Synthesis of fluorescent silica nanoparticles conjugated with RGD peptide for detection of invasive human breast cancer cells

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ABSTRACT

The objective of this research was to detect malignant human breast cancer cells using fluorescent Silica Nanoparticles (FSNPs) conjugated with RGD peptide that exhibits high affinity toward integrin receptors on cancer cells. The fluorescent silica nanoparticles (FSNPs) were synthesized by the Stöber method via controlled hydrolysis of tetraethylorthosilicate (TEOS) in a water/oil microemulsion in the presence of 3-aminopropyltriethoxysilane (APTS), fluorescein-5-isothiocyanate (FITC), and 3-(Trihydroxylsilyl)propylmethylphosphonate (THPMP). The FSNPs (70 nm in diameter) were checked for quality via TEM and fluorescence microscopy (515 nm), and confirmed to be consistent in shape and size. For tumor targeting, the FSNPs were conjugated to cyclo(Arg-Gly-Asp-D-Tyr-Cys) peptide (RGD) with the use of 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP) in DMSO. Both peptide-FSNP conjugates and FSNPs were added to MCF7 (benign breast cancer cells), MDA-MB 435 (transformed human breast cells), and MDA-MB 231 (metastasized breast cells) and after 2 hrs subjected to fluorescence microscopy. The FSNP-RGD peptide conjugates selectively got attached to the high concentration of integrins expressed on the surface of the cancer cells. In the case of the normal cells, the expression of integrin was low and hence such cells showed very few FSNPs on the cell surface. The results confirm that FSNP-RGD conjugates are excellent imaging tools for cancer detection. Since silica nanoparticles are inexpensive, readily synthesized, and relatively non-toxic, they afford a convenient method to identify malignant sights in cellular matrices.

Introduction

Functionalization of silica nanoparticles (SiNPs) is a simple, cheap and effective strategy for the preparation of multichromophoric detection systems. For example, dye-doped silica nanoparticles could be conveniently employed as cellular markers and tracked via their fluorescent properties. Compared to semiconductor nanocrystals (quantum dots such as CdTe QDs), SiNPs are far less toxic due to the lack of heavy metals. The non-cytotoxic properties along with their inherent dispersivity make SiNPs a superior material for microscopic imaging *in vivo*. Another advantage of SiNPs is the fact that their fluorescent properties can be altered with the judicious choice of different dyes. In this project, fluorescein was chosen as it is also a cheap and effective reagent for imaging (green fluorescence). Fluorescent Silica

Nanoparticles (FSNPs), are conveniently synthesized following the Stöber method which involves controlled hydrolysis of tri- and tetra-alkoxy silanes. In such synthesis, the dye can be incorporated within the SiNP by adding another silane in which the dye is covalently attached to the silicon center. The dye gets covalently attached to the Si-O-Si network and is tightly incorporated within the SiNPs (no leakage).

While FSNPs can be used for a variety of imaging purposes, in this project they were used for the detection of invasive human breast cancer cells. In such a pursuit, three human breast cancer cell lines namely, MCF-7, MDA-MB-231 and MDA-MB-435 (provided by UCSC drug screening laboratory) were selected. In these cancer cell lines, the extent of regulation of the integrin family of proteins (surface receptors responsible for metastasis) is known to vary significantly. In particular, the $\alpha_v\beta_3$ integrin protein is known to be overexpressed in the MDA-MB-231 and MDA-MB-435 cells, although the degree of expression does vary. This integrin is not present on the surface of MCF-7 cells (and hence can be used as a control). The major goal of this project was to conjugate FSNPs to an exogenous ligand that could distinguish the different levels of expression of this integrin. If the conjugate could specifically bind to the $\alpha_v\beta_3$ integrin, then one can both identify the cancer cells and determine the progression of metastasis.

