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J Nanopart Res. Author manuscript; available in PMC 2014 December 01.

Published in final edited form as:
J Nanopart Res. 2013 December 1; 15(12): 2133–. doi:10.1007/s11051-013-2133-0.

Comparison of nanowire pellicles for plasma membrane enrichment: coating nanowires on cell

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Abstract

A study is reported on the effect of nanowire density on the ease of pellicle formation and the enrichment of plasma membrane proteins for analysis by mass spectrometry. An optimized synthesis is reported for iron silicate nanowires with a narrow size range of 900 ±400 nm in length and 200 nm diameter. The nanowires were coated with Al_2O_3 and used to form pellicles around suspended multiple myeloma cells, which acted as a model for cells recovered from tissue samples. Lighter alumina-coated silica nanowires were also synthesized (Kim et al. 2013), which allowed a comparison of the construction of the two pellicles and of the effect of nanowire density on plasma membrane enrichment. Evidence is offered that the dense nanowire pellicle does not crush or distort these mammalian cells. Finally, the pellicles were incorporated into a mass-spectrometry-based proteomic workflow to analyze transmembrane proteins in the plasma membrane. In contrast to a prior comparison of the effect of density with nanoparticles pellicles (Choksawangkarn et al. 2013), nanowire density was not found to significantly affect the enrichment of the plasma membrane. However, nanowires with a favorable aspect for pellicle formation are more easily and reliably produced with iron silicate than with silica. Additionally, the method for pellicle formation was optimized through the use of iron silicate nanowires (ISNW), which is crucial to the improvement of PM protein enrichment and analysis.

Keywords

Plasma membrane enrichment; nanowire pellicles; nanowire cell coating; silica nanowires; iron silicate nanowires

Introduction

Due to their important role in communication and interactions between cells and their environment, plasma membrane (PM) proteins rank high among protein targets for the development of novel drugs (Overington et al. 2006). The successful study of PM proteins is critical to conducting pharmaceutical research, developing disease diagnostics and elucidating fundamental biochemistry (Wu and Yates, 2003). The most commonly used methods take advantage of the hydrophobicity of PM proteins (Speers and Wu 2007), or their accessibility to external chemical or physical probes (Elschenbroich et al. 2010). Another method takes advantage of the higher density of plasma membrane components of

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the cell, and relies upon differential gradient centrifugation for separation and creation of an enriched sample (Blackler et al. 2008) A fourth approach, an extension of the differential density method, has used cationic silica beads to coat the cell surface, which, after crosslinking with an anionic polymer, results in a stable nanoparticle pellicle coating the plasma membrane (Choksawangkarn et al. 2013, Rahbar and Fenselau 2004, Rahbar and Fenselau 2005, Chaney and Jacobson 1983, Prior et al. 2011, Li et al. 2009). The resultant pellicle is large, robust and, most importantly, of a much higher density than the remaining cellular components. The increased density allows for better separation of the plasma membrane by centrifugation following cell lysis, therefore more efficient PM protein enrichment can be achieved (Choksawangkarn et al. 2013). The method was originally developed by Chaney and Jacobson (1983) and later introduced for mass spectrometry-based proteomic analysis by Rahbar and Fenselau (2004). Although PM protein enrichment can be estimated based on the percentage of the total PM proteins identified, enrichment of transmembrane proteins is considered to be the more reliable metric (Choksawangkarn et al. 2013, Speers and Wu 2007) because the assignment of transmembrane proteins is unique. Thus far transmembrane enrichment has been observed to be two to three-fold compared to whole cell lysate (Kim et al. 2013, Choksawangkarn et al. 2013). While attempts have been made to improve PM enrichment using pellicles, issues of particle internalization and thus contamination, as well as the destruction of the pellicle before separation, hinder wider application, as has been discussed by Choksawangkarn et al. (2013). Toward the goal of a rugged method to enrich the plasma membrane it is necessary to determine the optimum conditions for PM pellicle formation. Beyond spherical nanoparticles, nanowire structures are expected to form more dense pellicles due to key structural characteristics. Nanowires exhibit high aspect ratios which allow for multipoint contact with other wires, and also multipoint binding to, the cell.

