Receptor-targeted quantum dots: fluorescent probes for brain tumor diagnosis

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1 Introduction

Despite aggressive treatment by surgery, radiotherapy, and chemotherapy, the median survival of patients diagnosed with glioblastoma multiforme (GBM) is approximately 12 months.\(^1\) Studies conducted on GBM have demonstrated that patient clinical outcome is closely related to the extent of surgical resection.\(^2\) Stereotactic image guidance technology has a limited sensitivity for defining low-cellularity infiltrative tumor cells.\(^3,4\) In the end, the best resources available to the neurosurgeon in determining tumor boundaries are their own experience and the standard intraoperative neuropathological evaluation of frozen sections. The neurosurgeons may terminate surgery or adjust the extent of the resection based on the identification of tumor in the margins. However, due to the infiltrative nature of GBM and the histologic similarity of some tumor cells to normal brain cells, the standard hematoxylin and eosin-stained intraoperative frozen sections are of limited utility in accurately assessing resection margins. Thus, one important challenge in brain tumor treatment is to develop strategies for accurate intraoperative identification of tumor cells in brain tissue.

In the post-operative setting, therapies targeted toward specific tumor-specific molecules or pathways are becoming increasingly available.\(^5\) Standard immunohistochemical procedures on paraffin tissue may be completed two to three days

Abstract. The intraoperative diagnosis of brain tumors and the timely evaluation of biomarkers that can guide therapy are hindered by the paucity of rapid adjunctive studies. This study evaluates the feasibility and specificity of using quantum dot-labeled antibodies for rapid visualization of epidermal growth factor receptor (EGFR) expression in human brain tumor cells and in surgical frozen section slides of glioma tissue. Streptavidin-coated quantum dots (QDs) were conjugated to anti-EGFR antibodies and incubated with target cultured tumor cells and tissues. The experiments were conducted first in human glioma tumor cell lines with elevated levels of EGFR expression (SKMG-3, U87) and then in frozen tissue sections of glioblastoma multiforme and of oligodendroglioma. The bioconjugated QDs used in the study were found to bind selectively to brain tumor cells expressing EGFR. QD complexed quickly to the cell membrane (less than 15 min), and binding was highly specific and depended on the expression level of EGFR on the cell membrane. Tissue experiments showed that only tumor specimens expressing EGFR were labeled in less than 30 min by QD complexes. These findings demonstrate that QD-labeled antibodies can provide a quick and accurate method for characterizing the presence or absence of a specific predictive biomarker. © 2007 Society of Photo-Optical Instrumentation Engineers.

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after surgery. Molecular tests may take one week or more to be completed and reported. To reduce the time to chemotherapy initiation, a second important goal then is to develop a generalizable methodology that might rapidly characterize the absence or presence of specific biomarker molecules that might predict response to therapy.

The epidermal growth factor receptor (EGFR) signaling pathway plays an important role in gliomagenesis. EGFR overexpression or amplification is found in as many as 40% to 50% of de novo high-grade astrocytomas or GBM. The expression level is relatively low in low-grade glioma and is absent in normal brain tissues. EGFR overexpression in patients with GBM correlates with disease progression, poor prognosis, and reduced sensitivity to some chemotherapy. The overexpression of EGFR has been reported to correlate with response to EGFR inhibitors. Therefore, the development of a rapid method for the detection of EGFR would be a useful paradigm for the timely assessment of biomarkers in general.

Recent studies have demonstrated that quantum dots (QDs) can be covalently linked with biorecognition molecules such as antibodies, peptides, nucleic acids, and other small molecules and used as fluorescent molecular probes in a variety of biomedical diagnostics. QDs have unique optical properties. When compared with organic fluorescent dyes, they are characterized by narrow and tunable emission, broad excitation, and photostability. In addition, QDs are extremely small, with a diameter generally less than 10 nm, which makes them much easier to cross biological membranes and reach intracellular targets. Bioconjugated QDs have the potential to monitor long-term intracellular processes at the single-molecule level and to target and detect multiple biomarkers. Modification of QD size and chemical composition allows for tuning their fluorescence emission toward the near-infrared (NIR) spectrum that in turn enables deep photon penetration into and out of tissue. Thus, they are an ideal candidate for in vivo fluorescence imaging studies. For example, QDs have been demonstrated to label breast cancer marker Her2 in living cells and EGFR in SiHa cervical cancer cells. They have been used to target tumor vasculature, and provide real-time guidance for cancer surgery in large animals.

