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*Full Length Research Paper*

# Preparation and characterization of human malignant melanoma ganglioside ScFv antibody conjugated quantum dot probe

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**Melanoma is a highly aggressive and deadly skin cancer. Few treatment options are available for patients with metastatic melanoma, and the global incidence of melanoma is increasing faster than that of other cancers. Therefore, it is vitally important to uncover and use genetic and epigenetic regulatory mechanisms at work during the development and progression of melanoma for better prevention, diagnosis and clinical management. The single-chain antibody (ScFv) has good stability, high antigen affinity, low molecular weight, blood and normal tissue clearance rate, faster and easier penetration into the tumor tissue, which made ScFv to have extremely wide range of application in tumor diagnosis, treatment and prevention. In this study, the prokaryotic expression vector with the anti-human melanoma gaglioside single chain variable fragment antibody gene (GD/ScFvMEL) was constructed, expressed in *Escherichia coli* and purified by cation-exchange chromatography. Purified GD/ScFvMEL was combined with quantum dots (QD), which was evaluated by fluorescence spectrophotometer and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Finally, GD/ScFvMEL coated with QD (QD-GD/ScFvMEL) was transformed into malignant melanoma A375 cell line and human gastric cancer MGC-803 cell line to analyze its function by fluorescence microscopy. It was demonstrated that QD-GD/ScFvMEL could specifically bind with melanoma A375 cells and could not bind with stomach cancer MGC-803 cells, which showed that QD-GD/ScFvMEL could be used as a probe to diagnose melanoma. These results suggested that QD-GD/ScFvMEL has been successfully generated and may serve as a probe to detect malignant melanoma.**

**Key words:** Melanoma, preparation, single-chain antibody, quantum dot, probe.

## INTRODUCTION

Malignant melanoma represents the most common form of fatal skin cancer and accounts for 1 to 3% of all neoplasias. Its incidence has grown world-wide over the past decades, especially, among the Caucasian population with intense sun exposure (Burkhart and Burkhart, 2009). The 5-year survival rate drops from 95% for patients with a maximum tumor thickness of 1 mm lacking metastases (pathologic stage 1) to below 10% for patients with visceral metastasis (pathologic stage 4) (Lasithiotakis et al., 2006; Gutzmer et al., 2008). Metastatic melanoma is largely resistant to known treatment modalities. In particular, the application of chemotherapeutics is not effective, due to the resistance of melanoma cells to systemic treatment with anti-cancer agents (Marcoval et al., 2008). Early intervention correlates with nearly 100% patient survival, but greater than 80% mortality is associated with advanced disease (Geller et al., 2007). Therefore, novel therapeutic options and diagnostic reagent was needed for diagnosis, treatment and prevention of malignant melanoma. Ganglioside (GLS) is a kind of expression surface antigen in melanoma cells (Tsao and Sober, 2005). It does not express itself in normal cell; however, it can be expressed

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in melanoma cells and the extent of GLS expression is relevant to change and transfer of malignant tumor (Ferrari et al., 2008). Therefore, GLS change could indirectly demonstrate variance of melanoma cells. In this study, we selected human malignant melanoma ganglioside single-chain antibody (ScFv) as target to study early diagnostic probe of melanoma.

Previous study demonstrated that the ScFv had low molecular weight, good stability, high antigen affinity, low molecular weight, blood and normal tissue clearance rate, faster and easier penetration into the tumor tissue (Bird et al., 1988; Huston et al., 1988), which made ScFv have an extremely wide range of applications in tumor diagnosis, treatment and prevention. Quantum dots (QDs) was more stable than conventional organic fluorescent dyes, and it could emit different colors of light simultaneously by using different sizes of QDs, thus, the living cells or the target molecules on its surface could be observed, which helps to research the biological characteristics of these molecules and their mutual relations at the same time (Jaiswal et al., 2003; Tokumasu and Dvorak, 2002). The QDs, especially water-soluble QD has become a research hotspot in the biomedical field. Therefore, in this study, anti-human melanoma ganglioside single chain variable fragment antibody gene (GD/ScFvMEL) coated with QD was studied to detect malignant melanoma by a series of biological and physics method.

