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A non-invasive detection technique of adenocarcinoma with the use of streptavidin-coated iron nanoparticles and biotinylated-antibodies and the primary-secondary approach

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The streptavidin and biotin interaction is one of the strongest non-covalent interactions in nature. As a result, this non-covalent interaction has been of great interest when it comes to biochemical assays, diagnosis of diseases, and cell-targeted drug delivery. Past research has proven that biotin-streptavidin is useful in biosensor development to improve the detection of a system when conjugated to nanoparticles. This study aims to prove that streptavidin-coated nanoparticles can be conjugated with biotinylated antibodies using the primary-secondary method to non-invasively detect adenocarcinoma in-vitro. While the use of nanoparticles is not uncommon to the diagnostics area of scientific research, the technique this research aims to investigate is a non-invasive one, utilizing the primary-secondary method. Specifically, the increased stability of fluorophores when bound to antibodies as opposed to nanoparticles directly can be indicative of the particles conjugated through the primary-secondary method's ability to specifically bind to overexpressed transferrin receptors in the A549 cell line (1, 2). In this paper, streptavidin-coated nanoparticles were conjugated with biotinylated anti-transferrin receptor antibodies and AlexaFluor-488 secondary antibodies were used to enable fluorescence-based detection. The efficiency of these particles were observed quantitatively through a plate reader and qualitatively through a fluorescence microscope. I demonstrated that these nanoparticles are able to specifically bind to the target proteins in this study. These findings contribute to the field of nanoparticle diagnostics and can be extended to different diseases caused by overexpression of proteins in the future. While this was conducted in-vitro, conjugates can be prepared to detect cancer in-vivo and can be tested with magnetic relaxometry in the future.

Keywords: Iron-oxide nanoparticles | primary-secondary method | diagnostics of cancer | A549 cell line | transferrin protein receptors | adenocarcinoma | biotin-streptavidin) | biotinylated antibodies

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Introduction

Nanotechnology is ever-growing across a wide plethora of fields, ranging from applied physics to medicine to biochemistry, especially due to the great potential it holds for research. Natural interactions, such as the one with streptavidin and biotin, are a vital aspect of nanotechnology because it simplifies conjugation between nanoparticles and proteins of interest. The streptavidin-biotin interaction is one of the strongest non-covalent interactions in nature, having a dissociation constant in the femtomolar range (3, 4). Since each streptavidin monomer binds to one biotin molecule, one streptavidin protein can bind up to four biotin protein molecules . Using this interaction, past research has been done on using nanotechnology in drug delivery, vaccines, and other pharmaceutical agents. Nanoparticles have become promising candidates for non-invasive detection tools for cancers that overexpress a specific protein. For example, researchers have engineered particles to target HER2 protein receptors with a variety of conjugation methods (5, 6). Such methods include conjugation of nanoparticles with engineered antibodies, streptavidin with Phosphor Integrated Dot fluorescent particles (PID), and nanoparticles with single-chain variable fragment antibodies (7, 8). Our study focuses on demonstrating the efficiency of the primary-secondary method of nanoparticle detection in A549 cells. Because fluorophores are not directly attached to the nanoparticles and are instead conjugated with the primary antibodies, the impact of fluorescence moieties on nanoparticle stability is reduced.

Lung adenocarcinoma affects almost 230,000 Americans a year, killing around 135,000 annually (9). Of these, 15% die as a result of late diagnoses, with 5% getting diagnosed only after their death (10). Late diagnoses are often accounted for by a lack of awareness for the disease and, unfortunately, by a fear of detection methods, highlighting the need for nanoparticles which are non-invasive, unlike bronchoscopies. For these reasons, our study focuses on the A549 cell line to study the efficiency of our nanoparticles.

