Silicon nanoneedles for drug delivery

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Abstract: Silicon nanoneedles are emerging as a strategy to negotiate the cell membrane and deliver drugs intracellularly. This chapter discusses the different strategies for silicon nanoneedle fabrication, drug loading, cell interfacing and drug delivery. The chapter also overviews four exemplary systems for nanoneedle-mediated intracellular drug delivery.

Key words: drug delivery, intracellular delivery, microfabrication, nanoneedles, nanowires.

8.1 Introduction

The efficiency of a drug delivery method, in general, depends on the internalisation of the drug being administered. In eukaryotic cells, there is physical segregation between the interior of the cell and the external environment by the plasma membrane, a structure composed of a phospholipid bilayer with embedded proteins that prevents unchecked influx and efflux of solutes from cells. Thus membrane permeability is one of the main constraints for the delivery of drugs with intracellular targets. Very few molecules that yield to specific parameters regarding molecular size, net charge and polarity are able to cross the plasma membrane by passive diffusion (Fischer et al., 2005). Indeed, and for good evolutionary reasons, the cell tightly controls the flux of the great majority of molecules (including biomolecules), preventing their unaided internalisation (Fischer et al., 2001). Because of these limitations, the vast majority of drugs with intracellular targets that are currently available on the market fall within the small molecule category, which are fairly permeable to the cell membrane, and can be internalised simply by imposing a concentration gradient across the cell membrane (Di et al., 2012; Sugano et al., 2010).

Recently, the efficacy of the use of antibodies for oncotherapy (Weiner et al., 2010; Adams and Weiner, 2005) and the potential of short interfering RNA (siRNA) in modulation of gene expression (Dykxhoorn et al., 2003) highlighted the vastly superior therapeutic potential of biologicals and renewed interest for delivery strategies that efficiently negotiate the plasmalemma. Biologicals are large and charged molecules that cannot negotiate the plasma membrane, requiring appropriate delivery strategies. The impact of biologicals in therapy is expected to
be revolutionary. Developing efficient and universal strategies to negotiate the cell membrane could benefit both currently available small molecule drugs, allowing use of lower dosages, and molecules with promising molecular interactions but which are currently unable to reach the cell at therapeutic concentrations.

Several methods of molecular transfer across the membrane into living cells have been developed, each with different characteristics in terms of cell viability and transfer efficiency (Stephens and Pepperkok, 2001). The methods can be broadly categorised into biochemical and physical delivery strategies.

8.1.1 Biochemical delivery strategies

Biochemical delivery strategies exploit cellular uptake and transport mechanisms to reach the intracellular environment and deliver their cargo. Cellular uptake results from chemical modification of the molecule’s surface or through encapsulation within a carrier that can act as mediator of transport (Chou et al., 2011). In the former, cell-permeable molecules are coupled to cell-impermeable drugs to shuttle them across the plasma membrane. Several modulators of cell permeability exist, some of which are commercially available (Stephens and Pepperkok, 2001). The broad category of cell-penetrating peptides (CPP) is extremely versatile and widely used to successfully translocate biomolecules, including plasmid DNA, oligonucleotides, siRNA, peptide nucleic acids, proteins and peptides, liposomes and nanoparticles both in vitro and in vivo (Morris et al., 2008). CPP are water-soluble, partly hydrophobic and/or rich in basic residues (Madani et al., 2011) consisting of approximately 10–30 amino acids (Fischer et al., 2005) that bind to their cargo either covalently or non-covalently (Morris et al., 2008). Over 30 CPP have been identified so far and their biological and biophysical characteristics are very different (Fischer et al., 2005) suggesting that, many routes of internalisation may exist. Although still debatable, endocytosis and direct penetration have been appointed as the two main routes of uptake (Madani et al., 2011). Although CPP are broadly employed to enhance cell permeability, there are restrictions to the size of the molecules they can transfer, and transporting proteins almost always causes their unfolding (Stephens and Pepperkok, 2001).