Beyond spherical nanoparticles, nanowire structures are expected to form even more dense pellicles due to two key structural characteristics. Nanowires exhibit high aspect ratios which allow for multipoint contact with, and stronger binding to, the cell. By forming a stronger interaction with the cell surface, nanowires may allow for the elimination of the crosslinking procedure that is currently used to stabilize the nanoparticle pellicle prior to cell lysis. Additionally, their larger size inhibits cell internalization, which minimizes the chance of binding intracellular proteins and contaminating enriched PM proteins. We have recently reported for the first time the use of silica nanowires in the construction of pellicles and a fundamental investigation of nanowire interactions with the cell surface (Kim et al. 2013). In that work, we successfully demonstrated complete coating of eukaryotic cells with silica nanowires and systematically verified that the cationic (i.e. alumina-coated) nanowires form mechanically robust pellicles and exhibit minimal cell internalization.

Increasing the density of nanoparticles used in pellicles has been shown to provide improved enrichment of plasma membranes and their proteins (Choksawangkarn et al. 2013). In the present study we evaluate the hypothesis that, by analogy to nanoparticles, nanowire pellicles of higher density will provide enhanced enrichment. Here the use of iron silicate nanowires (ISNW) is evaluated to improve pellicle construction and PM isolation. By incorporating iron into the nanostructure we generate a denser, heavier nanowire than the silicate wires, which in turn may allow enhanced separation of pellicle-coated plasma membrane fragments from the rest of the cell during centrifugation. In this report the ease of lysis of pellicle-covered cells is investigated, as well as the size of the pellicle fragments formed by lysis under high pressure. Finally, the efficacy of these novel pellicles is evaluated in analyses by mass spectrometry of transmembrane proteins.

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Materials & Methods

Materials

Anodic aluminum oxide (AAO) templates (Anodisc 47) with an average pore diameter of ~200 nm and thickness of ~60um were purchased from Whatman GmbH (Dassel, Germany). Deionized (DI) water was supplied by a Milli-QA10 system (EMD Millipore Corp., MA). Iron (III) chloride (FeCl3•6H2O, 97.0%) was purchased from Alfa Aesar (Ward Hill, MA). Tetraethyl orthosilicate (TEOS, 99.999%), Penicillin-Streptomycin solution, 2-(N-morpholino)ethanesulfonic acid (MES), poly(acrylic)acid, NaCl, imidazole, protease inhibitor cocktail, D-sorbitol, Na2CO3, KCl, urea, glutaraldehyde, NH4HCO3, dithiothreitol (DTT), iodoacetamide, Tris-HCl, sodium dodecyl sulfate (SDS) and beta-mercaptoethanol were purchased from Sigma- Aldrich (St. Louis, MO). Optima LC/MS grade acetonitrile, formic acid, and trifluoroacetic acid, were purchased from Thermo Fisher Scientific (Pittsburgh, PA). The enzymes trypsin and endoproteinase Lys-C were purchased from Promega (Madison, WI). C18 TopTips were purchased from Glygen Corp (Columbia, MD). Human multiple myeloma cell line RPMI 8226 and cell culture medium RPMI 1640 were purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). RC DC protein assay kits were obtained from Bio-Rad (Hercules, CA).

Synthesis of nanowires

Silica and iron silicate nanowires were synthesized through a sol-gel evaporation process as described in our previous work (Kim et al. 2013). Briefly, for silica nanowires, 1 M TEOS solution was prepared by adding 2.08 g tetraethyl orthosilicate (TEOS) to 3.95 g ethanol, 1.8 g water, and 3 g of 0.2M HCl. The solution was hydrolyzed at 60 °C for 1 hr, followed by immersing commercial AAO templates into properly adjusted amounts of the solution and drying at room temperature for 6 hrs then at 120 °C for 24 hrs, sequentially. The synthesis of ISNW followed the same process using a modifying TEOS solution of 1.35 g $FeCl_3$ $6H_2O$, 1.04 g TEOS, 3.95 g ethanol, 1.86g H_2O, and 1.5 g of 0.2M HCl. After sol-gel synthesis, the AAO templates were dissolved in 0.1 M sodium hydroxide to release free-standing nanowires. After washing these 60μm nanowires several times with DI water, a sonicator equipped with a microtip (Q500, QSONICA, LLC., CT) was employed to cut the nanowires into shorter pieces.

To cover the surface with an alumina layer, the nanowires were dispersed in 0.01 M $Al(NO_3)_3$/0.1M $K(NO_3)$ solution for 24 hrs. After rinsing with water, the alumina-coated nanowires were dispersed in a PMCBA buffered solution (see below) for prompt use in cell coating experiments. The nanowires were characterized by using a Hitachi SU-70 Field Emission SEM (Hitachi High-Technologies America, Inc., Gaithersburg, MD) and a JEOL JEM-2100F Field Emission TEM (JEOL USA, Inc., Peabody, MA) operating at 200 kV with scanning TEM capability and Oxford energy dispersive x-ray spectrometry, and a Zetasizer Nano ZS90 particle analyzer (Malvern Instruments Ltd, Worcestershire WR14 1XZ, UK).