A549 is an adenocarcinoma lung cancer cell line of alveolar basal epithelial cells created in 1972. Based on past research, it was determined that lung adenocarcinoma is found to have an overexpression of the transferrin receptor(11). Papers have demonstrated that transferrin is a successful representative receptor-mediated endocytic marker when conduct-

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ing its intake by the adenocarcinoma human alveolar basal epithelial cells in the A549 cell line (12). Transferrin receptors (TfR) are glycoproteins expressed on the cytoplasmic membrane of cells. In healthy lung tissue, alveolar epithelial (A549 cells) and bronchial glands do not express TfR, so for preliminary stages of detection, the antibody-conjugated nanoparticles can be used to determine if a biopsy is required, based on whether they bind to the receptors or not (13, 14). The nanoparticles would be detected through Magnetic Resonance Imaging (MRI) because they are paramagnetic and show up as dark contrast on T2-weighted images. This non-invasive method of detection will encourage early screening because it is common for individuals to avoid necessary check-ups due to invasive and uncomfortable detection techniques.

This paper details our shortcomings with streptavidin-phycoerythrin (PE), a protein fusion commonly used for detecting biotinylated antibodies. The plate reader used in our study had a low enough limit of detection for it to be unable to provide enough signal to demonstrate success with our engineered particles. Because of our instrument's limitations, we turned to using antibodies coated with Alexa-Fluor 488 fluorophores, which are more sensitive than streptavidin PE with the hardware we used.

In this study, we demonstrate the advantage of using iron-oxide nanoparticles conjugated to antibodies in active targeting applications. We describe the fabrication of engineered iron nanoparticles coated with streptavidin and functionalized with anti-transferrin antibodies to target transferrin receptors in human lung adenocarcinomas. The conjugation of fluorophores to the primary antibody prevents the nanoparticle from being unstable, allowing for the particle to be reliable in-vivo. The use of fluorescence and plate reader methods showed the engineered molecule specifically binds to the receptors. These nanoparticles can act as imaging agents in vitro and in vivo in MRI imaging, therapeutic applications, and targeted drug delivery.

Methods and Materials

Cell culture. For the culturing of cells, a standard 1:5 splitting protocol for adherent cells was used (15). The A549 cells were cultured with Ham's F-12K Nutrient Mixture with 10% streptomycin and 1% penicillin. At the beginning of the project, cells were harvested in T75 flasks and then transferred to Eppendorf tubes for the labeling in solution. However, it was soon realized that trypsin, which was used to detach the adherent cells, could be cleaving off the transferrin receptors. For this reason, we switched to plating the cells in 96-well plates and allowing them to grow prior to performing experiments on adherent cells. A Cell Drop manufactured by DeNovix was used to count cells. All procedures involving cells were conducted in a sterile environment.

Nanoparticle-Primary Antibody Conjugation. The conjugation process we used relied on the involvement of streptavidin and biotin due to their extraordinary affinity for each other. The streptavidin-coated iron oxide nanoparticles (1 mg/mL) were conjugated with biotinylated human anti-rabbit anti-transferrin receptor primary antibodies (0.6 mg/mL). In order to calculate the number of nanoparticles per mL, we used the stock concentration, volume of each nanoparticle (1.41×10^{-23} nm^3), $and the density of Fe2O3 (5,300 mg/cm^3)$.

We found that the number of nanoparticles in 1 mL was 3.75×10^{26} after using dimensional analysis. Initially, the plate reader was characterized. To do this, we measured nanoparticles that had been diluted 10-fold and 100-fold from the stock solution.

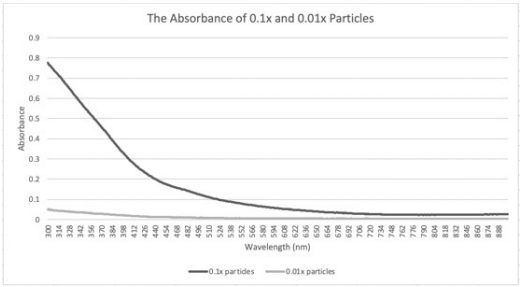

Fig. 1. In a 10-fold dilution, the nanoparticle signal is well within the linear range of the spectrometer. This solution had approximately3.75×10^{28} nanoparticles, while the 100-fold diluted solution had approximately 3.75×10^{29} nanoparticles. The absorbance is approaching zero in the 100-fold solution, suggesting that the plate reader is near its limit of detection. The results also demonstrate that the nanoparticles themselves have low sensitivity.