## MATERIALS AND METHODS

### Materials

The plasmid pGEX-4T-1-ScFv containing ScFv variable region was obtained from Pr. Baoling Wang (Department of Biochemistry, Fourth Military Medical University, China). The plasmid pET-28a (+), *Escherichia coli* strains BL21 (DE3) and DH5α was purchased from Takara (Japan). Malignant melanoma A375 cell line and human gastric cancer MGC-803 cell line were cultured at 37°C in 5% $CO_2$ in DMEM containing 1 g/L glucose, 2 mM glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 0.1 mg/ml streptomycin and 100 U of penicillin, and 10% Fetal Bovine Serum (FBS) (Invitrogen, USA).

### Construction, expression and purification of single-chain antibody (ScFv)

The ScFv gene of anti-human melanoma gaglioside was polymerase chain reaction (PCR)-amplified using pGEX-4T-1-ScFv as template and cloned into the pMD18-T vector (pMD18-T-scFV). The ScFv gene was subjected to deoxyribonucleic acid (DNA) sequencing, and the inserts were verified against the corresponding region of ScFv. After digestion with *Nde*I and *Xho*I, the resulting fragment was cloned into the *pET28a* (+) *Nde*I/ *Xho*I sites, yielding the expression vector pET-ScFv.

The expression plasmid pET-ScFv was transformed into Escherichia coli strain BL21. Cells were grown in luria broth (LB) containing 50 µg/ml kanamycin and was shaken at 37°C until the OD (600 nm) was 0.8. Then, ScFv production was induced by the addition of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sambrook and Russell, 2001). Four hours (4 h) after induction, 100 ml cultures were pelleted by centrifugation. The pelleted cells were thawed in ice, re-suspended in 20 mM Tris-Cl (pH 8.0), and lysed by sonication in ice-water bath. The lysate was clarified at 10,000 × g for 30 min at 4°C and both soluble fraction and pellet containing the insoluble fraction (inclusion bodies) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The supernatant was filtered (0.45 µm pores) and then ready for purification. ScFv purification from the crude cell extract was purified by Ni-NTA Superflow Cartridges according to protocols handbook.

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

Samples were incubated at 100°C for 5 min in sample buffer (0.25 M Tris-Cl, pH 6.8, 5% glycerol, 5% 2-mercaptoethanol, 3% SDS, and 0.2 mg/ml bromophenolblue) and then separated by SDS-PAGE (10% gels). For western blot, the proteins separated by electrophoresis were transferred to nitrocellulose membrane (Invitrogen) using a Mini TransBlot cell (Bio-Rad, USA), blocked in 5% bovine serum albumin (BSA) in Tris-buffer saline (TBS) (100 mM Tris, 0.9% NaCl, pH 7.5) and immune reacted with 1:3000 dilution of mouse anti-His monoclonal antibody (Takara, Japan), followed by HRP-labelled goat anti-mouse IgG (1:5000, Tiangen, China). Chromogenic-based detection was performed using tetramethylbenzidine (TMB) as a staining substrate.

### Preparation anti-human melanoma ganglioside single chain variable fragment antibody gene (GD/ScFvMEL) nano-probe

The purified protein was conjoined to quantum dots designated as nano-probe. In short: 600 µl prepared QDs were activated by 300 µl 1 - ethyl -3 - (3 - dimethylamino-propyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) at a concentration of 2 mg/ml, respectively for 5 min by ultrasound. Then 300 µl target protein (2 mg/ml) was added. The solution was allowed to react at room temperature for 2 h on a rotating mixer to obtain pET28a-GD/ScFvMEL-QDs. The final nano-probes was purified by using affinity chromatography to remove unbound QDs and stored at 4°C. The prepared nano-probes were analyzed by fluorescence spectrophotometer and SDS-PAGE electrophoresis.

### Nano-probe binding with melanoma cells

Malignant melanoma A375 cell line and human gastric cancer MGC-803 cell line were placed on 24-well chamber slides and cultured for 24 h in DEME with 10% (v/v) FCS at 37°C in a humidified 5% $CO_2$ incubator. Cells fixed in 4% paraformaldehyde for 15 min were blocked with 3% BSA for 10 min and incubated with nano-probe at 4°C overnight. After several washing steps, cells were analyzed with the Olympus IX71 fluorescent microscope (German). Photographs were taken using the scope-mounted camera (German).

