Biomaterials 35 (2014) 2971–2980

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Quantitative assessment of Tn antigen in breast tissue micro-arrays using CdSe aqueous quantum dots

Giang H.T. Au [a], Linette Mejias [b], Vanlila K. Swami [b], Ari D. Brooks [c], Wan Y. Shih [a], Wei-Heng Shih [d,*]

[a] School of Biomedical Engineering, Science and Health System, Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104, USA
[b] Department of Pathology and Laboratory Medicine, College of Medicine, Drexel University, 245 N. 15th Street, Philadelphia, PA 19102, USA
[c] Department of Surgery, College of Medicine, Drexel University, 245 N. 15th Street, Philadelphia, PA 19102, USA
[d] Department of Materials Science and Engineering, Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104, USA

ARTICLE INFO

Article history:
Received 15 November 2013
Accepted 13 December 2013
Available online 8 January 2014

Keywords:
Immunochemistry
Image analysis
Nanoparticle
Quantum dots
Breast cancer
Surgery margin

ABSTRACT

In this study, we examined the use of CdSe aqueous quantum dots (AQDs) each conjugated to three streptavidin as a fluorescent label to image Tn antigen expression in various breast tissues via a sandwich staining procedure where the primary monoclonal anti-Tn antibody was bound to the Tn antigen on the tissue, a biotin-labeled secondary antibody was bound to the primary anti-Tn antibody, and finally the streptavidin-conjugated AQDs were bound to the biotin on the secondary antibody. We evaluated the AQD staining of Tn antigen on tissue microarrays consisting of 395 cores from 115 cases including three tumor cores and one normal-tissue core from each breast cancer case and three tumor cores from each benign case. The results indicated AQD-Tn staining was positive in more than 90% of the cells in the cancer cores but not the cells in the normal-tissue cores and the benign tumor cores. As a result, AQD-Tn staining exhibited 95% sensitivity and 90% specificity in differentiating breast cancer against normal breast tissues and benign breast conditions. These results were better than the 90% sensitivity and 80% specificity exhibited by the corresponding horse radish peroxidase (HRP) staining using the same antibodies on the same tissues and those of previous studies that used different fluorescent labels to image Tn antigen. In addition to sensitivity and specificity, the current AQD-Tn staining with a definitive threshold was quantitative.

1. Introduction

Immunostaining is an integral part of pathological analysis for diagnostic and therapeutic decisions and monitoring [1,2]. Immunostaining is used to visualize cellular or tissue constituents (antigens) based on antigen–antibody interactions. Two main staining methods in immunostaining are immunohistochemical (IHC) staining which uses an enzyme such as horse radish peroxidase (HRP) to react with its substrate to produce a colored substance to show the molecules of interest and immunofluorescence (IF) which uses fluorescent molecules to light up the molecules of interest. HRP-labeled antibodies are widely used in pathological examination due to the stability and durability of the staining over a long period of time. However, the extent of the expression of the biomarkers often requires semi-quantitative evaluation. Many factors can affect the assessment including different scoring systems, amount of chemicals, etc. Determination of whether a tissue is positive or negative often depends on the experience and skill of the interpreter, possibly leading to inaccurate results. Since the late 1980s, computerized image analysis systems have been introduced and shown to be a more accurate means to quantify the image. However, the non-linear relationship that occurs at higher levels between the amount of the antigen and the absorption intensity of the chromogen used in HRP-IHC, diaminobenzidine (DAB), can result in inaccurate interpretations [1,3]. It remains a challenge to quantify HRP-IHC accurately. Recent approaches have explored the use of IF-based methods and fluorescent microscopy to better quantify protein expression in tissues [4,5]. Conventional organic fluorescent dyes have several limitations such as small Stokes shifts and difficulty in distinguishing positive fluorescent signals from auto-fluorescence of formalin-fixed-and-paraffin-embedded tissues [6]. Moreover, photo-bleaching is a major drawback of conventional fluorescent dyes which makes it difficult if not impossible to view the same region repeatedly [7].

Semiconductor nanoparticles such as quantum dots (QDs) are a new class of inorganic fluorophores that are made of inorganic

* Corresponding author.
E-mail address: shihwh@drexel.edu (W.-H. Shih).