While testing the question with just these conjugates, there were two tubes: one with 100 µL of nanoparticles (negative control) and the other with 106 µL of NP-antibody conjugates; this conjugate solution had 100 µL of nanoparticles and 6 µL of antibodies, meaning there would be approximately 9 antibodies per nanoparticle (based on the nanomolar concentrations of each material $\rightarrow 3.3 \times 10^{-5}$ M for the antibodies: 2.2×10^{-5} M of the nanoparticles becomes a 1:1.5 ratio). 100 µL of cell solution was added to these tubes. There were approximately 4×10^{-6} cells in this solution, meaning there were approximately 4×10^{-12} transferrin receptors to be detected. After 15 minutes of interaction, these tubes were centrifuged for 10 minutes at 15,000 revolutions per minute (RPM). The supernatants of these tubes were then run under the plate reader.

Streptavidin PE. To test the conjugation of the particles, efficiency of the primary antibodies, and attachment of the particles to the receptors, Streptavidin Phycoerythrin (PE), which has an excitation wavelength of 488 nanometers (nm) and an emission wavelength of 525 nm, was used. The Streptavidin PE would act as a substitute for the nanoparticle and would determine if the streptavidin-biotin interaction works and whether the primary antibody is able to attach to the transferrin receptors. For this experiment, cells were first plated in a 96-well plate in order to avoid the chances of trypsin cleaving off the transferrin receptors. While labeling the cells, the standard primary-secondary approach was

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used and cells were harvested in a 96-well plate.

Once cells were washed with PBS three times, 4 µL of primary antibodies were added and allowed to incubate for 20 minutes at room temperature. The cells were washed again with PBS three times. Then, 6 µL of Streptavidin PE solution was added and was allowed to incubate for 30 minutes at room temperature. The 96-well plate was later inserted into a plate reader to measure the fluorescence signal in each sample. We used a black plastic plate with a transparent bottom layer to avoid fluorescence spillover between wells.

All washing steps were conducted to ensure that the signal received by the plate reader was solely due to the fact that the conjugated particles were able to attach to the receptors.

Secondary Antibody. Data from the Streptavidin PE experiment indicated that our plate reader was not optimized for measuring fluorescence signals from PE. Because of this we switched to AlexaFluor 488-conjugated goat anti-rabbit antibodies as the fluorescence profile of AF488 more closely matched the fluorescence filters available to us. The antibody has an excitation wavelength of 495 nm and an emission wavelength of 510 nm. For this assay, the primary-secondary approach was used and cells were harvested in 96-well plates.

Prior to the assay, the nanoparticles and primary antibodies were conjugated through centrifugation to facilitate the primary-secondary process. These conjugates were allowed to interact for 24 hours before they were conjugated to the secondary antibodies. Once cells were washed with PBS three times, approximately 104 µL of the nanoparticle-antibody conjugate solution was added and allowed to incubate for 20 minutes at room temperature. The cells were washed again with PBS three times. Then, 4 µL of secondary antibody solution was added and allowed to incubate for 30 minutes at room temperature. The 96-well plate was later inserted into a plate reader to get data.

Results

Streptavidin PE. The primary-secondary method should result in conjugated molecules bound specifically to the transferrin receptors in the A549 cells. As a result of the ideal outcome of the primary-secondary conjugation process, we expected the fluorescent emission signal to be highest for the wells with the Streptavidin PE-Antibody nanoparticle.

The lack of difference between signals received from the blanks and the streptavidin PE conjugate indicated that it had low sensitivity. To ensure that streptavidin PE without a probe does not stick to the cells, there was a negative control (Streptavidin PE with wash). All data was collected in triplicate and the graph demonstrates the average signal each of the groups emitted (Figure 3). The error bars are derived from the standard deviation values.