## RESULTS

### Construction, expression and purification of the recombinant single-chain antibody (scFv)

The genes of scFv were amplified by PCR using pGEX-4T-1-ScFv as template and cloned into the pMD18-T

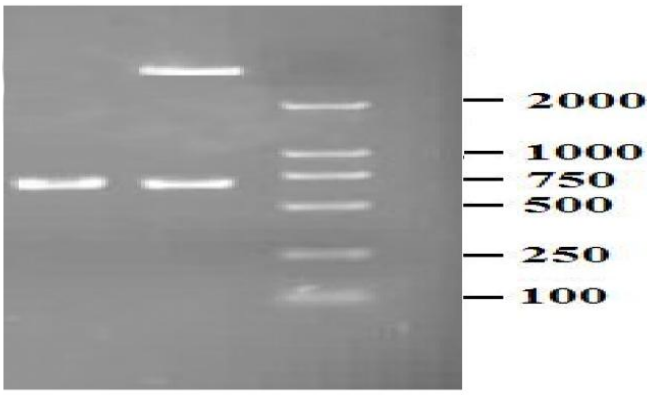


**Figure 1.** PCR and restriction analysis of *pET28*-GD/ScFvMEL. M: Mark; DL2000; Line 1, *pET28*-GD/ScFvMEL PCR product; Lane 2, *pET28*-GD/ScFvMEL digested by *Nde*I and *Xho*I.

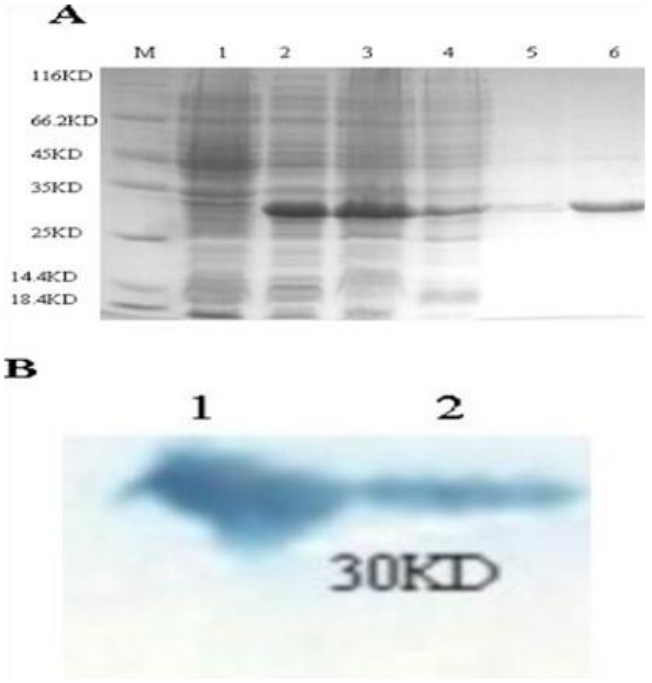


**Figure 2.** *pET28*-GD/ScFvMEL expression in *E. coli* BL21(DE3). A, SDS-PAGE analysis of the expression of pET28-GD/ScFvMEL; M, standard molecular weight markers; Lane 1, total protein of uninduced *pET28*-GD/ScFvMEL by IPTG; Lane 2, total protein of induced *pET28*- GD/ScFvMEL by IPTG; Lane 3, Supernatant of the bacteria lysate; Lane 4, GD/ScFvMEL after purification by nickel-NTA metal affinity chromatography; Lane 5, eluent; Lane 6, final purified *pET28*-GD/ScFvMEL; B, western blotting analysis of the pET28- GD/ScFvMEL protein; Lane 1, *pET28*- GD/ScFvMEL total protein of induced cell lysate; Lane 2: ,final purified *pET28*- GD/ScFvMEL.

vector. The pMD18-T-scFV was digested with *Nde*I and *Xho*I and cloned separately into the plasmid PET28 predigested with the same restriction enzymes, in order to construct prokaryotic expression plasmid PET-ScFv DNA sequencing. Enzyme digestion confirmed the successful constructions (Figure 1).

