**BmK CT-conjugated fluorescence nanodiamond as potential glioma-targeted imaging and drug**

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**ABSTRACT**

The development of glioma-specific nanoparticles is an area of intense research recently. Fluorescent nanodiamonds (FND) as optical probes to image biological systems have attracted considerable attention. In this paper, the glioma-specific nanoparticles FND-conjugated with BmK CT, a key chlorotoxin-like peptide isolated from the scorpion venom of *Buthus martensi* Karsch, were developed. The receptor-mediated uptake of FND-BmK CT bioconjugates into rat C6 glioma cells and direct tumor visualization were confirmed by confocal fluorescence assay. Moreover, FND-BmK CT had high inhibition rate during the migration of rat C6 glioma cells by in vitro wound healing assay. These glioma-specific multifunctional nanoparticles FND-BmK CT might be responsible for the development of more effective therapeutic agents in clinical treatment of glioma.

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1. Introduction

Nanoparticles have been investigated as drug delivery vehicles, contrast agents and multifunctional devices for patient care [1]. The development of glioma-specific nanoparticles is an area of intense research in the last two years [2–11]. Interestingly, nanodiamonds (NDs) have emerged as a new candidate material due to the obvious advantage of non-cytotoxicity, chemical stability and high affinity to biomolecules [12]. Comprehensive bioassays were performed to assess and confirm that the endocytotic NDs clusters were carried in cytoplasm and did not interfere with the normal cellular functions including cell division and differentiation [13]. In addition, the fluorescence nanodiamonds (FNDs) from defect centers showed no sign of photobleaching even after surface functionalized with various biomolecules, indicating a negligible surface effect caused by chemical interactions, FNDs have been explored for ND–protein interactions and imaging [14–19].

Tumor-targeted delivery, biocompatibility, stability are technological challenges in the development of effective nanoparticle (NP)-based diagnostic and therapeutic treatments [5,20]. Chlorotoxin (Cltx or CTX), one of the key toxins in scorpion *Leiurus quinquestriatus* venom, has been shown to bind specifically to glioma cell surface as a specific chloride channel blocker [21,22]. Invasion of glioma cells into adjacent brain structures occurs through the activation of multigenic programs, including matrix metalloproteases (MMPs) such as MMP-2, which degrades extracellular matrix to overcome the extracellular matrix barrier at the invasive fronts of tumors. Results from matrigel invasion assay demonstrate that Cltx can inhibit the enzymatic activity of MMP-2 which is involved in cell migration and specifically up-regulated in gliomas [23]. Similarly, the significance of BmK CT, a key chlorotoxin-like peptide isolated from the scorpion venom of *Buthus martensi* Karsch, has been well documented as a novel blocker of chloride channel and matrix metalloproteinase-2 (MMP-2) [24,25].

Here, we developed a type of glioma-specific multifunctional nanoparticle FND-BmK CT which was designed to bind and inhibit the activity of the MMP-2 endopeptidase, and to induce endocytosis of the lipid rafts, subsequently limiting invasive cell activities.

2. Materials and methods

2.1. Materials

Rat C6 glioma cells were kindly provided by Prof. Wang (Institute of Clinical Medicine, Renmin Hospital, Yunnan Medical College, Shijian, Hubei, PR China). DMEM/high glucose was purchased from Gibco Life Technologies (NY, U.S.A.). Fetal bovine serum (FBS) was purchased from the Institute of Hematology (Hang Zhou, PR China). N2-α-Nitroliatriacetic acid (NTA) agarose was from Qiagen (Germany). All chemicals and reagents were analytical grade. Red fluorescence nanodiamonds (FNDs, mean size of 140 nm) were provided as a gift (Institute of Atomic and Molecular Sciences, Academia Sinica, Taiwan).

2.2. Expression and purification of BmK CT

Expression and purification of recombinant BmK CT were performed as we described previously [25]. Briefly, His6-tagged-BmK CT
was expressed in *E. coli* BL21 (DE3). Cells were harvested, ultrasonicated and then centrifugated. The supernatant was loaded on to a Ni²⁺-NTA affinity column and after washed thoroughly with wash buffer the fusion protein His₆-tagged-BmK CT was eluted with elution buffer (20 mM Tris–HCl, 300 mM imidazole and 500 mM NaCl, pH 9.0).

2.3. Synthesis of FND-BmK CT

The couple of BmK CT with FND was performed according to followed standard protocols reported in literatures [15]. The BmK CT-coupled FND bioconjugates were produced via the formation of amide after FND surface functionalized with carboxyl groups (Fig. 1). Briefly, acid-treated FND particles (2.0 mg) were suspended in phosphate buffered saline (PBS, pH 7.4, 1.0 ml) and sonicated for about 30 min. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (8.0 mg) was then added to the suspension to activate the surface carboxyl groups, followed by addition of BmK CT (1.0 mg) into the solution. BmK CT was finally activated and chemically linked to the carboxyl groups of FND. The resulting mixture was vortexed gently overnight. Subsequently, the BmK CT-coupled FND particles were separated by centrifugation, washed in deionized water and kept in PBS (pH 7.4, NaCl 140 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1.8 mM) at 4 °C. The amount of protein adsorbed was determined from the difference between protein concentrations before and after addition of the FND samples into the solution. We achieved about 25% coupling of BmK CT on the FNDs.

2.4. Cell culture

Rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic. Cultures were incubated at 37 °C in a humidified incubator maintained at 5% CO₂.