Liposome encapsulation is an alternative biochemical approach to membrane translocation. Particularly their cationic formulations, which overcome some limitations of CPP, can introduce with high efficiency a variety of molecules (e.g. DNA, RNA, proteins, etc.) inside cells. Liposomes can vary in size and morphology (Balazs and Godbey, 2011), enabling the encapsulation of molecules and even nanoparticles without significant size restrictions (Stephens and Pepperkok, 2001). Moreover, the positive charge of cationic liposomes favours interaction with negatively charged backbones. As the plasma membrane is also negatively charged, interaction between the liposome and the plasma
membrane enhance delivery. The most accepted concept is that cationic liposomes enter cells through adsorptive endocytosis and fusion with endosomal membranes then occurs, leading to cargo release (Sharma and Sharma, 1997). Thus, this internalisation pathway avoids lysosomal degradation. Liposome fusion might, however, interfere with lipid metabolism (Stephens and Pepperkok, 2001). Other formulations of liposomes are not as efficient for delivery of biomolecules: neutral liposomes have limited interaction with cells while anionic liposomes are electrostatically repelled by the negatively charged cell membrane (Balazs and Godbey, 2011). However, when internalisation through endocytosis occurs, a liposome can be delivered by the endosome into the lysosome or, in the case of pH-sensitive liposomes, it can induce endosome destabilisation, which results in drug delivery into the cytoplasm (Torchilin, 2005).

An alternative biochemical strategy employs viruses as drug carriers. Viruses can encapsulate genetic material by self-assembly of their coat proteins into a capsid after recombinant expression. This unique characteristic has been exploited to develop drug delivery systems, which are generally named virus-like particles (VLP). The genome of a virus is enclosed in the capsid, stabilised by electrostatic interaction with the basic polypeptide domains of the coat proteins. In a similar fashion, suitably charged moieties can be entrapped within VLP (Garcea and Gissmann, 2004). Obvious limitations are then the charge of the cargo to be encapsulated and its size. Although most of the VLP being developed for human application are typically not human pathogens (Manchester and Singh, 2006), they still can potentially exhibit pathogenicity.

8.1.2 Physical delivery strategies

Physical methods of delivery comprise direct transfer methods, where there is direct access to the intracellular compartment, or plasma membrane permeabilisation methods, which uses detergents, UV light, pore-forming toxins or electrical pulses to enhance the permeability of the plasma membrane and therefore introduce the cargo by passive diffusion (Stephens and Pepperkok, 2001). Among permeabilisation methods, electroporation is the most widely adopted. Electroporation involves transient increase in the plasma membrane permeability resulting from application of an external electric field (Neumann et al., 1982). Briefly, electroporation uses short high-voltage pulses that just surpass the capacitance of the plasma membrane, thus creating transient pores. This reversible state permits the diffusion of small molecules or electrophoretic transfection of other molecules (Gehl, 2003), including DNA, proteins, etc. Besides being able to address a large range of compounds to be internalised, it is available to a wide range of cell types. It has proven beneficial both in vitro and in vivo (Gehl, 2003) and exhibits high efficiency when cells are in suspension but requires specialised equipment and may lead to cell death as a result of the
electrical pulse (Chou et al., 2011; Stephens and Pepperkok, 2001). Moreover, when used in vivo, it may cause decreased blood flow in the pulsed areas (Gehl, 2003).

Direct access to the intracellular compartment is advantageous and results in most cases in high efficiency of transfection. Microinjection is a conceptually simple technique where a glass micropipette with a tip of less than 0.5 μm injects the sample into the cell with the guidance of an optical microscope. Because it is a direct approach, it can reach high transfer efficiencies and survival rates (Celis, 1984). Furthermore, it allows co-injection of several distinct compounds and has few restrictions in terms of cell or payload type. However, because of its sequential nature, it possesses low throughput, is technically challenging and expensive (Stephens and Pepperkok, 2001). Microinjection’s unmet potential for universal, low-toxicity intracellular delivery of molecules has stimulated further research into direct injection strategies. Nanoneedles conceptually originate as a miniaturisation of microneedles. They enhance microneedles’ superior ability for localised, painless, minimally invasive drug delivery. Further, nanoneedles grant direct physical access to the cytosol with minimal disruption to the activity of cells, while they enable interaction with cellular components at the nanoscale.

8.1.3 Nanoneedle platforms

Nanoneedles are broadly definable as high aspect ratio structures with diameter at the nanoscale. The basic requirements for nanoneedles are similar to, and to some extent less stringent than, those for vertically aligned nanowires. Hence established techniques for nanowires can generate nanoneedles. Alongside the approaches adopted from the semiconductor industry, specific fabrication techniques are emerging that facilitate engineering of application-specific nanoneedles.