*E. coli* BL21 strain containing plasmid pET-ScFv was induced with IPTG, and recombinant protein ScFv was identified by 10% SDS-PAGE with a molecular mass of about 27 kDa as expected (Figure 2). pET-ScFv protein was expressed in *E. coli* in the inclusion body, and purification was performed using the Ni-NTA purification system. The process of purification was monitored by SDS-PAGE (Figure 2). The specificity of the purified ScFv protein was verified by western blotting with anti-His (Figure 2).

## Preparation and characterization of of the nano-probe

The prepared nano-probes were analyzed by fluorescence spectrophotometer and SDS-PAGE electrophoresis. The fluorescence peak of QDs shifted to the left at 20 nm after QDs was conjoined with GD/ScFvMEL by analysis of fluorescence spectra (Figure 3). This demonstrated that QDs was successfully marked on the GD/ScFvMEL. Moreover, fluorescence intensity of the labeled QDs increased a little, which showed that protein enhanced QDs with fluorescence signals, was consistent with previous study (Bakalova et al., 2004). SDS-PAGE analysis of the nano-probes results showed that GD/ScFvMEL was about 29 kDa (*pET28a*-GD/ScFvMEL while the prepared probe was located in the sample hole and cannot enter the gel, which further demonstrates that the purified GD/ScFvMEL antibody was effectively conjugated with CdTe quantum dots. These results implied that GD/ScFvMEL-QDs nano-probe were successfully prepared.

## Analysis of the specificity of nano-probe to tumor cell

A375 melanoma cells and gastric cancer MGC-803 cells were incubated with nano-probe, and results were observed under a fluorescence microscope. As shown in Figure 4, A375 melanoma cells incubated with nano-probe could emit strong fluorescence signal intensity, which showed that nano-probe could specifically bind to melanoma A375 cells and gastric cancer MGC-803 cells incubated with nano-probe cells could emit faint fluorescence signal intensity. This implied that gastric cancer MGC-803 cells did not specifically bind to nano-probe and no observable non-specific adsorption occurred. These results that showed that prepared nano-probe could specifically bind to melanoma cells.

## DISCUSSION

In the current study, prokaryotic vector were constructed with the anti-human melanoma gaglioside. GD/ScFvMEL

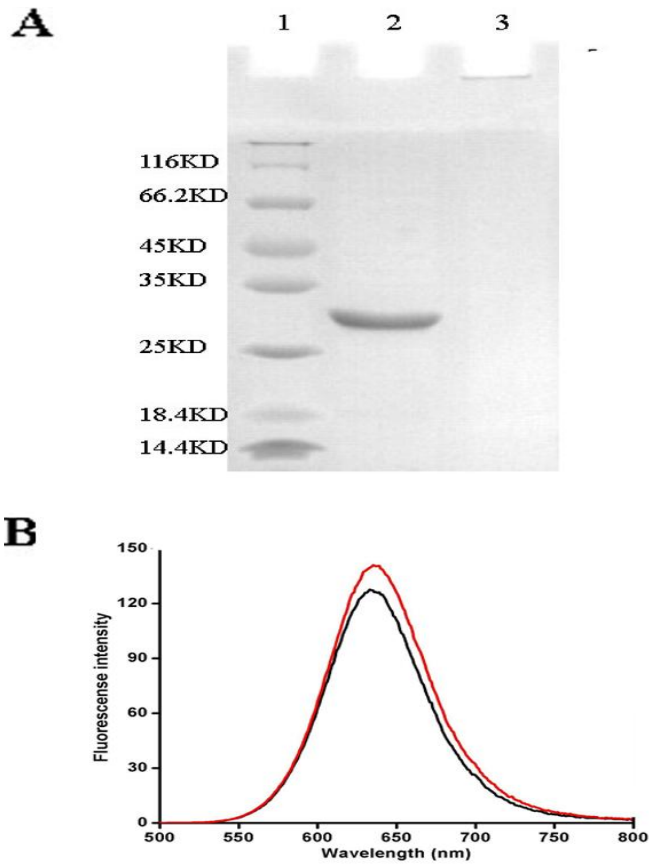


**Figure 3**. Analysis of prepared nano-probes by photoluminscent spectra (A) and SDS-PAGE (B). A, Fluorescence intensity of QDs before (black) and after (red) binding to GD/ScFvMEL. B: Line 1, marker; Line 2, *pET28*-GD/ScFvMEL; Line 3, *pET28*-GD/ScFvMEL-QDs.