2.5. Confocal fluorescence assay of internalization of FND-BmK CT by C6 glioma cells

After 24 h of culturing, an adequate number of cells were randomly selected from the cell lines and digested with 0.25% trypsin, and subcultured in 6-well plates. Three wells were randomly selected from each group and marked as FND only without fetal bovine serum (FBS), FND only with FBS, FND-BmK CT with FBS, respectively.

The cultured cells were treated with FND or FND-BmK CT at the concentration of 5 μg/ml. Then, the cultures were incubated at 37 °C in a humidified incubator maintained at 5% CO₂ for 5 h after fully mixed for 0.5 h. C6 cells were fixed and permeabilized in cold 4% (w/v) paraformaldehyde for 10 min and allowed to adhere onto cover slips. Cells were washed in PBS (pH 7.4, NaCl 140 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1.8 mM) for three times and observed using a Olympus FV1000 Laser Scanning Confocal Microscope (Japan).

2.6. In vitro wound healing assay

C6 cells were used for examining the effect of FND, BmK CT and FND-BmK CT on cell migration. C6 cells were plated in 24-multiwell microtiter plates and grown to a confluent monolayer. The “scratch” was introduced by scraping the monolayer with a p200 pipette tip and was then washed three times with cell culture medium to remove cell debris. FND, BmK CT and FND-BmK CT were diluted in cell culture medium to the final concentrations of 5 μg/ml, 1 μg/ml, 5 μg/ml, respectively. Percent inhibition rate (IR%) was calculated according to the following equation: IR% = (1 – T/C) × 100%, where T and C represented the mean migration distance of the treated group and the control group, respectively.

3. Results and discussion

3.1. Determination of the location in the cell for FND-BmK CT

Tumor-targeted ligands are important in mediating NPs to recognize and bind the receptors at the cell surface. Also, for preparing tumor-specific nanoprobes, small peptides were useful because they provide better cellular uptake and tissue penetration when introduced to animals *in vivo*. BmK CT can be used as the platform for diagnostic imaging and treatment of glioma because: i) this small peptide has compact structure; ii) it has the ability to penetrate the blood brain barrier and more readily overcomes the limitations of widely used antibodies that are bulky and exhibit limited tissue penetration and cellular uptake when introduced *in vivo*; iii) it has the anti-glioma activity in inhibiting glioma invasion or metastasis. As shown in Fig. 2, assembled red upconversion fluorescence nanodiamonds could be detected in the cytoplasm of C6 cells when treated with FND without FBS and FND-BmK CT, respectively, for 5 h at the particle concentration of 5 μg/ml, which demonstrated that MMP-2-mediated uptake of FND-BmK CT bioconjugated into rat C6 glioma cells and direct tumor visualization were actualized by this delivery system. Moreover, FND had low glioma intracellular localization when the cell cultures supplemented with 10% FBS, which could confirm the high glioma specificity of FND-BmK CT.

3.2. In vitro wound healing assay

To test the effect of FND-BmK CT on astrocytoma cell migration, C6 cells were incubated with FND, BmK CT and FND-BmK CT, and the migration of cells was assessed by an *in vitro* wound healing assay. As shown in Fig. 3, the average inhibition rate of FND-BmK CT was 32% which was higher than that of FND or BmK CT. These two experiments showed that FNDs uptake into the C6 cells were enhanced specifically due to BmK CT.

To our knowledge, CdTe [26], Ga₂O₃ [27], MnO, MnFe₂O₄ [28,29], DySiO₃ [30], gold nanoparticles (AuNPs) [29], TiO₂, ZnO, ZnS, silica and polymer nanoparticles [31], PLGA [32] and nanodiamond [33,34] have been investigated as drug delivery vehicles. Iron oxide, multifunctional superparamagnetic and NaYF₄:Yb, Er upconversion NPs-conjugated with chlorotoxin (CTX) exhibited a high affinity for gliomas and direct tumor visualization. However, the biocompatibility of these NPs is a critical issue. Although ND is a biocompatibility and promising candidate as various cargos, including DNA, proteins, and drugs for cancer therapy, more studies are still needed before their clinical application.

4. Summary

In the present study, the glioma-specific multifunctional nanoparticles FND-conjugated with BmK CT are developed and confirmed to be an effective strategy for glioma control. Current nanoparticle-based therapeutic strategies for glioma treatment are mainly based on delivery of chemotherapeutic agents to induce apoptosis or DNA/siRNA to regulate oncogene expression. So, the glioma-specific multifunctional nanoparticles based on FND-BmK CT delivery vehicle might be responsible for the development of more effective therapeutic agents in clinical treatment of glioma. Also, partly due to its access...
to MMP-2, FND-BmK CT delivery vehicle might represent a future therapeutic option for a wide variety of tumors, including prostate cancer, intestinal cancer and sarcoma.

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References


Fig. 2. Images of pristine FND and FND-BmK CT internalized by C6 cells with or without FBS. The images displayed are confocal fluorescence (left), differential interference contrast (middle), and the merge images (right): A) FND only without FBS; B) FND only with FBS; C) FND-BmK CT with FBS. The cells were incubated with FND or FND-BmK CT for 5 h at a particle concentration of 5 μg/ml in this particular experiment. The arrows showed the assembled FNDs which were absorbed by C6 cells. (Bar, 20 μm).

Fig. 3. FND-BmK CT decreased C6 cells migration rate. Migration of cells was routinely monitored after confluent monolayers were gently scratched with a plastic pipet tip in the wound assay. Inhibition rates of cell migration were detected 8 h after treatment with FND, BmK CT and FND-BmK CT at the concentrations of 5 μg/ml, 1 μg/ml, 5 μg/ml, respectively.

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