Delivery of Shiga Toxin 1 A Subunit into Epithelial Cells Using Silica-Based Nanowires

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ABSTRACT

Silica-based nanowires were used to introduce the Shiga toxin 1 A subunit (StxA1) into bovine and human epithelial cells. We extended our previous technology, which employs fibronectin (Fn) as a ligand to induce integrin-mediated uptake of nanowires by coating nanowires with StxA1 and Fn. Without its counterpart, B subunits, the protein synthesis inhibitor StxA1, cannot enter eukaryotic cells without the aid of Fn-coated nanowires. The bonding strengths of StxA1and Fn to the surface of nanowires were investigated by X-ray photoelectron spectroscopy. This technique demonstrated some complex interactions between Fn, StxA1, and the nanowires. Neutral red cytotoxicity assays and field emission scanning electron microscopy demonstrated that the StxA1-Fn-nanowire complexes were effectively internalized and resulted in epithelial cell death. This study shows that nanowires can be used to carry StxA1 or potentially other toxic or therapeutic agents into eukaryotic cells. Ongoing studies are aimed toward specific cell targeting and increasing the functionality of the StxA1.

Keywords: silica nanowires, drug delivery, Shiga toxin 1, fibronectin

1 INTRODUCTION

There is a great deal of interest in developing biocompatible nanomaterials to deliver drugs or genes to cells for therapeutic purposes. Nanomaterials as drug carriers have merits over classical drug delivery systems due to their small size and large surface area. These characteristics may permit them to carry drugs across physiological barriers or into tumors with enhanced biocompatibility, solubility and controlled drug release [1, 2]. Properly functionalized nanomaterials, therefore, could potentially deliver drugs to target cells or organelles with maximum efficacy, reduced dose, and reduced nonspecific or systemic toxicity [1, 2].

We previously reported the use of fibronectin (Fn) as a molecular bridge to induce integrin-dependent internalization of nanowires (NWs) into bovine mammary epithelial cells (MAC-T) [3]. In the present study, NWs coated with Fn delivered recombinant A subunit of Shiga toxin type 1 (StxA1) into cultured human laryngeal epithelial cells (HEp-2) and MAC-T cells.

Shiga toxin type 1 is a classical AB_5 toxin, consisting of one StxA1 (toxic enzyme) and five B (receptor binding) subunits. StxA1 has N-glycosidase activity which renders the ribosome unable to interact with the aminoacyl-tRNA, thus inhibiting protein synthesis [4]. In this study we selected the recombinant StxA1 as a model cytotoxic agent because StxA1 is unable to enter cells in the absence of its counterpart B subunits.

2 MATERIALS AND METHODS

2.1 Fabrication of NWs

Silica NWs used in this study were grown on Si (100) substrates sputtered iron as a catalyst, in a Lindberg/Blue three-zone programmable tube furnace. High purity Si powder was placed in an alumina boat inside the tube. Growth of the NWs occurred at a temperature of 1130°C in the presence of an Argon flow of approximately 200 ml/min for a duration of 1 h. Oxygen for the growth reaction was supplied by ambient O_2 released from the porous alumina during growth. The lengths of the NWs were converted into shorter segments in the presence of HPLC grade methanol in order to enhance internalization.

2.2 Purification of recombinant StxA1

E. coli SY327(pSC25), harboring the cloned structural gene encoding StxA1, were harvested in the logarithmic phase of growth, periplasmic proteins were extracted by treatment with polymyxin B (50 µg/ml), precipitated by ammonium sulfate precipitation (80% saturation) on ice, dialyzed, and adsorbed to a Matrex Gel Green A agarose column (Amicon) equilibrated with 10 mM phosphate-buffered saline (PBS) (pH 7.4). The recombinant toxin subunit protein eluted as a single protein peak was dialyzed exhaustively against 10 mM PBS, and concentrations were ascertained using a Bio-Rad microassay.