Integrins are heterodimeric cell surface receptors [Figure 1] that mediate adhesion between cells and communication with the extracellular matrix.[1] They bind strongly to ligands that contain an exposed arginine-glycine-aspartate (RGD) moiety. These integrins are involved in cell growth, migration, and survival. The integrins, if not properly regulated can lead to thrombosis, inflammation and cancer. As stated before, integrins (such as the $\alpha_v\beta_3$ integrin) have been demonstrated to be present in high concentration on cancer cell surfaces (such as metastatic human breast cancer cells). Since integrins are overexpressed in malignant cells, various exogenous agents can be delivered to cancer cells via the strong integrin-RGD interaction. In this project, the high affinity of the overexpressed $\alpha_v\beta_3$ integrin (on cancer cell surface) for the RGD motif has been exploited for the detection of metastatic human breast cancer cells by conjugating the RGD peptide with the FSNPs. As described below, the RGD-decorated FSNPs indeed identified cancer cells via attachment to the cell surface due to strong RGD-$\alpha_v\beta_3$ integrin interaction. Since the integrin concentration is significantly lower on the surface of the benign MCF-7 cells, very few RGD-FSNPs were noted on such cells. The detection method is thus proven to be quite effective in identifying invasive human breast cancer cells.

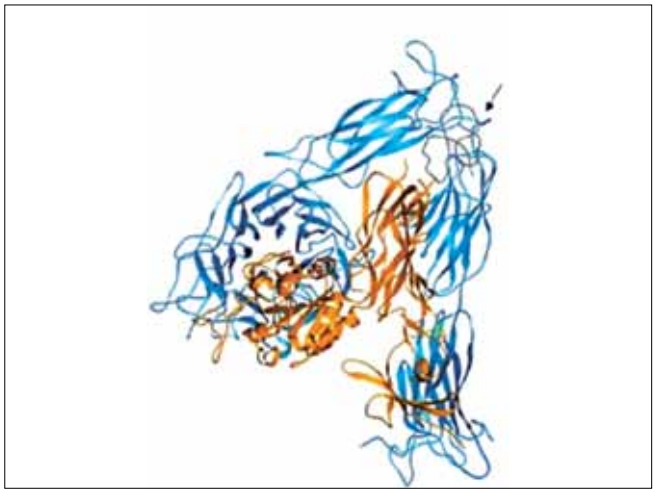

Figure 1: Structure of $\alpha_v\beta_3$ integrin showing the α_v units in blue and the β_3 units in orange

Investigative Questions

1) Are RGD-peptide conjugated fluorescent silica nanoparticles (RGD-FSNPs) stable and fluorescent over long periods of time? Are they uniform in size?

2) Can RGD-FSNPs be used to distinguish MDA-MB-231 (metastasized human breast cancer) and MDA-MB-435 (transformed human breast cancer) cells from benign MCF-7 breast cancer cells?

Materials and Methods

A. Preparation of the Fluorescent Silica Nanoparticles (FSNP)

The FSNPs were synthesized by the Stöber method[2] via controlled hydrolysis of tetraethylorthosilicate (TEOS) in a water/oil microemulsion in the presence of 3-aminopropyltriethoxysilane (APTS), fluorescein-5-isothiocyanate (FITC), and 3-(Trihydroxylsilyl) propylmethylphosphonate (THPMP). APTS was used in excess in order to decorate the surface of the FSNPs with -NH_2 groups (for further conjugation with RGD peptide). Since such -NH_2 groups will be present as -NH_3^+ in physiological buffers (pH 7.4) and reduce the ζ-potential (thus promoting coagulation of FSNPs), THPMP was also added to the reaction mixture. The THPMP makes negatively-

charged chemically inert methylphosphonate groups available on the FSNP surface thereby increasing the ζ-potential.

First, the FITC-APTS conjugate was synthesized by mixing 69 mg of APTS with 5.25 mg of FITC in 1 mL of absolute ethanol under dry N_2 atmosphere. The mixture was stirred for 24 h. During the synthesis, the FITC-APTS conjugate was protected from light to prevent photobleaching. This conjugate solution was used as the fluorescent silane reagent. A water-in-oil emulsion was prepared by mixing 7.7 mL of cyclohexane (oil), 1.77 gm of TX-100 (surfactant), 1.6 mL of n-hexanol (cosurfactant) and 0.34 mL of DI water in a 30 mL rb flask for 30 min. With an interval of 10 min between 2 successive additions, 50µl of FITC-APTS conjugate, 100µl TEOS and 100µl ammonium hydroxide was added. After 30 min of stirring, 15µl of THPMP was added and the mixture was stirring was continued at room temperature. After 24 h, the microemulsion system was destabilized by adding denatured ethanol (roughly 12 mL), and the FSNPs were collected by centrifugation (2000 RPM, 10 min). The FSNPs were then repeatedly washed and centrifuged 4 times with ethanol and 2 times with DI water. For redispersing FSNPs, each centrifugation step was followed by vortexing and sonication. The final FSNPs in DI water were stored in the dark to prevent photobleaching and later checked for quality by Tranmission Electron Microscopy (TEM) imaging.