Cell growth, harvest and construction of the pellicle

RPMI 8226 human multiple myeloma cells were grown in RPMI 1640 media with bovine serum and prepared for coating as previously reported (Choksawangkarn et al. 2013, Rahbar and Fenselau 2004). Iron silicate and silica nanowire pellicles were constructed and evaluated in parallel to allow for a more accurate and direct comparison. Alumina coated iron silicate or silica nanowires were suspended in a coating buffer composed of 800 mM sorbitol, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 150 mM NaCl, pH 5.3, and washed cells introduced dropwise. The entire suspension of cells and wires was incubated at

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4°C for 15 minutes. All unbound wires were removed and samples washed, via centrifugation, in the fashion previously mentioned above. For cross-linking the coated cells were added dropwise to 10mg/mL poly(acrylic)acid in the coating buffer, and this suspension was incubated for 15 minutes at 4° C. Excess poly(acrylic)acid was removed and samples washed, via centrifugation, as previously discussed. Once coated and cross-linked, cells were lysed via nitrogen cavitation at 1800 psi for 30 minutes following a 30 minute incubation in lysis buffer (2.5 mM imidazole with a protease inhibitor cocktail). This process was repeated up to three times to encourage extensive lysis of the cells. Following lysis, the pellicle fragments were separated from the remainder of the cell portions via low speed centrifugation (100 xg for 7 minutes). The pellicle was washed three times with lysis buffer, 1 M Na_2CO_3, and 1 M KCl respectively. The pellicles were resuspended in 2% SDS, 62.5 mM Tris-HCl, and 5% β-mercaptoethanol and proteins extracted at 100°C for 5 minutes via a laboratory grade microwave (CEM Corporation, Matthews, NC). For samples not cross-linked, all procedures were the same with the subtraction of the poly(acrylic)acid crosslinking and associated washing steps. Protein concentration was determined with an RCDC protein assay kit.

Preparation of samples for scanning electron microscopy (SEM)

For each experiment seven samples were prepared for SEM: intact cells, nanowire-coated cells (silica coated and iron silicate coated), poly(acrylic)acid cross-linked cells (silica coated and cross-linked and iron silicate coated and cross-linked), and pellicle fragments as reported previously (Kim et al. 2013). SEM images were obtained using a Hitachi SU-70 Field Emission SEM.

Proteomic Analysis

Iron silica and silica samples were processed in parallel. Protein precipitation was performed using a chloroform/methanol method following the published procedure (Wessel and Flugge 1984). Following tryptic digestion, peptides were identified from tandem mass spectra obtained by LC-MS/MS using previously reported conditions on an LTQ-orbitrap (ThermoFischer, San Jose CA) (Kim et al. 2013, Choksawangkarn et al. 2013) and the PepArML program (Edwards et al. 2009) to search a reference set of human proteins from the UniProtKnowledgeBase (March 2012 version). Protein identifications required identification of two unique peptides identified with <10% false discovery rate. A global parsimony analysis was applied to ensure that at least two distinct peptides per proteins were not shared with other retained proteins. Subcellular localization of each protein was assigned by in-house software, which determines the location, including transmembrane, based on the information available from the UniProt Human Gene Ontology Annotation (GOA) (Ashburner et al. 2009).

Results and discussion

Successful application of nanowires to the surface of living cells (Fig. 1a) relies heavily on nanowire size, surface properties, and minimization of contamination by chemicals (*e.g.*, surfactants) used during synthesis. We have minimized the possibility of contamination by using the template-based sol-gel method, which involves minimal toxic chemicals and no surfactants. With this simple method, we were able to mass produce iron silicate nanowires (ISNW) measuring ~200 nm in diameter and 60 μm in length (Fig. 1b). In our previous work with silica nanowires (Kim et al. 2013) we demonstrated that nanowire length greatly affected cell surface coverage. Specifically, it was reported that shorter nanowires resulted in enhanced coverage of the curved cell surface. Therefore we sonicated the ISNW to generate 900 ± 400 nm length pieces (Fig. 1c). Fig. 1d depicts the relatively narrow size

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distribution of the ISNW compared to that of silica nanowires, which may be attributed to the weaker strength of the composite nanowire.