2.3 X-ray Photoelectron Spectroscopy (XPS)

To investigate the interfacial chemistry between and among bonded Fn, StxA1, and NWs, XPS was performed in a vacuum chamber equipped with the Mg K_α emission line (1253 eV) and a hemispherical energy analyzer. During the XPS measurements, the NW samples were exposed to a

low energy (500 eV) electron beam using an electron flood gun to eliminate any spurious charging of the sample. If electron neutralization of the NWs was not performed, binding energy shifts of the core level states as large as 10 eV were observed. After initial spectra of non-coated NWs on Si substrate were acquired, wafers of NWs were incubated with StxA1 (300 ng/ml) and bovine Fn (100 nM) (Calbiochem) overnight at room temperature (RT). A wafer of NWs was coated with StxA1 and then Fn sequentially, and another wafer was treated with both simultaneously. Following addition of each ligand, NWs were washed with deionized (DI) water, dried, and subjected to XPS measurements.

2.4 Neutral Red Cytotoxicity Assay

NW suspensions in DI water were sterilized by autoclaving. To obtain NWs coated with StxA1 and Fn (NW-StxA1-Fn), NWs were processed in two steps. First, NWs were mixed with StxA1 in DI water (previously adjusted to pH 9.0 with 0.01 N NaOH), and incubated overnight at RT to generate NWs coated with StxA1 (NW-StxA1). After restoration of the NW-StxA1 suspension pH to 7.0 by adding DI water, the NW-StxA1 suspension was incubated with Fn overnight at RT. Analogous NW preparations coated only with StxA1 or Fn (NW-StxA1 and NW-Fn, respectively) were obtained by omitting the incubation steps with Fn or StxA1, respectively from the procedure described above. NWs processed through the coating procedure but with proteins omitted were prepared as controls.

For the cytotoxicity assay measured by neutral red, MAC-T and HEp-2 cells were treated by addition of NW-StxA1, NW-Fn, or NW-StxA1-Fn suspensions, and incubated at 37°C under 6% CO_2 for various lengths of time (24, 48, or 72 h). The NW:cell ratios used in this study were 2,000:1, 1,000:1, and 500:1, and the final concentrations of StxA1 and Fn were 300 ng/ml and 3 ng/ml, and 5 nM, respectively. Following incubation, the cells were stained with neutral red media (10 μg/ml of neutral red in DMEM medium) for 3 h, washed with fixative solution (1% $CaCl_2$ and 1% formaldehyde) and treated with 1% acetic acid in 50% ethanol solution. Absorption was measured at 540 nm using an ultra microplate reader, and OD values were presented as percentages of cell controls.

2.5 Field Emission Scanning Electron Microscopy (FESEM)

Cells were grown on Thermanox coverslips (Costar) in 24 well plates and incubated with NW-StxA1-Fn (300 nM StxA1 and 5 nM Fn) under the same regimen described for the cytotoxicity assay. After incubating for 48 h, dead cells detached from the plate bottom were collected, transferred to, and dried on, another clean Thermanox coverslip. These and the original coverslips as well as cell control coverslips were fixed and processed for FESEM [3].

3 RESULTS AND DISCUSSION

3.1 XPS Analysis

To acquire initial spectra of non-coated NWs, the Si 2p, O 1s, and C 1s core levels were examined [3]. The Si 2p core level of the NW consists of a predominant peak at 103.6 eV (spectrum (a), Fig. 1A), which corresponds to the Si^{4+} expected for SiO_2 [3]. The O 1s core level of the as-grown NWs is at a binding energy of 534.3 eV (spectrum (a), Fig. 1B). The C 1s core level of the NWs is at a binding energy of 285.5 eV (spectrum (a), Fig. 1C). The low signal-to-noise ratio of the C 1s core level is indicative of residual amounts of C on the surface of the NWs. Therefore, taken with the binding energy of the Si 2p core level of the NW, we conclude that the surface stoichiometry of the NWs used in this experiment is SiO_2.