Although QDs have been used in some cancer research and diagnostic applications, the use of QDs to target diagnostic and predictive molecular markers in GBM has not been extensively investigated. In this study, we first evaluate, in cell culture experiments, the kinetics of QD-labeled antibody binding to the EGFR receptor, the stability of fluorescence, and the sensitivity to levels of EGFR expression. Subsequently, the QD-based labeling technique is validated on surgical tissues by comparison to standard immunohistochemical staining of corresponding paraffin tissue.

2 Material and Methods

2.1 Samples

2.1.1 Cell lines

Three human cancer cell lines were used in this study: U87 human glioma; SKMG-3 human glioma; and MCF-7 human breast cancer cell lines. The U87 and MCF-7 cells were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS), 50 units/mL penicillin G, 50 μg/mL streptomycin, and 2 mM L-glutamine and incubated at 37 °C with 5% CO2. The SKMG-3 cells were grown in DMEM and incubated at 37 °C with 5% CO2. The SKMG-3 cell line retains the highest amplified EGFR genes in vitro among all three cell lines used in this study. In comparison to the SKMG-3 cell line, the U87 cell line has an intermediate EGFR expression level and MCF-7 has low EGFR expression. All cells were subcultured 24 hours before experiments. All sets of experiments involving cell lines were conducted (repeated) 10 times.

2.1.2 Tissue specimens

At the time of surgery, intraoperative frozen sections were prepared. The human brain tissue specimens were histologically confirmed as glioblastoma multiforme (3 samples), oligodendroglioma (2 samples), and cerebral cortex (1 sample). The brain tumor and cerebral cortex samples were obtained with the approval of the Cedars-Sinai Institutional Review Board.

2.2 Targeting Strategy and Preparation of Anti-EGFR Conjugated QDs

The targeted delivery was designed to enable investigation of both binding and internalization of QDs in brain tumor cells (Fig. 1). Initially, the anti-EGFR antibodies were attached to QDs streptavidin conjugates to form anti-EGFR-QD complexes. Then the complexes were mixed with the glioma cells. The biotin and streptavidin act as a stable bridge for attaching anti-EGFR to the QDs. The noncovalent binding of biotin to streptavidin in aqueous solution is essentially irreversible and extremely stable over a wide range of temperatures and pHs. Then, the cells were incubated with the anti-EGFR-QD complexes. The delivery of QD complexes into tumor cells was based on an EGFR mediated endocytosis mechanism. Following this strategy, the biotinylated human monoclonal anti-EGFR antibody (Biodesign International) targeted against the extracellular domain of EGFR and QD 525 streptavidin conjugates (Quantum Dot Corporation) were diluted to 2 ng/mL and 20 nM, respectively, in PBS containing 2.5% (wt/vol) BSA, and then kept at room temperature for 30 min before mixing with cells.
2.3 Labeling of Cells and Frozen Tissue with Quantum Dot Conjugates

Cultured live cells were first washed with PBS, then blocked with PBS containing 2.5% BSA for 30 min at 37 °C with 5% CO₂, and incubated sequentially with QD-anti-EGFR complexes in PBS containing 2.5% BSA for 15, 30 min, and 2 hours at 37 °C with 5% CO₂ before observation. Unbound QD-anti-EGFR complexes were removed by washing three times with PBS. The cells were observed immediately after removing unbound QD-anti-EGFR complexes. The frozen tissue slices were labeled in a similar fashion to the cells with anti-EGFR conjugated QDs.

2.4 Preparation of Frozen Section and Paraffin Slides and Immunostaining

Frozen sections of tumor and human normal cerebral cortex were cut in a −20 °C cryostat and air-dried. Slides were then stored at −70 °C until used for quantum dot assays. Tissue from each tumor was also fixed in 10% buffered formalin and processed into paraffin blocks. Slides were prepared from these blocks and stained with hematoxylin and eosin as well as for EGFR. The immunostaining for EGFR was performed with the EGFR pharmDx immunohistochemistry kit (mouse monoclonal anti-human EGFR, clone 2-18c9), on a Dako Autostainer (Dako, Carpinteria, CA).

