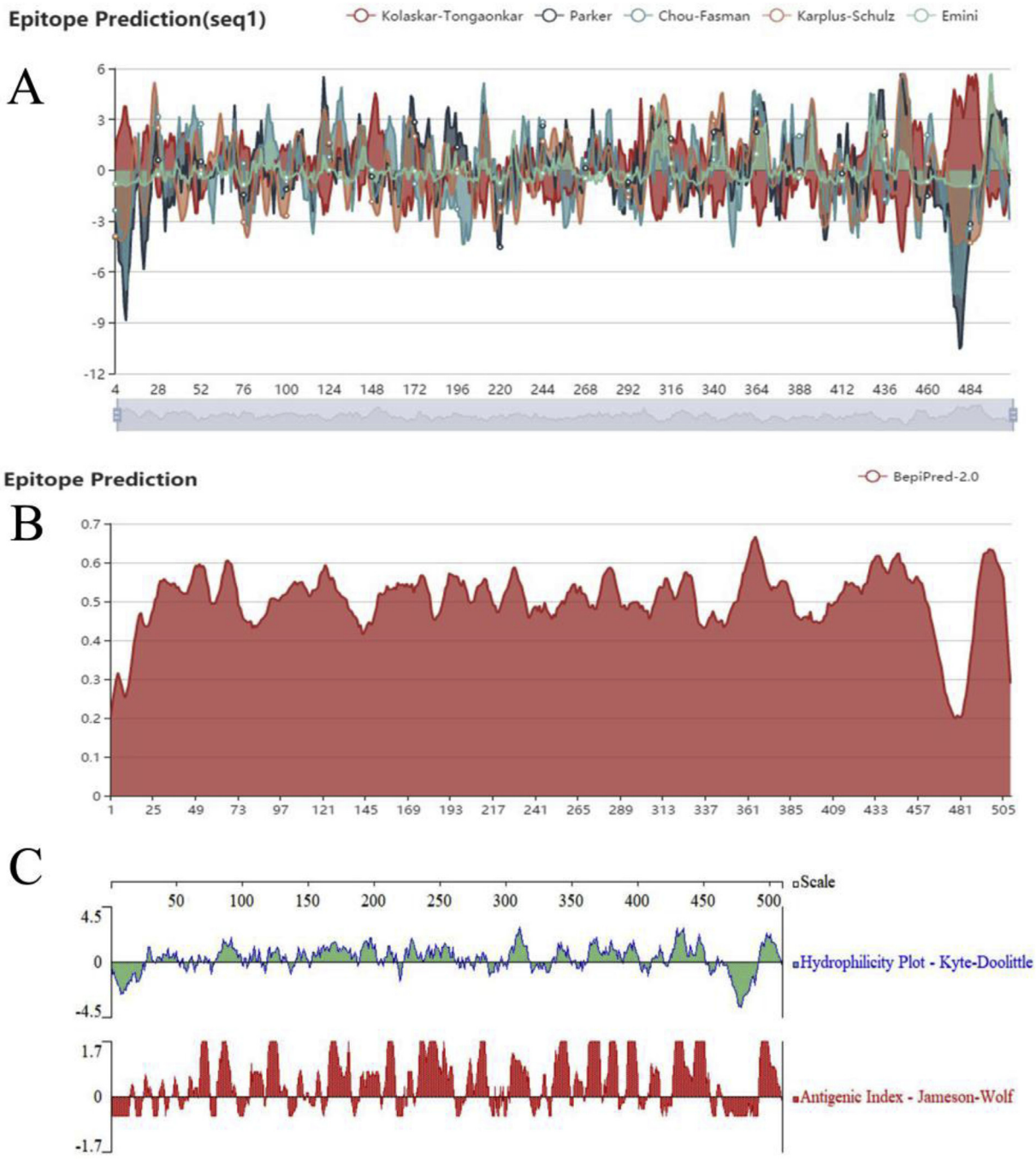

Fig. 2. Prediction of antigenic domain of SVCV G protein.

groups are much higer than fish vaccinated with naked subunit vaccine.