8.2 Strategies for nanoneedle fabrication

8.2.1 Vapour–liquid–solid (VLS) growth of nanoneedles

Vapour–liquid–solid (VLS) growth of silicon is established and largely employed in the synthesis of vertically aligned nanowires (Levitt, 1970) (Fig. 8.1(a)). In VLS, metal nanoparticles (usually Au) are heated above the eutectic temperature for the metal-silicon system in the presence of a silicon gas source (usually SiH₄ or SiCl₄). In such conditions a liquid nanodrop of metal–silicon alloy forms. The gas feeding the eutectic nanodrop supersaturates it with silicon, inducing the nucleation of solid Si. Once formed, the solid–liquid interface acts as growth interface catalysing continued incorporation of Si into the newly formed lattice. The metal nanoparticle rides the tip of the nanowire while it forms. This synthetic process can form straight cylindrical nanowires of constant diameter with lengths of hundreds of μm. The nanowire can grow either through incorporation of silicon
8.1 Fabrication strategies for nanoneedles. (a) In vapour–liquid–solid (VLS) synthesis, Si is incorporated from the vapour phase into a liquid eutectic Au-Si alloy nanodroplet. When the concentration of Si overcomes saturation for the eutectic, the Si nucleates in solid phase. This process is sustained as long as gaseous silicon is provided, forming silicon nanowires (pictured below). (b) In metal-assisted chemical etch (MACE), a noble metal mesh deposited on top of silicon catalyses the etching of silicon nanowires in an oxidizing solution of HF. Controlling the etching parameters results in solid or porous nanowires (pictured below). (c) Focused ion beam (FIB) can shape an already existing atomic force microscope (AFM) tip into a nanoneedle by milling the excess material (pictured below). (d) Microfabrication allows forming hollow nanoneedles (pictured below) by first etching deep nanopores into a Si membrane, conformably coating them in dielectric and then etching the top surface of the dielectric alongside the selective etch of silicon. Images reproduced with permission from Shalek et al., 2010, Chiappini et al., 2010, Han et al., 2005b and Peer et al., 2012 respectively. (All scale bars = 1 µm.)

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at the interface between the droplet and the wire (liquid/solid interface), or between the gas and the wire (vapour/solid interface) (Lu and Lieber, 2006). Incorporation at the liquid/solid interface results in nanowire elongation, whereas incorporation at the vapour/solid interface results in its thickening. Which mechanism dominates during the growth process is determined by the conditions during synthesis. Pressure, gas flow rate, temperature and the nature of the reacting species and their gaseous byproducts all influence the growth (Kolasinski, 2006). With silanes, low temperatures favour nanowire elongation, reducing the thermal dissociation of the gas. Addition of hydrogen gas also promotes elongation either by passivating the nanowire surface through hydrogen termination (Wu et al., 2004), or by preventing the dissociation of silane (Greytak et al., 2004; Wang et al., 2003). Careful control of the synthesis conditions allows the formation of uniform nanowires without diameter variation (see Chapter 2 for additional details).

VLS combined with lithography allows tight control over nanoneedle diameter (down to a few nanometres), their density and their arrangement over the carrier substrate (Fan et al., 2006). Variants of the basic chemical vapour deposition (CVD) VLS principle generate the precursor gaseous Si by laser ablation (Morales, 1998), plasma generation (Hofmann et al., 2003), and molecular beam epitaxy (Liu et al., 1999). Other strategies employ Si precursor in solution (Heitsch et al., 2008; Holmes, 2000) or in solid (Wong et al., 2005) form. These alternatives were developed to address specific requirements such as growth temperature, integration, uniformity and doping, but share the same underlying formation/growth principle of VLS. Overall, VLS and associated techniques are extremely powerful tools to synthesize solid silicon nanowires. VLS integration with microfabrication allows for fine control of the diameter, length and arrangement of the nanowires. Even though VLS requires tightly controlled and harsh synthetic conditions, it is a highly reproducible, versatile, scalable and established technique.