2.5 Fluorescence Microscopy

The cells stained using QDs were cultured in an 8-well Lab-Tek cover glass chamber (Nalge Nunc International, Naperville, IL) in PBS during observation. Fluorescence images were obtained with an inverted Zeiss Axiovert 200M fluorescence microscopy equipped with a Zeiss AxioCam MRm (CCD monochrome, 1300 × 1030 pixel, 1 ms–20 s shutter time, UV-NIR spectral range, Z-stack). The CCD camera was controlled by Zeiss image acquisition software AxioVision (Carl Zeiss MicroImaging, Inc., Thornwood, NY) through a PCI interface. The excitation filter was 425/45 nm. The emission was collected using a 525/30-nm filter. Image acquisition, processing, and analysis were conducted using the AxioVision 3.1 software package. A 63× water-immersion objective was used to evaluate the internalization and subcellular localization of QDs in live cells. False-color fluorescence images were obtained at excitation 425/45 nm with a 525/20-nm bandpass filter.

3 Results

3.1 Experiments in Tumor Cell Lines: Anti-EGFR Conjugated QDs Binding and Internalization

3.1.1 Binding and internalization dynamics

Direct microscopic observations showed that within a short duration (15-min incubation), small amounts of QDs were observed in the intracellular of the EGFR-overexpressing glioma cell line (SKMG-3). The QDs were initially (within 30 min) primarily localized to the cell membrane [Fig. 2(a)]. At 2 hours, they were mainly internalized [Figs. 3(a) and 3(b)] and uniformly distributed in the cytoplasm (Fig. 4). In addition, as the observation time increased, the fluorescence intensity on the cell membrane decreased. These results suggest that QD-labeled anti-EGFR antibodies first bind to the cell membrane and then are brought into cells together via EGFR-mediated endocytosis. After internalization, instead of diffusing into cytoplasm, the QDs were observed as being restricted to the small vesicles (most likely early endosomes as previously reported). Our results indicate that the QD complexes used in this study did not impair the biological process of the anti-EGFR-mediated endocytosis. The intensity of QD fluorescence inside the SKMG-3 cells continued to increase until reaching a plateau after 2 hours of incubation. These observations were consistently found in all 10 sets of experiments involving the cell lines.
3.1.2 Photostability
The fluorescence emission of QDs did not visually decreased during multiple exposures to excitation light including 3-dimensional optical sectioning (Fig. 4). This finding indicates no or minimal photobleaching while exposing QDs to the light source. Cells loaded with QDs did not show obvious alterations in cell growth and division. After 24 hours, the QDs accumulated in a small region of the cytoplasm [Fig. 5(a)]. However, the fluorescence intensity of QDs decreased significantly, suggesting loss of the stabilizing ligands on the QD’s surface in the biological environment. Similar results were recorded after 48 hours [Fig. 5(b)]. The fluorescence of QDs inside cells was detected in a few cells even after 7 days. These results are in line with previous studies, suggesting that QDs can act as fluorescent probes for long-term cellular studies and tracking of cells.

3.1.3 EGFR expression-level dependence
Performing similar experiments, fluorescence of QDs was also clearly detected in the U87 cell line [Fig. 3(b)], which has an intermediate EGFR expression level. The fluorescence level was much weaker when compared to SKMG-3 cells [Fig. 3(a)]. Furthermore, no QDs were detectable in MCF-7, which have virtually no EGFRs on their membrane [Fig. 4(c)], indicating that the amount of QDs in entered cells depends on the level of expression of membrane EGFR.

3.1.4 Receptor-mediated uptake
Cells incubated with only anti-EGFR [Fig. 6(a)] or only QD525 streptavidin conjugates [Fig. 6(b)] had no detectable QDs fluorescence inside the target cells on their cell membranes. These results further support and confirm that bioconjugated QDs attach and enter the cells through anti-EGFR-mediated binding to cell membrane EGFRs.

3.2 Tissue Specimens (Frozen Sections): Anti-EGFR Conjugated QD Binding
Figure 7 depicts the binding specificity of conjugated QD to frozen sections expressing EGFR. Binding of QDs to GBM [Fig. 7(b)] and oligodendroglioma [Fig. 7(c)] was observed, tumors known to express EGFR, but not to normal human cerebral cortex. The presence of the EGFR in these investigated GBM and oligodendroglioma samples was confirmed by the immunohistochemistry analysis (Fig. 7, left panels). In contrast, EGFR was undetectable in the human cerebral cortex. These results suggest that antibodies against EGFR conjugated with QDs are able to distinguish EGFR-expressing tumor tissue from normal tissue.
4 Discussion

The experiments reported here, conducted in both human brain tumor cell culture and human brain tumor tissue specimens, show that bioconjugated QDs can selectively bind to cells expressing EGFR. In addition, the experiments involving various human cell lines demonstrate that the binding of QDs complexes to cell membrane depends on the expression level of EGFR on the cell membrane and that QD uptake is mediated by EGFR. To our knowledge, this study demonstrates for the first time that QDs can be specifically targeted to EGFR expressed in primary brain tumors and enable detection of these tumors with cellular resolution.