3.5. Changes of enzyme activities in vaccinated fish

As shown in Fig. 5, complement C3 activity, total antioxidant activity (T-AOC), alkaline phosphatase activity (AKP) and acid phosphatase activity (ACP) in different treatment groups (G-3, G, SWCNTs-G-3 and SWCNTs-G) and control groups (PBS, pET32a and SWCNTs) were recorded. The results showed that complement C3 activity, T-AOC activity, AKP activity and ACP activity of the vaccinated groups were significantly higher than those of the control groups ($P < 0.01$). In addition, except T-AOC activity G-3 could induce similar strong enzyme activities as G in regardless of SWCNTs.

3.6. Immune-related genes expression

To further determine the immuneprotective effect between G-3 and G. We verified the expression levels of immune-related genes (*TNF-α*, *IL-10*, *Cxca*, *IgM*, *CD4*, *MHC-II*, *IFNg2b* and *I-IFN*) in kidney tissues by qRT-PCR analysis at 21 days post-vaccination. As Fig. 6 shown, all above genes were significantly up-regulated (4.6–37.4 times) in vaccinated carps, when compared with control groups (PBS, pET32a and SWCNTs). Moreover, carps vaccinated with G-3 induce similar levels of immune-related genes expression with that of G.

3.7. Protection of vaccinated fish

At the end of monitoring period (21 days post-vaccination), the

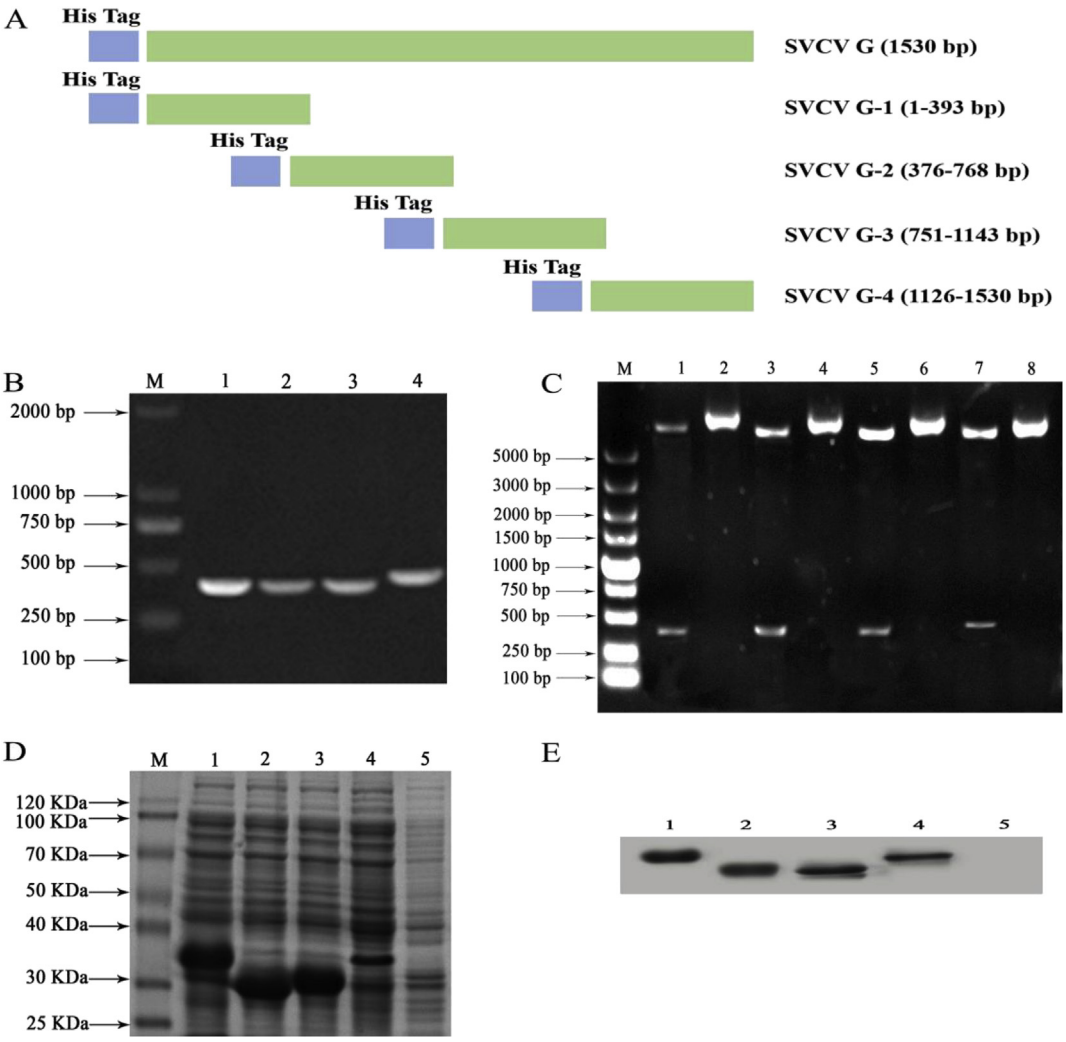

Fig. 3. Analysis of 4 truncated recombinant G proteins: (A) Schematic illustration of recombinant plasmids expressing truncated forms of G protein. (B) RT-PCR amplification of 4 truncated *G*: lane M, DNA marker; lane 1, *G*-1; lane 2, *G*-2; lane 3, *G*-3; lane 4, *G*-4. (C) Double enzymes digestion of 4 truncated recombinant G plasmids: lane M, DNA marker; lane 1, pET32a-G-1; lane 2, pET32a-G-2; lane 3, pET32a-G-3; lane 4, pET32a-G-4. (D) SDS-PAGE analysis of 4 recombinant G proteins: lane M, protein marker; lane 1, G-1; lane 2, G-2; lane 3, G-3; lane 4, G-4; lane 5, pET32a. (E) Western blot analysis of 4 recombinant protein: lane 1, G-1; lane 2, G-2; lane 3, G-3; lane 4, G-4; lane 5, pET32a.

relative percentage survival (RPS) were analyzed (Table 3). Vaccinated groups (G-3 groups, G groups, SWCNTs-G-3 and SWCNTs-G) showed a significant improved survival compared with control groups (PBS, pET32a and SWCNTs group). What's more, the highest RPS in G-3 groups were 42.31% which is similar with that in G groups (48.08%). Notably, the RPS in SWCNTs-G-3 group is similar to the RPS in SWCNTs-G group.