Coating the NWs with StxA1 resulted in a 1.4 eV shift of the Si 2p core level to a higher binding energy (Fig. 1A). Subsequent coating with Fn resulted in a secondary shift of 0.5 eV to a final binding energy of 105.5 eV.

Coating the NWs with StxA1 resulted in no significant change in the binding energy of the O 1s core level of the NWs (Fig. 1B). Subsequent coating with Fn resulted in a single feature at a binding energy of 533.1 eV corresponding to the O of Fn.

The C 1s core level of the StxA1 coating on the NWs is at a binding energy of 286.9 eV (Fig. 1C). Spectrum c of Fig. 1C was de-convoluted into two features at binding energies of 286.6 eV and 289.5 eV, respectively.

The effects of coating the NWs with StxA1 are consistent with our previous results of coating the NWs with Fn in that the Si 2p core level shifted to higher binding energy while the O 1s core level remained unchanged. This result suggests that like Fn, StxA1 binds primarily to the Si sites on the NW surface.

The spectral results of coating the NWs simultaneously with Fn and StxA1 demonstrate some complex interactions between Fn, StxA1, and the NWs.

3.2 Cytotoxic Assay Analysis

Upon internalization of NWs functionalized with StxA1 and Fn into HEp-2 and MAC-T cells, the most effective killing activity was observed in HEp-2 cells (Fig. 2A and 2B). In the cytotoxic assay, higher NW:cell ratios caused more severe cytotoxicity implying that StxA1 can be carried by NWs in a dose-dependent manner. An initial ratio of 500:1 (NW:cell) was only weakly toxic, and it was subsequently determined that NW:cell ratios over 1,000:1 were required to deliver sufficient amounts of StxA1 to cause more severe effects on the whole HEp-2 cell population. A maximum reduction in the viability of HEp-2 cells (96.0%) was induced by treatment with NW-StxA1-Fn
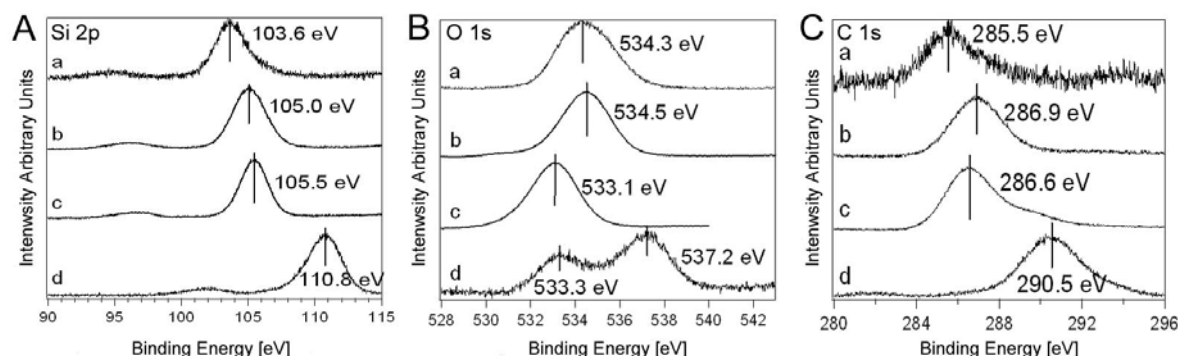
Figure 1. XPS spectra for NWs or NWs coated with StxA1 or StxA1 and Fn. (A) Si 2p spectra. (B) O 1s spectra. (C) C 1s spectra. Energy levels of (a) non-coated NWs, (b) NWs coated with StxA1 only, (c) NWs coated first with StxA1 and then with Fn, and (d) NWs coated simultaneously with Fn and StxA1.

(300 ng/ml StxA1) at a NW:cell ratio of 2,000:1. NW-StxA1-Fn coated with the low concentrations of StxA1 (3 ng/ml) were able to kill approximately 80% of the HEp-2 cells after 72 h, although the mortality rate was not as efficient as when higher concentrations of StxA1 (300 ng/ml) were employed. The cytotoxicity caused by NW-StxA1-Fn complexes was due to StxA1; most cells (>95%) treated with the NW or NW-Fn remained viable during the treatment periods (Fig. 2).