B. Conjugation of the RGD peptide to the FSNPs

A batch of 25 mg of FSNP was dispersed in 1 mM Tris-citrate buffer (pH 7.4). Next, 25 mg of SPDP was dissolved in 0.5 mL of DMSO and the solution was added to the FSNP suspension. The mixture was then stirred at room temperature (under dark conditions) for 12 h. Next, the reaction mixture was spun down (2000 RPM, 10 min) to obtain the FSNP-SPDP conjugates as a fluorescent pellet. This pellet was resuspended in the same buffer and once again spun down (to remove any excess SPDP). The pellet was finally dispersed in 7 mL of tris-citrate (pH 7.4) buffer and to it was added a solution of 5 mg of cyclo(Arg-Gly-Asp-DTyr-Cys)[3] in 0.2 mL of DMSO. The mixture was stirred overnight at room temperature (under dark conditions). Next morning, the reaction mixture was centrifuged at 1700 RPM for 10 min to collect the RGD-FSNPs. They were finally resuspended in DI water and stored in the dark. A small batch of the peptide-conjugated nanoparticles was used to obtain TEM images [Figure 2]. Also, their fluorescence spectrum (Perkin Elmer Fluorescence Spectrometer) showed no variation in fluorescence characteristics [Figure 3] following RGD conjugation.[4]