Using dimensional analysis, we were able to convert the results we got from the plate reader to the number of Streptavidin PEs per well. This data is depicted in Figure 4.

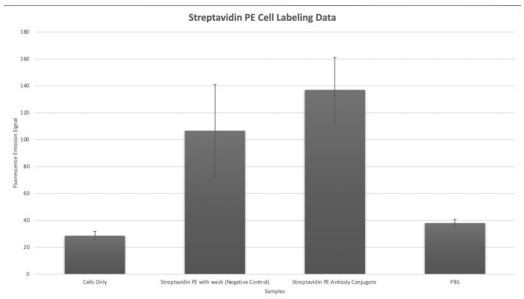

Fig. 2. Fluorescence Emission Signals of Streptavidin PE Samples. All data were collected in triplicate. While the data was suggestive, the measured signal for all samples was very low and the difference between the negative control and the positive sample was not statistically significant.

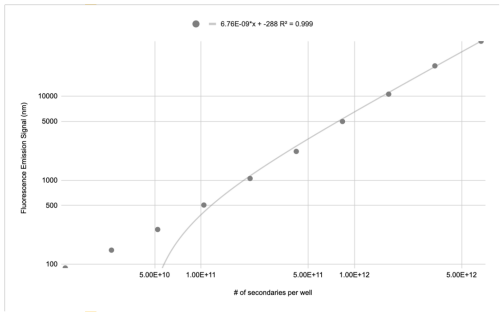

Fig. 3. Streptavidin PE Standard Curve data. Data were converted from the plate reader using dimensional analysis The dots represent a fitted line to the equation $(6.76 \times 10^{-9}$x - 288) which has an R-squared value of 0.999. The measured fluorescence is plotted with the solid line and begins to drop away from the fitted line at 1e11 secondary antibodies per well, suggesting a lower limit of detection.

Secondary Antibody. This experiment involved the conjugation of primary antibodies, secondary antibodies, and the nanoparticles, which were allowed to interact with the A549 cells through the primary-secondary method. We expected signals from the AlexaFluor488 antibodies to overall be stronger than the Streptavidin PE signals as a result of the filters available in our plate reader. Wells with the fully conjugated nanoparticle were expected to emit a higher fluorescent signal, as well.

Data demonstrated that the wells containing the NP-Ab conjugates and the secondary antibodies emitted significantly more signal than the blanks (Figure 5). To ensure that the secondary antibodies without a probe do not stick to the cells, there was a negative control (secondary antibody with wash). The same was done with the nanoparticle-secondary antibody wells to test whether they have properties that allow them to stick to the cells and to each other without a primary antibody.

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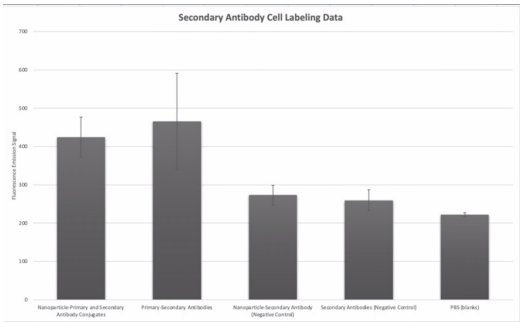

Fig. 4. Secondary Antibody data produced by the plate reader. All data were collected in triplicate. This graph demonstrates the average signal collected from each sample and the error bars are derived from the standard deviation.

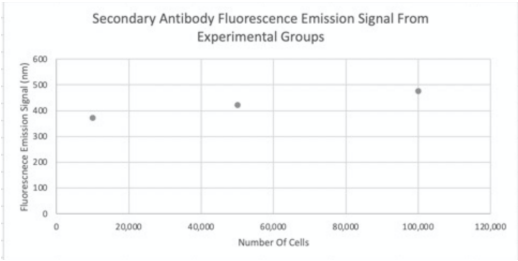

Fig. 5. Secondary antibody emission signal from experimental groups. This data shows the actual values of the fluorescence emission signals of the experimental group that contained different numbers of cells.