To observe the cell morphology during the cytotoxicity assay, photo images of the cells were visualized microscopically (data not shown). The visual change in HEp-2 cell viability coincided with results of neutral red cytotoxic assays. HEp-2 cells killed by NW-StxA1-Fn were easily rounded and could be differentiated from live cells.

In contrast to the high cytotoxicity of NW-StxA1-Fn in HEp-2 cells, the cytotoxicity in MAC-T cells was notably different. The cytotoxicity of StxA1 to MAC-T cells was not so high as to HEp-2 cells overall (Fig. 2C and 2D). MAC-T cells treated with non-coated NW or NW-Fn were not affected (Fig. 2C and 2D); however, the cytotoxicity observed among MAC-T cells treated with NW-StxA1-Fn was moderate and 44.2% of the cells remained alive with the highest concentration of treatment (Fig. 2C). In addition, cytotoxicity toward the MAC-T cells reached a plateau at 24 h and did not increase beyond this time in contrast to cytotoxicity kinetics observed with HEp-2 cells. This implies that only limited number of MAC-T cells was intoxicated by the NW-StxA1-Fn. It is suspected that the difference in cytotoxicity between the two types of cells was due to the hindered internalization of NW-StxA1-Fn complexes by endogenous Fn. Unlike HEp-2 cells, noted for lack of Fn expression, MAC-T cells produce endogenous Fn [5]. Fn is a multifunctional protein; small quantities of the protein stimulate the invasion of bacteria but higher concentrations reduce the internalization [5]. The endogenous Fn produced by MAC-T cells and NW-StxA1-Fn complexes likely competed with one another, and the saturation of integrins with soluble Fn presumptively hindered access of NW-StxA1-Fn complexes to the integrin

receptors. We previously reported the uptake of Fn-coated NWs by MAC-T cells with an enhanced efficacy relative to non-coated NWs [3]. It is clear that binding 5 nM of Fn to NW-StxA1 complexes can increase the internalization of the complexes into HEp-2 as well as MAC-T cells, by 1.78- and 1.32-fold, respectively. However, the fold differences and statistic analyses show that adding Fn is more effective for HEp-2 cells than for MAC-T cells.
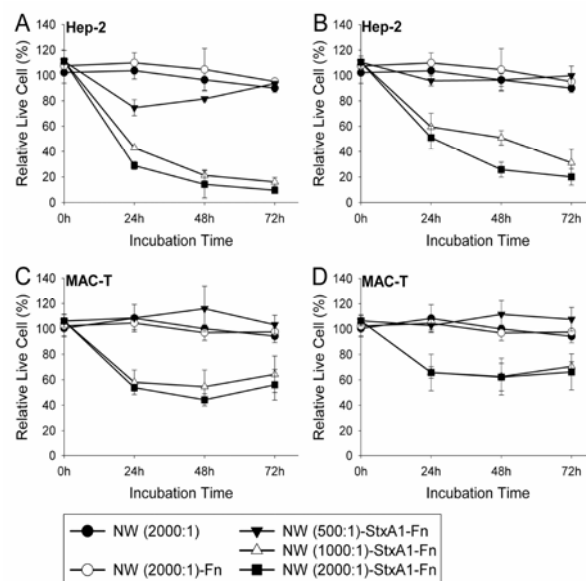