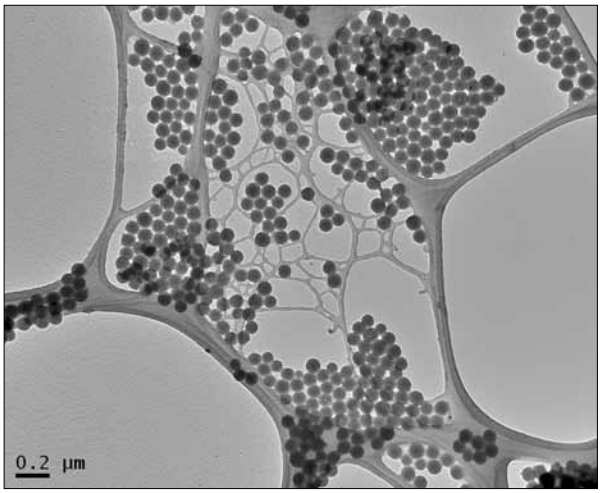

Figure 2: ITEM image of the FSNPs

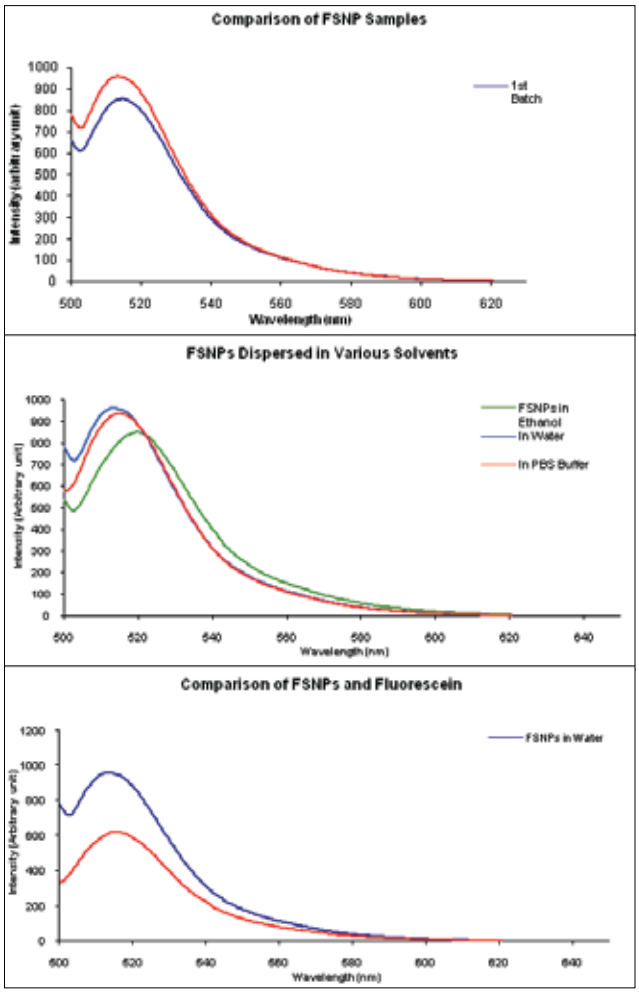

Figure 3: Fluorescence spectra of the various samples

C. Cell experiments

All three cell lines were placed in 8-well plates with 0.5 mL of DMEM growth medium and incubated in 5% CO_2 incubator for 72 h. After the cells grew to confluency (checked by visible-light microscopy), 0.25 mL of the medium was pipetted out from each well and 0.25 mL of the RGD-FSNP solution was added. The trays were placed back into the incubator for 2 h in order to allow the NPs to interact with the cells. Next, all the liquid was aspirated off and each well was carefully washed 2 times with 0.1x PBS buffer. The cells were then fixed with paraformalin fixer. After 15 min, the wells were again washed 2 times with the PBS buffer, 1 time with the quenching buffer (containing glycine) and 1 time with DI water. Next, the cells were stained with DAPI and the well covers were removed. Fluoro-Mount drops were added on each cell cluster and slide covers were placed on the slides. The trays were then allowed to dry for 45 min. Finally, the four sides of the slides were sealed with transparent nail polish. These slides were stored in the refrigerator. A parallel set of experiments was carried out with three trays of the same cell lines and FSNPs without RGD by following the same procedure. These served as controls for my experiment. The cells were visualized with the aid of a Zeiss HAL 100 fluorescent microscope.[5,6]

Results

A. TEM Images of the FSNPs

Dilute solutions of the FSNPs in ethanol were spread on TEM slides, dried under vacuum, and the TEM images were taken using a JEOL 1200 EX instrument (Dr. Yang of UCSC provided help in such measurements). The images [Figure 2] clearly showed that the particles were all spherical and of uniform (~ 70 nm) diameter.

B. Fluorescence spectra of FSNPs[7]

C. Fluorescence spectra and TEM images of the RGD-FSNPs

D. Results of cell experiments

The merged fluorescent images clearly showed increased NP interactions with MDA-MB-231 and MDA-MB-435 cells while MCF-7 cells exhibited minimal interactions. NP association with the cells was specific to each cluster on the slide with few NPs noted outside the cell-covered areas (showing no adhesion to the slide floors). It was also evident that there were greater NP interactions with the highly invasive MDA-MB-231 cells which are known to exhibit higher expression levels of $\alpha_v\beta_3$ integrin on their cell surfaces. With FSNPs without RGD, some interactions with the cell surfaces were noted. However, the FSNPs did not show specificity.

Conclusions

The results shown in Figure 4 clearly indicate that the RGD-FSNPs are stable in water and exhibit strong fluorescence. Experiments also showed that the intensity does not change over days of storage. Figure 5 confirms that the RGD-FSNPs can identify cancer cells on the basis of $\alpha_v\beta_3$ integrin concentrations on cell surfaces. For example, while MCF-7 cells with an inherently minimal concentration of $\alpha_v\beta_3$ integrins show very few RGD-FSNPs on their surfaces, MDA-MB-231 cells exhibit strong