In order to achieve a strong, nonspecific electrostatic interaction with the negatively charged cell surface, the ISNW pieces were modified with an alumina layer. This was confirmed using energy dispersive X-ray spectroscopy (EDS). Zeta potential analysis of ISNWs in coating buffer before and after alumina coating reveal a dramatic shift from −35mV to +30mV, respectively, analogous to previous observations on alumina-coated silica nanowires (Kim et al. 2013).

These nanowires were then used to coat freshly harvested multiple myeloma cells. Approximately 6×10^7 cells (Fig. 2a) were added dropwise to ~5×10^{11} alumina-coated ISNWs suspended in the coating buffer solution, and incubated for 15 min at 4° C. It is clear from Fig. 2b that complete coverage of the cell with cationic ISNWs is achieved during the incubation period. A magnified image of the nanowire-coated cell (Fig. 2c) reveals that the nanowires are interwoven across the surface, forming a dense nanowire pellicle. By analogy to our previous work (Kim et al. 2013, Choksawangkarn et al. 2013) the cationic nanowire-coated cells were added dropwise to a polyacrylic acid solution in the coating buffer (pH 5.3), which generated a cross-linked pellicle around the cell. The resulting cells appear to have a denser nanowire coating compared to the non-cross-linked cells (Fig. 2d and e). However, cross-linking resulted in the aggregation of cells due to the strong networking of the anionic polymer coating.

Notably, the addition of the pellicle does not cause crush the plasma membrane or damage the cell, as seen in the optical micrograph in Fig. 2f. Even after 70 minutes the ISNW-coated cell membranes were clearly visible and circular. This is meaningful since the cell membrane must remain intact to allow for proper pellicle attachment and selective isolation. The internalization of individual nanowires into cells can also degrade the selective isolation of PM proteins. However, it was confirmed in the previous study that cationic alumina-coated nanowires are rarely internalized by the cells (Kim et al. 2013).

A major objective of this work was to identify aspects of the process of pellicle formation that can be optimized to improve PM protein enrichment. A primary concern is the extent of cell lysis, since lysis is required for centrifugal isolation of pieces of the pellicle/plasma membrane, and unlysed cells limit both sensitivity and selectivity. It was previously determined that lysis of cells with silica nanowire pellicles proceeds well at 1800 psi (Kim et al. 2013) therefore this pressure was used throughout the present study. Under this condition the degree of lysis of cells coated with cross-linked ISNW pellicles was comparable to that of cells coated with cross-linked silica nanowires (Fig. 3a). Given that the high aspect ratio of the nanowires allows for multipoint contact, and thus greater interaction, with the cell surface, the stability of the ISNW pellicle was studied in the absence of cross-linking. When ISNW pellicles were formed without cross-linking, cells were less resistant to lysis and significantly smaller membrane fragments were recovered (Fig. 3b–e). Therefore cross-linking generates a tougher pellicle that is less likely to fragment. In order to determine if the size of the fragments has any effect on the enrichment, transmembrane protein enrichment was analyzed from cells with ISNW pellicles that were both cross-linked and not cross-linked. Samples enriched with cross-linked and not cross-linked silica nanowires were also analyzed in order to compare the effect of density.

In order to evaluate protein enrichment using the four isolated pellicle samples, proteins were processed in parallel: solubilized from the fragments, digested with Lys-C and trypsin, and analyzed by LC/MS/MS. Proteins were also analyzed from whole cell lysate as a control. Plasma membrane enrichment was determined based on the number of

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transmembrane proteins identified. Table 1 indicates that transmembrane proteins are enriched approximately two-fold in all four nanowire-coated samples compared to whole cell lysate. Enrichment is 10 % higher in the two cross-linked samples than in the corresponding samples whose pellicles were not cross-linked. Within the ISNW pair and the silica nanowire pair the cross-linked samples produced more total protein identifications, perhaps because larger fragments are more readily pelleted by centrifugation.

The hypothesis for this effort to improve sample preparation for analysis by mass spectrometry was that increasing the density of the nanowire pellicle would improve enrichment of the plasma membrane, as it has for nanoparticle pellicles (Choksawangkarn et al. 2013). Table 1 indicates that enrichments are similar, though the heavier pellicle can be said to trend higher. The Venn diagram in Fig. 4 shows that 78 % of the total proteins identified in the smaller cross-linked silica list overlap with proteins identified in the larger ISNW list, while overlap of transmembrane proteins increases to 88%, consistent with enrichment of the plasma membrane by both pellicles. This trend suggests the nanowire pellicle is indeed enriching for the plasma membrane proteome, as reproducibility between wire types is higher in the protein category considered to be a more reliable representation of enrichment values (transmembrane proteins).