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http://dx.doi.org/10.1016/j.biomaterials.2013.12.034

materials. They exhibit high fluorescence intensity, high resistance to photo-bleaching [8] and a wide range of possible emission wavelengths. Their size is between 2 and 10 nm, which is comparable to green fluorescent protein (GFP) [9]. By changing the particle size of the same material, the emission wavelength is tunable making it possible to image multiple markers simultaneously on the same pathological sites [10]. They have the potential to be an excellent fluorescent molecular probe to image biomolecules when combined with specific receptors to the biomolecules of interest. Bioimaging applications of QDs have been demonstrated in cell labeling and tracking [11,12], cell proliferation [13], *in vivo* sentinel lymph node mapping in a pig [14], *in vivo* brain imaging in mice [15], molecular beacons for DNA detection [16–18] and *in vivo* tumor detection in mice [19,20]. For specific imaging of a target antigen, QDs can be coupled with an antibody to detect the target antigen on the cell surface. Recent studies have demonstrated such QD-based IF staining in molecular pathology for HER2 expression in breast cancer tissues [21,22]. The advantages of such QD-based IF staining for molecular pathology include brighter fluorescent signals over organic dyes, therefore, a better signal-to-noise ratio, and better detection sensitivity and accuracy than conventional IHC [21,23].

However, commercial QDs are made in an organic solvent (OQDs hereafter). To be used in bioimaging, OQDs must undergo solvent and ligand exchange. The intrinsic disadvantage of such solvent and ligand exchange is that the amount of capping molecules on the OQDs is insufficient for optimal colloidal stability and biomolecule conjugation. Typically, a large amount of proteins [24] must be used in order to bind the protein on the OQDs, rendering OQD IF staining not cost-effective. We have pioneered an aqueous synthesis process in which QDs can be made directly in water with 3-mercaptopropionic acid (MPA) as the capping molecule [24–28] (Aqueous quantum dots (AQDs) hereafter). Using such approach, we have made bright MPA-capped CdSe AQDs from a nominal MPA:Cd:Se = 4:3:1 with a high quantum yield of 70% [24]. Furthermore, these CdSe AQDs could conjugate to streptavidin (SA) with a high SA-conjugation efficiency of 75% (i.e., 75 of every 100 SA molecules in the solution were conjugated to AQDs) to form a SA-AQD complex consisting of one AQD with 3.4 SA with high imaging efficacy [24] as opposed to a 3% SA-conjugation efficiency exhibited by OQDs to form a SA-OQD complex consisting of one OQD with 2.7 SA with similar imaging efficacy [24]. These results illustrated that AQDs needed only one twentieth of the SA needed by OQDs to achieve the same imaging efficacy [24]. As protein is typically the cost limiting factor, AQDs' ability to conjugate to SA with high SA conjugation efficacy offers great potential to use AQDs for cost-effective IF staining in molecular pathology applications.

The goal of this study is to examine the efficacy of CdSe AQD-based IF imaging method for molecular pathology applications. Specifically, we will use CdSe AQDs to image Tn antigen, a pan-carcinoma biomarker [29,30] in breast tissues and compare the results with those of the corresponding HRP-IHC staining. Tn antigen is a truncated O-linked core glycan linked to the serine or threonine of mucin 1 (MUC1) [31–35]. It is a tumor-associated carbohydrate antigen (TACA) present only on epithelial cancer cells due to the lack of the elongation of the core glycan by β1-3 D-galactosyltransferase and α2-6 sialyltransferase enzymes [36,37]. That is, in normal cells Tn antigen is hidden by the additional sugar residues attached to it whereas Tn antigen is exposed in cancer cells. Tn antigen has been observed in more than 90% of human epithelial cancers [38–41]. Tn antigen has been shown to be present in most breast cancers including invasive ductal carcinomas (IDC), invasive lobular carcinomas (ILC) and ductal carcinomas *in situ* (DCIS) [30,42] but absent in a broad range of normal adult tissues including normal breast tissues [43–46]. Tn antigen is also present in some benign breast lesions such as atypical ductal hyperplasia (ADH). However, ADH is considered a cancer precursor [42,44].

We will quantitatively determine the sensitivity and specificity of the AQD-based IF imaging in differentiating breast cancer against normal breast tissues and benign breast tumors and compare these results with those of the corresponding HRP-based IHC probes. Both the AQD IF and the HRP IHC methods will be tested on the same tissue microarrays (TMAs) of breast tissues from 115 patients including 58 malignant cases (stages 0-III) and 57 benign cases. For the AQD-based IF imaging, we will use MPA-capped CdSe AQDs each conjugated to 3.4 SA [24] as the fluorescent label for a three-step indirect staining strategy consisting of (1) binding of the primary antibody to the cancer cells, (2) binding of the biotinylated goat anti-mouse antibody to the primary antibody, and (3) binding of the SA-conjugated AQDs to the biotinylated goat anti-mouse antibody.