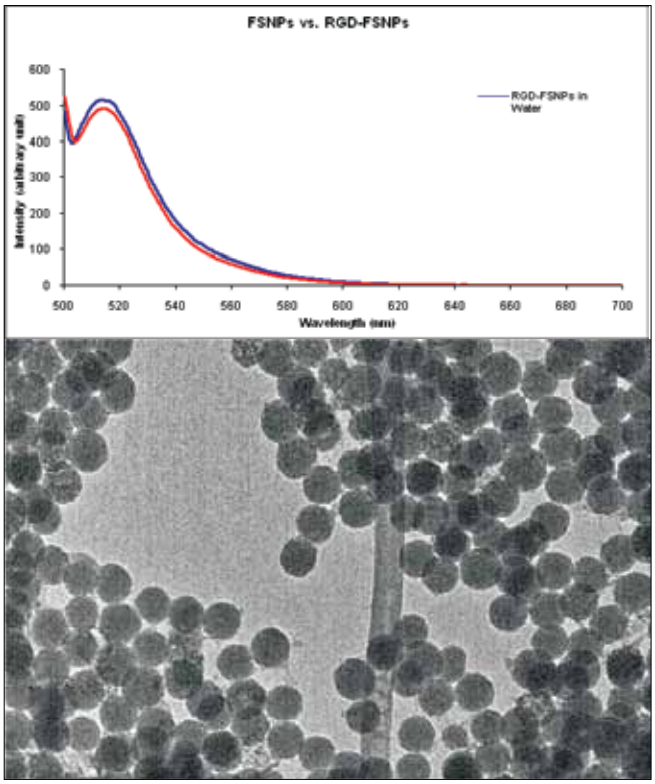

Figure 4: Fluorescence spectrum and TEM image of RGD-FSNPs

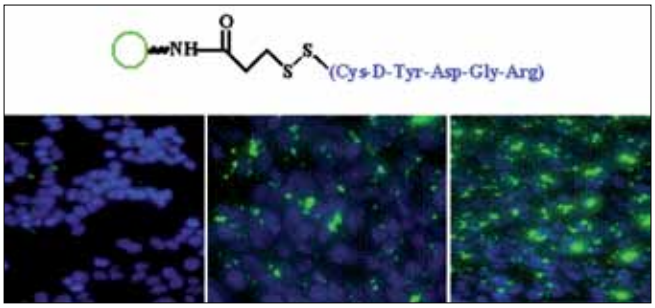

Figure 5: Images of the three cancer cell lines showing increased interactions with the RGD-FSNPs in the more invasive lines

association with RGD-FSNPs. It is therefore possible that RGD-FSNPs could be employed to distinguish between cancer cells with varying concentrations of $\alpha_v\beta_3$ integrin and hence their likelihood of metastasis.

Broader impact of this work

Breast cancer is the second leading cause of cancer deaths in women today (after lung cancer) and is the most common cancer among women.[8] According to the American Cancer Society, about 1.3 million women will be diagnosed with breast cancer annually worldwide and about 456,000 will die from the disease. Metastasis (to bone, liver and brain) is the primary cause of death in human breast cancer.[9] While the breast cancer rate has risen in the last 30 years in western countries, breast cancer deaths have been dropping steadily since 1990 thanks to better treatments and early detection. Clearly, there is a need for tools for early and effective detection of breast cancer.

The RGD-FSNPs in this project has been synthesized by using very simple chemical steps performed under mild conditions (room temperature, simple centrifugation). The synthetic steps employ no severely toxic materials (like H_2Te in CdTe quantum dot synthesis) and silica-based materials are known to have low cytotoxicity. Since the fluorescein dye is trapped within the silica NPs, no photobleaching is observed with these FSNPs. The synthetic protocol affords particles of uniform size and they are stable over months. This detection tool is therefore noteworthy for its easy preparation, sensitivity, and ability to distinguish human breast cancer cells with varying degrees of $\alpha_v\beta_3$ integrin regulation. The integrin has been implicated in the pathology of metastatic breast tumors. The detection system described here demonstrates its ability to distinguish between breast cancer cells of different kinds in terms of their invasiveness (metastatic tendency). Further research on this detection system could lead to a cheap, effective, and non-toxic method for *in vivo* imaging of invasive breast cancers.

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About the Author

Shamik Mascharak is 16 years old and lives in Santa Cruz, California. In his free time, he enjoys playing drums and guitar, as well as doing Tae Kwon Do (where he holds a second degree black belt). He also participates in a number of extracurricular activities such as Mock Trial and the local Kuumbwa Jazz Honor Band (where he is the drummer). Upon graduating, he wants to attend college and eventually become a researcher in a medical field.