Conclusions

A surfactant-free synthesis has been optimized for iron silicate nanowires that form interwoven pellicles around the outside of human cells. This dense pellicle does not crush the cell, and allows enrichment of the plasma membrane by differential centrifugation, compared to whole cell lysate. Contrary to expectation, the heavier composite pellicle provided enrichment only slightly greater than that provided by the lighter silica nanowire pellicle. However, the novel ISNW offer other advantages. Notably, it is easier to form nanowires from the composite with lengths in the range for strong pellicle formation. Since nanowires are rarely endocytosed, the collection of intracellular proteins by ISNW pellicles is minimized and selectivity is improved compared to nanoparticle pellicles. In addition, advantageous magnetic isolation of plasma membrane fragments has been reported using spherical nanoparticles of similar “magnetite” composition (Zhang et al. 2011, Li et al. 2009) and should also be applicable to plasma membrane fragments coated with iron silica nanowires..

Acknowledgments

The authors thank Dr. Yan Wang, Director of Proteomic Core Facility, Maryland Pathogen Research Institute, University of Maryland, College Park, for advice on the proteomic analyses; Dr. Nathan Edwards, Department of Biochemistry and Molecular & Cellular Biology, Georgetown University, for supporting our bioinformatics; Tim Maugel, Director of Laboratory for Biological Ultrastructure, University of Maryland, College Park for assisting with SEM processing. We acknowledge the NispLab of the Maryland NanoCenter, supported in part by the National Science Foundation as an MRSEC Shared Experimental Facility. The research reported here was supported by a grant from the National Institutes of Health, GM 021248. WC acknowledges a Royal Thai Government Fellowship.

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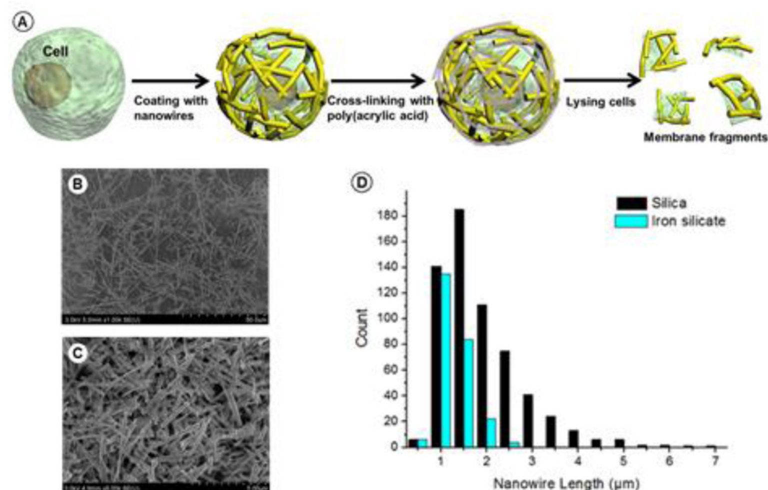

Fig. 1.

a) Schematic illustration of the coating process of cells with positively charged nanowires. Multiple myeloma cells with a negatively charged surface are incubated with positively charged nanowires in order to coat cell surfaces with nanowires through nonspecific electrostatic interaction. The nanowires on the cell are cross-linked to give a dense and stable pellicle. The cells are finally lysed under high pressure. Adapted from reference 1. b) SEM image of ISNW after dissolving AAO templates and c) SEM image of ISNW after cutting the nanowires by sonication (900 ± 400 nm). d) Length distribution of iron silicate and silica nanowires after cutting. The scale bars in B, C are 5 μm.

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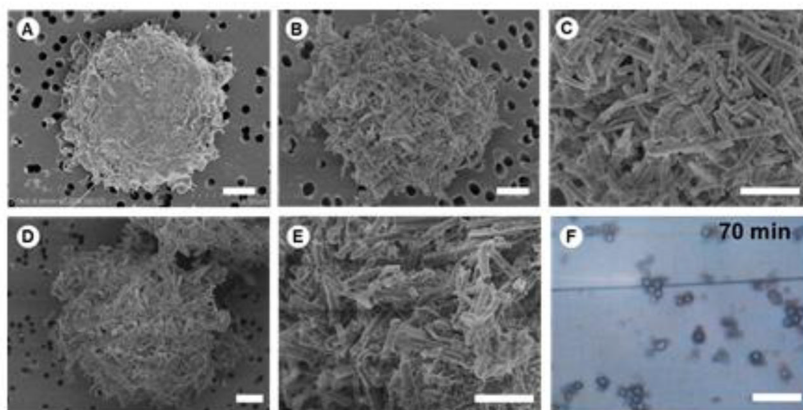

Fig. 2.