Figure 2. Cytotoxic effect of StxA1 delivered into HEp-2 and MAC-T cells by coated and non-coated NWs. NWs were tested at NW:cell ratios of 500:1, 1,000:1, and 2,000:1 with differing amounts of StxA1: 300 ng/ml (A and C) and 3 ng/ml (B and D). Except for non-coated controls, the NWs were also coated with 5 nM of Fn. The cells were incubated with the NWs for various times, followed by determination of neutral red uptake. Results shown are from a single representative experiment which was performed twice.
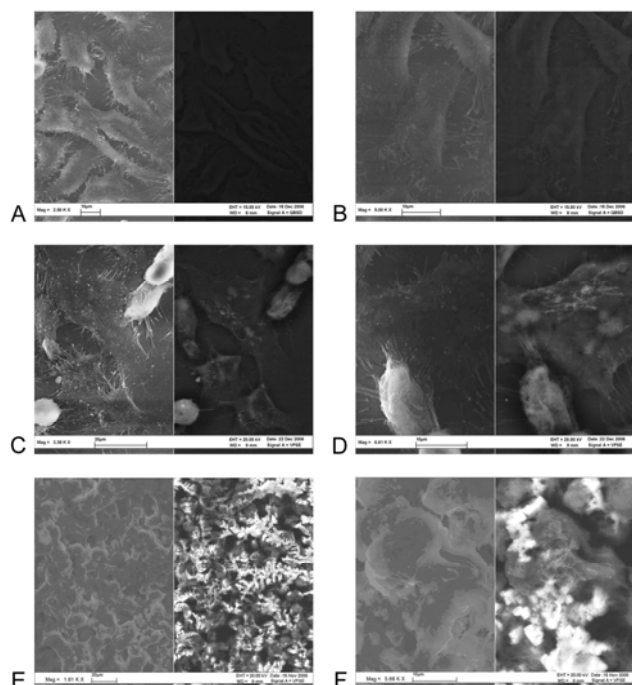
Figure 3. FESEM images of HEp-2 cells. Each image displays two imaging modes: SEI (left) and BEI (right). Cells treated with NW-StxA1-Fn (300 ng/ml StxA1 and 5 nM Fn) were processed at 48 h after treatment. The NW:cell ratio was 2,000:1. (A and B): HEp-2 cell control with low magnification (A) and high magnification (B). (C and D): HEp-2 cells on the original growth plate with low magnification (C) and high magnification (D). (E and F): Detached cells from the growth plate with low magnification (E) and high magnification (F). Detached cells from the growth plate were lrecovered during the processing steps for FESEM.

3.3   FESEM Analysis

FESEM analysis revealed that the morphology of the HEp-2 control cells were elongated and attached to the coverslips, while the cells treated with NW-StxA1-Fn were markedly different from the control (Fig. 3). Approximately 50% of the HEp-2 cells attached to the coverslips, and all the cells detached post-treatment with NW-StxA1-Fn were rounded (Fig. 3C, 3D, 3E and 3F). The rounding of cells in Figure 3C, 3D, 3E, and 3F was indicative of dead, dying, or apoptotoc cells.

Because the escape depth of secondary electrons is only on the order of 20 nm, the left section of each image only provides information on the surface and a depth of no more than 20 nm into the cells. The escape depth of the backscattered electrons is approximately 760 nm. Therefore the contrast between the two sections in each image are used to detect subsurface NWs apparent in backscattered electronic imaging (BEI) but not visible in secondary electronic imaging (SEI). The rounding of the cells due to

cell death causes an increase in the density of these cells and therefore they appear brighter than viable cells in the FESEM images. In some cases the brightening of dead cells was sufficient to hinder the detection of internalized NWs. However internalized NWs were apparent in all the cells treated with NW-StxA1-Fn in which this brightening effect was not sufficient to interfere with detection as show in Fig. 3D and 3F. The presence of internalized NWs in the cells post-treatment with NW-StxA1-Fn, taken with the cytotoxicity results, leads us to conclude that cell death is a direct result of StxA1 delivery.

4   CONCLUSION

This study indicates that NWs can be used to deliver cytotoxic agents into target cells efficiently. The potential uses of this technology include immunotherapy for various diseases such as cancer, autoimmunity, or infectious diseases. Ongoing studies include improved functionalization of nanowires aimed at increasing internalization efficiency and replacing Fn with various ligands that will promote internalization by specific target cells.

5   ACKNOWLEDGEMENT

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