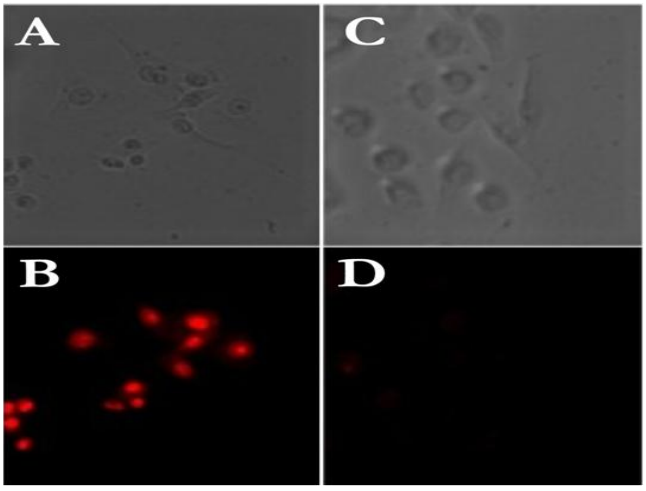


**Figure 4.** Melanoma A375 cells and gastric cancer MGC-803 cells were treated with *pET28*-GD/ScFvMEL-QDs under the light microscope (LM) and fluorescent microscope (FM). A, A375 cells under LM; C, MGC-803 cells under LM; B, A375 cells under fluorescence microscopy; D, MGC-803 cells under fluorescence microscopy.

gene fragments were constructed in prokaryotic vector using pET28a (+) vector and expressed recombinant vector (pET28-GD/ScFvMEL) in *E. coli*, and then expression product was purified by Ni-NTA Superflow Cartridges. Finally, purified recombinant vector (pET28-GD/ ScFvMEL) combined with nano-particles quantum dots to the GD/ScFvMEL were used to prepare a new nano-probe and explore its possibilities as new target molecules in *in vitro* imaging and in the diagnosis of melanoma. The current study would lay a foundation to its early clinical investigations and applications, which helps to cure melanoma.

Previous studies have demonstrated tumour binding in melanoma and other cancers following administration of whole monoclonal antibodies (Buraggi, 1986; Siccardi, 1990). When used for *in vivo* tumor targeting, whole MAbs have shown limitations due to their high molecular weight (-150kDa), which include slow blood clearance and high apparent background accumulation in blood-rich organs (such as liver and lung) which limit their use in tumor. Single chain Fv antibody fragments (ScFvs) have been developed and are about one-sixth the size of whole MAbs which exhibit improved bio-distribution, rapid blood clearance and improved tumour penetration as a result (Bird et al., 1988; Huston et al., 1988). The ScFvs have been widely used in diagnosis and treatment of some cancer, such as ovarian cancer (Li et al., 2010), nasopharyngeal carcinoma (He et al., 2004), lung cancer (Tamura et al., 2008), gastric cancer (Zhikui et al., 2010), bladder cancer (Tsai et al., 2009), colon cancer (Coelho et al., 2007) and prostate cancer (Liu et al., 2009). However, *in vivo* low absolute amounts of these ScFV were found to be located close to the tumour and non-specific background accumulation was conserved (Kang et al., 2000) purely increasing the dose of ScFv and administration did not improved contrast. Therefore, ScFv needed to be binded to other materials to overcome non-specific background and improve targeting. Quantum dots (QDs) make it possible to tag a variety of biological molecules, each with a crystal of a different size (Wu et al., 2003). Thus, in the current study, we made use of QDs technology binding to ScFv for the diagnosis of melanoma. The results showed that QDs could bind to ScFv and emit red fluorescence in melanoma cells, which help to detect ScFv for further usage in other disease.

In this study, we have successfully prepared an anti-human melanoma ganglioside single chain antibody CdTe QD nano-probe, which can specifically bind melanoma cells. However, how to use prepared nono-probe in melanoma cells of live animals is largely unknown. Elucidation of this question will depend on further studies of the binding kinetics of melanoma cells and the fields of molecular imaging. In conclusion, our data have shown for the first time that prepared anti-human melanoma ganglioside single chain antibody CdTe quantum dot nano-probe could specifically bind melanoma cells, which lay a foundation to malignant melanoma early clinical investigations and applications.

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