4.1 Nanoparticles in Neurosurgery

Over the past decade, in an effort to improve the effectiveness of brain tumor resection, a variety of fluorescent dyes (e.g., indocyanine green, fluorescein conjugated to human serum albumin or to sodium, and 5-aminolevulinate) were employed to enhance the specificity of optical detection of these tumors and thus improve surgical outcome. However, to date the efficacy of these organic dyes in intraoperative detection of tumor margins has been limited due to various factors including lack of specificity, photobleaching, limited circulation time, and consistency of the delivery/pharmacokinetic pattern. To solve these problems, inorganic fluorescent contrast agents may be more robust fluorescent probes for brain tumor delineation as well as for diagnosis of other tumor types. Recent advances in synthesis and bioconjugation of nanoparticles have resulted in new classes of molecular probes that are multifunctional or multimodal. A few recent studies showed the fabrication of multimodal nanoparticles that can be used in the delineation of brain tumors and that can be detected with both magnetic resonance imaging (MRI) and optical devices. These include a functionalized iron nanoparticle bound to fluorescent molecule Cy5.5 and chlorotoxin (a peptide with high affinity to matrix metalloproteinase-2 typically upregulated in various gliomas) and a nontargeted multimodal superparamagnetic iron oxide-based nanoparticle CLIO-Cy5.5. These nanoparticles showed potential for tumor visualization and co-registration with MRI. Unlike this study, however, they were not designed to target a specific receptor molecule.

4.2 Quantum Dots: Fluorescent Probe to Target Glioma Cell Membrane Receptors

Recently, QDs were also proposed as potential optical molecular probes to aid in the surgical resection of brain tumors. Preliminary results using semiconductor QDs were reported by a few research groups, including ours. The results reported here extend on our early report and demonstrate that QDs with appropriate functional groups are able to highlight brain tumor in both cell culture and surgical tissue by specifically targeting an overexpressed cancer marker, EGFR. Our findings provide insights into the molecular mechanism involved in bioconjugated QD binding and uptake and show that nonspecific QD labeling is unlikely to occur in normal tissue or tumors that lack EGFR expression. Furthermore, the method is specific and rapid.

In addition, these results are consistent with earlier reports that have demonstrated that QDs with stable polymer coating do not affect cell division and other physiological functions. The functionalized QDs used in this study also did not appear to interfere with the receptor mediated endocytosis, viability, and growth of target cells. We found no morphological change among cells loaded or not loaded with QDs. These findings confirm early reports that QDs can be used for long-term cell tracking. For this study, we used commercially available QDs and relatively simple protocols to target specific receptors on the cell surface. Taking advantage of unique surface chemistry and optical and electronic properties such as size- and composition-tunable fluorescence, the emission of QDs multimodal imaging can be advanced. For example, recent reports demonstrated synthesis of nanoparticles composed of a quantum dot coated with paramagnetic and pegylated lipids. The bimodal character, high relaxivity, and specificity of QDs suggest that they may be excellent multifunctional/multimodal probes for molecular imaging of brain tumors in future studies.

4.3 Quantum Dots Aid in Fluorescence-Guided Brain Tumor Resection and Intraoperative Neuropathology

The high quantum yield and photostability of QDs make these nanoparticles excellent fluorescent probes that can be detected using existing fluorescence-based spectroscopy and imaging technologies. Advances in such optical technologies have allowed development of compact and portable devices that play an important role in screening and diagnosis of cancer, both in vivo and in vitro. In addition, they allow for remote tissue investigations using fiber optic probes and integration with neurosurgical navigation tools. For example, by taking advantage of brain tissue autofluorescence, recent studies have shown the potential of fluorescence spectroscopy to distinguish the normal brain tissues from gliomas of various grades. Also, fluorescence imaging-guided systems have been custom-built and integrated in operating micro-