Discussion

Streptavidin PE Data Analysis. As previously mentioned, we observed that the streptavidin PE had low sensitivity, leading to only slight differences in signal observed between blank wells and wells containing the conjugates. A T-test was conducted with the Streptavidin PE Antibody Conjugate value and the Streptavidin PE with wash value. The p-value was 0.139, which was suggestive but not significant.

According to the equation derived from this standard curve (Figure 4), the wells with the Streptavidin PE-antibody conjugate had 6.29×10^{10} PEs detected, but this was very near the limit of detection. While this shows that the experiment could have worked, we could not make any concrete conclusions because the sensitivity of our plate reader to Streptavidin PE was too low.

Primary-Secondary Data Analysis. A 3x serial dilution was conducted to get the Secondary Antibody standard curve data (Figure 6). According to the standard curve equation, (x + 142)/(8.57×10^{-11}) (where x is the data received from the plate reader), the experimental well with 10,000 cells had approximately 6.01×10^{12} secondary antibodies, while the experimental well with 50,000 cells had approximately 6.59×10^{12} secondary antibodies, and the experimental well with 100,000 cells had approximately 7.22×10^{12} secondary antibodies. The fact that the number of secondary antibodies detected increased as the number of cells increased proves that the novel probe was able to accurately attach to the target proteins. Based on these results, an assumption can be made as to how many transferrin receptors were detected by the particles. If for every primary antibody there is one receptor and for every secondary, there is one primary, there would be the same number of receptors detected as the number of secondary antibodies detected. This would mean that 6.01×10^8 receptors were detected per cell in the experimental well with 10,000 cells, 1.318×10^8 receptors per cell in the experimental well with 50,000 cells, and 7.22×10^8 receptors per cell in the experimental well with 100,000 cells. The standard curve method provided this information, making it an ideal method when dealing with data from a plate reader. Data also proved that neither the nanoparticles nor secondary antibodies had their own sticking properties, meaning the signals received from these experimental groups were truly due to the fact that the particles were able to detect the transferrin receptors in the A549 lung cancer cells.

Data proves that the primary-secondary technique is the most efficient in detecting overexpressed transferrin receptors. Because there is a significant difference in signals between the blanks and control groups, reliable conclusions can be made based on the data.

To demonstrate that the data is statistically significant, a T-test was conducted using Microsoft Excel between two samples: the nanoparticle primary and secondary antibody conjugates value and the nanoparticle-secondary antibody (negative control) value. In this case we measured the fluorescence signal from triplicate samples that were exposed to either the conjugated nanoparticles and the secondary antibody or the secondary antibody alone. The p-value of 0.005 proves that the data is statistically significant and there was substantially higher fluorescence signal from the test sample, suggesting strong binding of the nanoparticle to the cells and relatively little background binding.

Conclusion

In this study, we examined the efficiency of the streptavidin-biotin interaction between nanoparticles and antibodies using the primary-secondary method for detecting the overexpression of transferrin receptors. In the end, results indicate that the nanoparticles were able to successfully and non-specifically bind to the receptors. Our results, which are statistically significant, support the use of the primary-secondary antibody method and the streptavidin-biotin interaction allowed iron-oxide nanoparticles to be conjugated with primary antibodies and specifically bind to transferrin receptors on the surface of cancer cells. Because this study was conducted solely in-vitro, future work regarding this research can be done in-vivo and using T2-weighted MRI. The nanoparticles could be used as contrast agents and can help determine their potential for clinical use (16, 17). Conducting this protocol in-vivo is critical to examine any potential negative effects of the nanoparticles and/or antibodies. This can further prove that nanoparticles can be applied clinically in the field of medicine and healthcare.

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Author Contributions The author of this paper conducted all assays and experiments individually.

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