4. Discussion

As the causative agent of spring viremia of carp (SVC), SVCV still imperil the development of aquaculture, in particular cyprinid farming industry [18–20]. As a promise prophylaxis, vaccination is commonly considered as a major improvement to induce immune protective effect against fish disease [21–26]. Up to now, the reported SVCV vaccine exert limited protection [2]. Our previous study showed the *matrix* gene of SVCV could be used as an antigen for DNA vaccine constructs with the highest protection rate of 33.8% in common carp [26]. Among the five structural proteins of SVCV, glycoprotein is commonly considered as the antigen protein for vaccine construction. Previously, we reported a single-walled carbon nanotubes (SWCNTs) based subunit vaccine with the highest protection rate of 71.1%, which is much higher than the protection rate in naked subunit vaccine (48.6%). Although an efficient vaccine carrier (SWCNTs) were used to enhance the efficacy of subunit vaccine, the vaccine effect is not ideal [13]. Hence other means to enhance the vaccine effect is needed.

The antigen of our previous SVCV subunit vaccines is a 60 kDa G protein, however, the domain antigen protein is far less than 60 KDa, which means most of the protein in our previous vaccine is redundant [15]. In a subunit vaccine, the higher content of antigen protein contains, the higher immune effect of vaccine will be. Strikingly, the dominant antigenic epitope was screened and evaluated to further enhance the efficient of SVCV vaccine. In this study, combined with bioinformation and immune response assay, we truncated the SVCV G protein into 4 parts. Antibody response assay were used to found the dominant antigenic epitope between these 4 segments. This study indicated that G-3 could induce strong immune response which is similar to G (positive control). To further confirm the efficacy of G-3, we vaccinated carp with G-3, G, SWCNTs-G-3 and SWCNTs-G, and evaluated the immune response (serum antibody production, enzyme activity and immune-related genes expression) in vaccinated fish.