Fig. 7 Images of frozen tissue sections of (a) human cerebral cortex, (b) GBM, (c) oligodendroglioma. Tissue samples were incubated with anti-EGFR conjugated QDs for 30 min at room temperature before imaging. Conventional immunohistochemistry of adjacent slide (left panels); fluorescence image of anti-EGFR conjugated QDs (middle panels); B&W image of the QDs stained slides (right panels).
scopes to guide tumor resection and photodynamic therapy. Consequently, existing fluorescence-based devices should easily be extended to a wide range of applications from the detection of autofluorescence in brain tissues to the detection of exogenous fluorescent probes such as QDs targeted to biomarkers expressed in brain tumors. In addition, the long-lived fluorescence lifetime of QDs enables the use of time-resolved or time-gated imaging to discriminate against autofluorescence (typically characterized by short lifetimes). Fluorescence-guided surgery/intervention systems can be easily customized to record distinct spectral and temporal windows. Thus, the high affinity to molecular targets combined with the high quantum efficiency of bioconjugated QDs and their long-lived fluorescence emission properties can improve both the sensitivity and specificity of optical detection and has the potential to enhance the delineation of brain tumor cells from normal tissue in vivo.

Generally, the QD probes can be delivered in vivo to tumors in animal models by passive and active targeting mechanisms. We acknowledge, however, that delivery of QDs to brain tumors in vivo in humans for intraoperative imaging of brain tumor margin purposes faces a few challenges. These include QD biocompatibility, circulation, and cytotoxicity. Efforts toward solving these issues are currently being addressed by several research groups, and a pertinent review was recently reported. While in vivo imaging studies in animal tumor models (not only in mice but also in large animals) using QDs have yielded very impressive results, the short- and long-term cytotoxicity of QDs may pose legitimate questions when such probes are to be used in humans. Nevertheless, the potential cytotoxicity of QDs may be of interest when using them as therapeutic agents.

The high fluorescence stability (resistance to photobleaching), when compared with organic dyes, makes QDs excellent probes for imaging biological samples. This study showed that bioconjugated complexes of QDs bind with high specificity to cells expressing EGFR in human brain tissue (frozen) sections within 30 min or less; these results suggest that QDs might serve as fluorescence probes for intraoperative frozen section diagnosis. Such a short timespan would be more than acceptable for post-operative guidance of targeted therapy. However, further optimization of processing time is desirable for intraoperative uses. It remains to determine the minimum time required for the anti-EGFR antibody conjugated QDs to bind to the EGFRs expressed on cell surface and to test, in a similar fashion, the use of other antibodies against well-known glial marker-associated proteins such as glial fibrillary acidic protein (GFAP). Development of QD-labeled antibodies against melanoma, lymphoma, and carcinoma markers would assist the distinction of these malignancies from a poorly differentiated high-grade glioma. Nevertheless, this study demonstrates for the first time the potential of QDs as fluorescent probes for the intraoperative identification of a brain tumor biomarker.

5 Conclusion

With their unique characteristics and intrinsic optical advantages, bioconjugated QDs have the ability to detect various biomarkers and localize tumors and tumor margins. To achieve high specificity and efficiency in our study, we used QDs conjugated with antibodies that recognize a readily accessible membrane receptor, EGFR. QD-labeled antibodies were shown to actively attach to cells that overexpressed EGFR. Besides anti-EGFR antibodies, other antibodies against brain tumor biomarkers could be linked readily to QDs through a similar streptavidin-biotin approach. Recently, the presence of PTK and of EGFRVIII, a mutant form of EGFR, in GBM has been strongly associated with response to EGFR tyrosine-kinase inhibitors and are potentially desirable targets for QD-labeled antibodies. Another potential target is the O6-methylguanine-DNA methyltransferase (MGMT) gene product, as high levels of MGMT expression have been reported to correlate with resistance to temozolomide.

In addition, the unique optical properties of quantum dots make them ideal sensors for simultaneous monitoring of several targets. Considering the broad excitation and narrow emission of QDs, multicolor and multitarget imaging for diagnosis of brain tumor can possibly be achieved. Moreover, the large surface of QDs allows the attachment of a large number of functional groups for diagnostic and therapeutic purposes. With an additional conjugation step, the QDs could serve not only as a fluorescent probe but also as a drug carrier to bring therapeutic agents to brain tumors.

In summary, over the past few years, quantum dots have gained recognition as nanoprobes that can play an important role in cancer diagnostics and therapy including nanoneurosurgery. The receptor-target approach for recognition and imaging of glioma cells reported here is an important step toward understanding how fluorescent quantum dots can be applied to the intraoperative diagnosis of brain tumors and, in particular, the recognition of biomarkers expressed in GBM.

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