2. Materials and methods

2.1. Cell line and cell culture

The MDA-MB-231 (ATCC) is a human breast cancer cell line obtained from Dr. M. J. Reginato of Drexel University of College of Medicine. MDA-MB-231 cells were maintained in DMEM high glucose medium supplemented with 10% fetal bovine serum (Bioexpress, Kaysville, UT), 1% penicillin and streptomycin (Mediatech Inc., Manassas, VA) and cultured at 37 °C in a 5% CO_2 incubator.

2.2. Tissue micro-arrays (TMAs)

Paraffin-embedded tissue blocks from each case were reviewed by co-author, pathologist Linette Mejias (LM), to identify appropriate areas to be included in the TMAs. When an appropriate area was identified, a 2-mm diameter punch was made and the tissue was randomly placed in a TMA block as a core. For each malignant case, we included 3 punches from the malignant region and a punch from the normal breast region. For each benign case, we included three punches from the benign tumor region. A TMA contained 50–60 cores from at least 15 patients randomly distributed within the TMA to minimize potential correlation from core to core. There were a total of 115 patients including 58 breast cancer patients of various breast cancer types and stages and 57 patients with benign breast pathology. There were 395 cores that were successfully made from a total of 403 punches.

2.3. Aqueous quantum dots conjugation

The synthesis of MPA-capped CdSe AQDs followed those of MPA-capped CdS and MPA-capped ZnS [25,47] with an optimal nominal MPA:Cd:Se ratio of 4:3:1 [24]. In what follows all CdSe AQDs were made with this molar ratio. In Fig. 1a we show the photoluminescence excitation (PLE) spectrum (blue) and photoluminescence (PL) spectrum (red) of the CdSe AQDs, which indicates that the peak of the PLE was at 460 nm and the peak of the PL was at 610 nm. In what follows, the window of the excitation filter was 460 ± 20 nm as indicated by the green shade and that of the emission filter was 600 nm long pass as indicated by the orange shade in Fig. 1a. The transmission electron microscopy (JEOL JEM2100) (TEM) image of the CdSe AQD is shown in Fig. 1b which indicated that the CdSe AQDs was crystalline with a size of about 3 nm. Freshly made AQDs suspension was first stored in a refrigerator (4 °C) overnight followed by the removal of the free MPA by centrifugation with a 10 kDa filter (Millipore Co., Beillerica, MA) at 3000 rpm for 10 min three times. After each centrifugation the volume of the suspension was restored by adding pH = 7.0 borate buffer. N-ethyl-N′-dimethylaminopropyl-carbodiimide (EDC) (Thermo Scientific, Rockford, IL, USA) and N-hydroxysuccinimide (NHS) (Thermo Scientific, Rockford, IL, USA) were used to facilitate the peptide bond formation between a primary amine of the SA and a carboxyl on the AQD. First, 4 mg of EDC and 6 mg of NHS were dissolved in 1 ml of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (TEKNOVA, Hollister, CA) at pH = 6.5. 2 mM of EDC and 5 mM of NHS were added to the suspension of the AQDs at 1.07 μM particle concentration at pH = 7.0 in borate buffer. The reaction was incubated for 15 min at room temperature followed by the addition of 2-mercaptoethanol (20 mM) to quench the EDC. The suspension was then run through a desalting column (Zeba Spin 7 KW, Pierce, Rockford, IL, USA) to remove unbound reagents and electrolytes in the suspension. The suspension was then mixed with an SA solution at room temperature and pH = 7.0 for 2 h. The unused NHS esters bound on the AQD surface were then quenched by hydroxylamine hydrochloride (10 mM) (Sigma–Aldrich, St. Louis, MO, USA). Unconjugated AQDs and SAs were then removed by microcentrifugation at 12,000 rpm with a 100 kDa filter (Millipore) for 5 min five times. After each microcentrifugation, the volume of the suspension was restored with a 50 mM borate buffer solution of pH 8.3. After five consecutive microcentrifugations, the suspension was filtered through a syringe