The results shown the similar results that G-3 reached similar immune response to G regardless of SWCNTs, which confirmed that G-3 might be the antigenic region. Cytokines play a key role in host innate

Table 2
Antibody titers of 4 truncated recombinant G protein.

Serum dilution（×10²）	2	4	8	16	32	64	128	blank	negative
absorbance $A_{405\ nm}$									
G	3.47	3.14	1.07	0.84	0.47	0.18	0.12	0.11	0.13
G-1	1.43	1.40	0.25	0.22	0.17	0.15	0.12	0.09	0.14
G-2	3.75	3.44	0.56	0.29	0.21	0.13	0.08	0.07	0.12
G-3	2.97	2.59	1.61	0.58	0.39	0.16	0.11	0.05	0.09
G-4	1.54	1.33	0.28	0.17	0.14	0.11	0.09	0.05	0.14
△A									
G	168.00	151.50	48.00	36.50	18.00	3.50	0.50		
G-1	26.80	26.20	3.20	2.60	1.60	1.20	0.60		
G-2	73.60	67.40	9.80	4.40	2.80	1.20	0.20		
G-3	73.00	63.50	39.00	13.25	8.50	2.75	1.50		
G-4	16.56	14.22	2.56	1.33	1.00	0.67	0.44		

Positive results are indicated in green

immunity and are indispensable for recruitment and activation of macrophage, neutrophil, and lymphocyte to the infection sites for pathogen elimination [27,28]. We analyzed the immune-related genes (*TNF-α*, *IL-10*, *Cxca*, *IgM*, Cxca, *CD4*, *MHC-II*, *IFNg2b*, *I-IFN*) expression in vaccinated fish. All these genes were significantly up-regulated in vaccinated carps, when compared with control groups. Moreover, fish vaccinated with G-3 induced similar immune-related genes expression with G vaccinated fish. The induction of *TNF-α* was apparently up-regulated in different forms of vaccinated fish [28]. The *TNF-α* is one of the main pro-inflammatory cytokines produced in response to a broad type of bacterial, viral and fungal infections, and has a crucial role in activating and orchestrating the immune response in order to protect the host organism from pathogens [29]. Among these genes, *TNF-α*, *IL-10* and type *I-IFN* have been reported play a significant role in the initiation and regulation of the inflammatory process and serve as an important component of innate immunity [30]. The type *I-IFN* is a complex group of cytokines and in charge of cell growth and differentiation in the hematopoietic and immune system, which serves as the first line of defense against viral infection [31–33]. *IFNg2b* belongs to Type II IFN which is largely secreted by T cell and natural killer cells,