SEM images of multiple myeloma cells. a) intact cell, b) cell coated with alumina-coated iron silicate nanowires, c) magnified image of the iron silicate NWs-coated cell, d) iron silicate nanowires-coated cell after crosslinking with PAA, e) magnified SEM image of the cell surface from the sample in Fig. 2d, and f) Optical microscope images of multiple myeloma cells aged for 70 min after coating with iron silicate nanowires. ISNW are shown as fuzzy spots around cells due to their refractive index. The scale bars are 2 μm for a, b, c, d, and e, and 50 μm for f.

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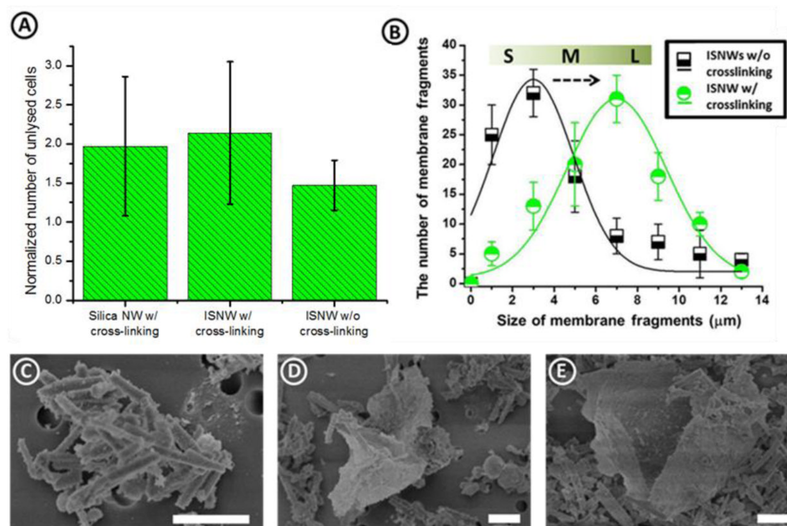

Fig. 3.

a) Normalized number of unlysed cells after lysing silica nanowire-coated cells and iron silicate nanowire-coated cells under 1800 psi. The normalized values were determined by averaging the number of unlysed cells identified in 8 different 100 μm × 100 μm areas as reported in (1). b) Size distribution of membrane fragments after lysing iron silicate NWs-coated cells under 1800 psi. c, d and e) Representative SEM images for membrane fragments with different sizes in order of small (S), medium (M) and large (L). The scale bar is 2 m.

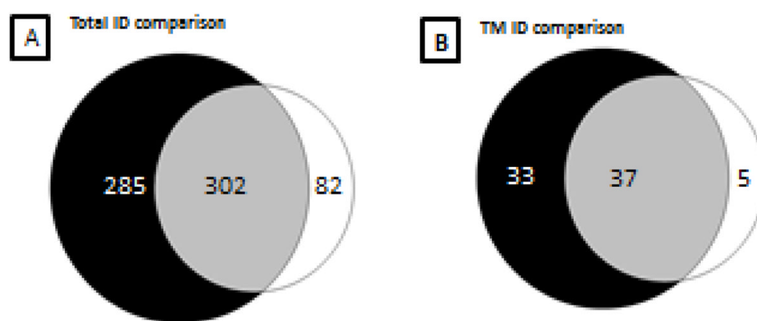

Fig. 4.
Comparison of (a) total proteins identified and (b) the transmembrane (TM) proteins identified. Pellicles constructed using cross-linked iron silicate nanowires are indicated by black circles, while white indicates cross-linked silica nanowire pellicle construction.

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Table 1

Transmembrane proteins enriched by iron silicate and silica nanowire pellicles

Pellicle construction method	Total proteins with GO annotation	% Transmembrane proteins (std. dev)*
Iron silicate NWs with cross-linking	587	11.9 (0.6)
Iron silicate NWs without cross-linking	322	10.8(0.7)
Silica NWs with cross-linking	384	11.0 (0.7)
Silica NWs without cross-linking	244	10.1(0.6)
Whole cell lysate	589	5.5 (0.7)