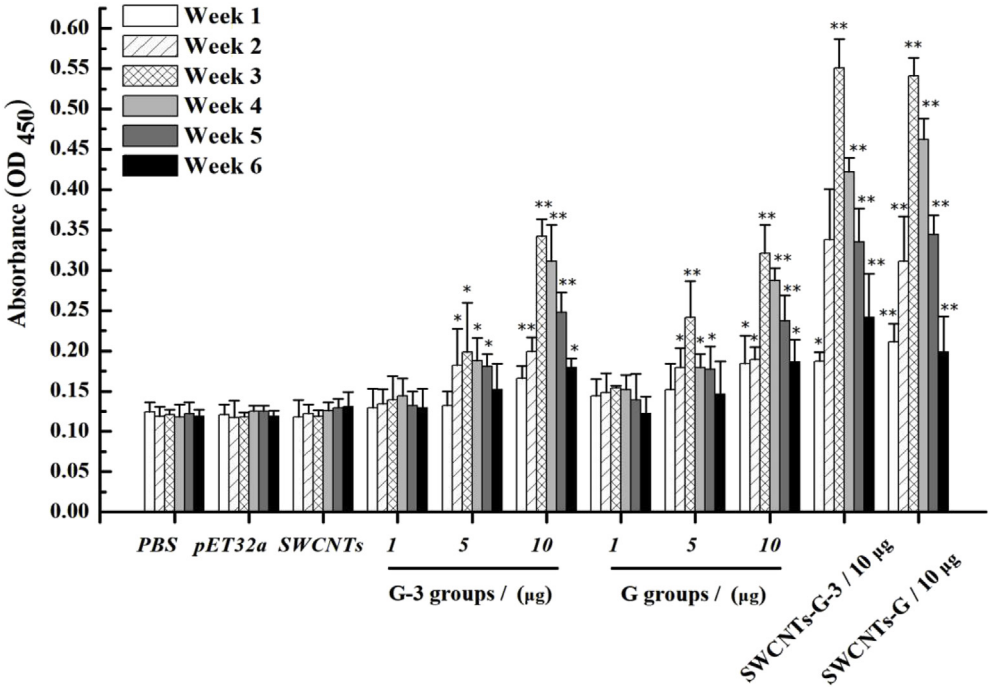

Fig. 4. Serum antibody production in vaccinated common carps. Sera were collected from the fish at 1–6 weeks post-vaccination. Data are means for three assays and presented as the means ± SD. $^{**}P < 0.01$; $^{*}P < 0.05$.

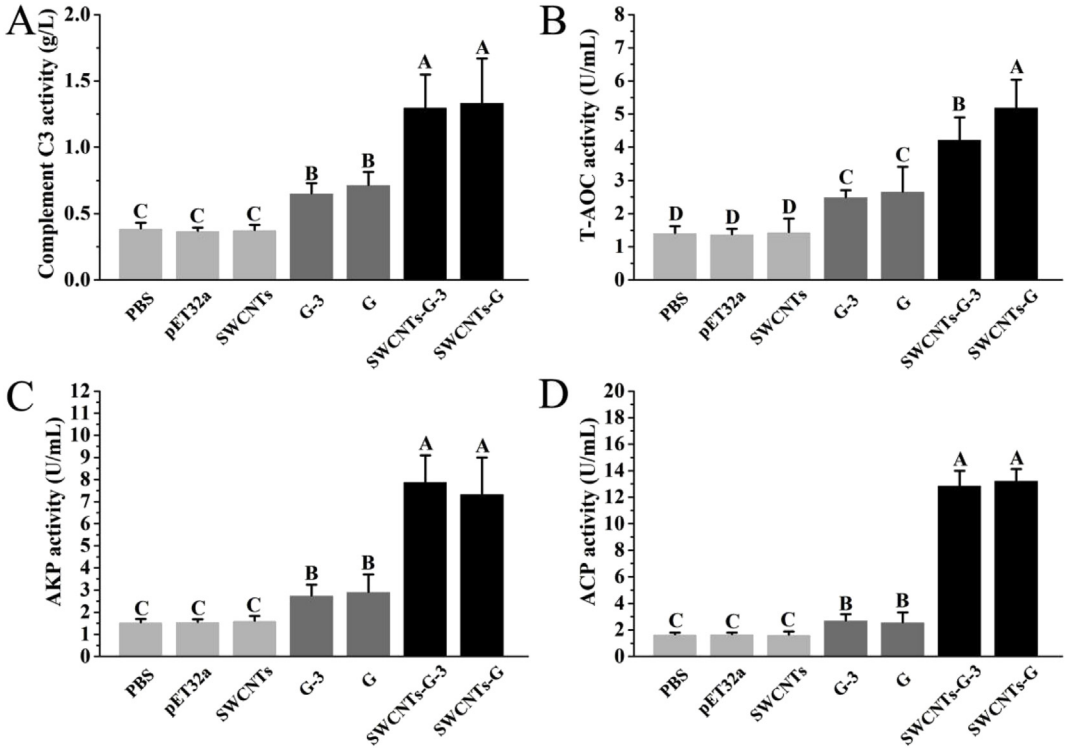

Fig. 5. Changes of immune parameters post-immunization at 21 days: (A) Complement C3 activity; (B) Total antioxidant activity; (C) Alkaline phosphatase activity; (D) Acid phosphatase activity. Data are means for three assays and presented as the means ± SD. Values with different letters are significant ($P < 0.01$).

and plays an essential part in natural immunity of the host [34]. Thus, activation of innate immunity may condition the initiation of specific adaptive immune responses [35]. The *IgM* expression was increased significantly in vaccinated fish kidney. It is known that *IgM* is a major component of the humoral immune system of teleost fish, regarded as the first antibody [36]. Some investigators have reported that the *IgM* expression would be intensively increase in many tissues and organs from the second week after immunization and maintained almost one month, which as corresponding with our study [37]. *MHC-II* are the markers reflecting the antigen presentation, the higher expression levels of *MHC-II* lead to increased advantages in terms of antigen presentation [38,39]. Therefore, the induction of cytokines, interferons, adaptive immune-related and antigen presenting response indicated that G-3 might be the dominant antigenic region of SVCV.

5. Conclusion

In summary, our results show that G-3 might be the dominant antigenic region of SVCV. This study lays a foundation for developing vaccine and immunodiagnostic techniques.

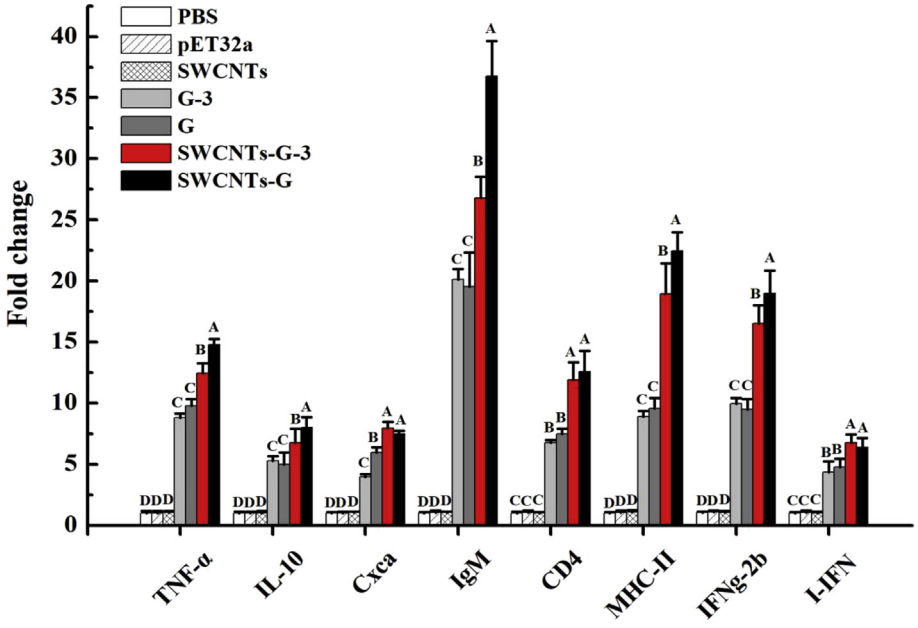

Fig. 6. Analysis of immune-related genes expression in kidney of fish immersed with PBS, pET32a, SWCNTs, G-3, G, SWCNTs-G-3 and SWCNTs-G at 21 days after vaccination. Common carps (6 fish/group) were sampled and the kidney were pooled and processed to determine the expression of *TNF-α*, *IL-10*, *Cxca*, *IgM*, *CD4*, *MHC-II*, *IFNg2b*, *I-IFN* genes by qRT-PCR. The *β-actin* was used as an internal reference. Data are means for three assays and presented as the means ± SD. Values with different letters are significant ($P < 0.01$).

Table 3
Relative percentage survival (RPS) of fish challenged with SVCV.

Fish injected	Relative percentage survival (RPS)
PBS	0%
pET32a	0%
SWCNTs	0%
G-3 (1 μg)	3.85%
G-3 (5 μg)	36.54%
G-3 (10 μg)	42.31%
G (1 μg)	9.62%
G (5 μg)	32.69%
G (10 μg)	48.08%
SWCNTs-G-3 (10 μg)	69.23%
SWCNTs-G (10 μg)	73.08%

Consent for publication

All author agrees to be published.

Declaration of competing interest

The authors declare that they have no competing interests.

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