8.2.2 Metal-assisted chemical etch of nanoneedles

Metal-assisted chemical etch (MACE) has risen to prominence in the past decade as a simple, low-investment strategy for wet anisotropic etch of silicon forming vertically aligned silicon nanowires (Fig. 8.1(b)) (Chiappini et al., 2010; Hochbaum et al., 2009; Huang et al., 2007; Peng et al., 2006). In MACE, a (noble) metal (usually silver or gold) deposited on a silicon substrate and immersed in an oxidizing solution of hydrofluoric acid, catalyses Si etching in the immediate vicinity of the metal itself, as a result of highly localised electrochemical dissolution of silicon (Chartier et al., 2008; Li and Bohn, 2000). Silicon nanowires form by MACE following deposition of a random mesh of metal nanoparticles, obtained through electroless deposition from metal salt precursors. MACE is a versatile technique that integrates with conventional photolithography and microfabrication (Chiappini et al., 2010), as well as with more specialised
nanofabrication strategies such as nanosphere lithography (Chiappini et al., 2010; Huang et al., 2007), interference lithography (Choi et al., 2008), anodised alumina (Huang et al., 2008) and block copolymer templating (Chang et al., 2009). This integration enables the synthesis of ordered arrays of high aspect ratio nanoneedles with a range of diameters comparable with those accessible to VLS: 10 nm to several microns. Lithographic patterning also permits arbitrary selection of the needles’ pitch and arrangement, within the limitations in resolution dictated by the specific lithographic technique employed. Selection of the etching conditions and combination with post-synthesis dry etches can shape the needles along their major axis, determining the sidewall angle and enabling tunable shapes from cylindrical to conical, with extremely sharp tips.

A major feature of MACE is the possibility to directly form porous silicon (pSi) nanoneedles under appropriate etch conditions (Chiappini et al., 2010). By selecting substrate resistivity, oxidant concentration and etch temperature, it is possible to finely control the porosity, pore size and crystalline orientation of the nanoneedles formed. By varying the oxidant concentration over time, it is possible to control the nanoneedle porosity along its axis, forming nanoneedles with multiple segments of different porosities. Thanks to their biodegradability (Chiappini et al., 2010; Anderson et al., 2003; Canham, 1995), biocompatibility (Piret et al., 2011; Goh et al., 2007; Low et al., 2006; Chin et al., 2001) and enhanced surface area (Herino, 1987), pSi nanoneedles are appealing for those biomedical applications where sustained release, delivery of large payloads, biocompatibility/biodegradability or protection of the payload from the external environment are concerned. These concerns are especially felt in vivo and for all those in vitro applications where nanoneedle treatment does not constitute an endpoint, such as the study of cellular pathways or cellular reprogramming for in vivo implantation. In these applications where pSi is favoured, and in all settings where VLS is impractical for integration or investment issues, MACE is strongly positioned as a reliable, versatile and low-cost strategy for the synthesis of nanoneedles.

8.2.3 Focused ion beam etch of nanoneedles

Focused ion beam (FIB) etching is a strategy that can sharpen existing microstructures into high aspect ratio nanostructure (Fig. 8.1(c)). With FIB, a beam of heavy ions (usually gallium) physically mills the surface of materials. The beam of ions can be focused and directed to desired areas on the sample with nanometric precision, to generate features of arbitrary geometry at the nanoscale. FIB controls the etch rate and etch resolution by tuning the ion current intensity. Higher currents mill faster but have lower resolution, and tend to increase the amount of re-deposited material and/or melt the surrounding material. FIB is a direct writing technique, where each needle must be milled individually. FIB is thus inherently low throughput and unsuitable for large-scale manufacturing, but
combined with scanning electron microscopy (SEM) in a dual beam, SEM-FIB allows for high precision shaping of existing micro- and nano-structures that cannot be handled with conventional microfabrication (Han et al., 2005b). FIB has been very successful for the sharpening of existing atomic force microscope (AFM) tips to form AFM-operated nanoneedles with diameters smaller than 50 nm. AFM-operated tips can be employed for the nuclear insertion of genetic material into hard-to-transfect, or rare cells owing to the low cytotoxicity and high transfection rate. Furthermore, the AFM-operated needle is suitable for delivery of drugs to certain specific cells within a culture, as it integrates with optical imaging of the area of interest.

8.2.4 Microfabrication of hollow nanoneedles

Conventional microfabrication strategies are at a disadvantage when trying to create high aspect ratio nanostructures into silicon, and cannot compete with the strategies outlined above for nanoneedle synthesis. Mostly the limitations arise from the challenges associated with the dry etching of silicon structures with nanoscale cross-sections and high aspect ratios (Woldering et al., 2008). Microfabrication, however, provides simple strategies to form hollow needles, which have been successfully used to interface cells with a drug reservoir for extended periods of time, thus enabling sustained or repeated drug delivery (Fig. 8.1(d)) (Peer et al., 2012; VanDersarle et al., 2012). Hollow nanoneedle synthesis starts with an array of nanopores conformably covered with a thin dielectric film. The film is then etched away from the horizontal surfaces and remains as a lining shell over the walls of the pores. Finally, selectively etching the material around the pore forms a hollow needle. Although this strategy is quite effective to reach the desired scope, the diameter of the resulting needles is quite large compared with the other strategies outlined, and the needles thus formed require surfactants to mediate drug delivery. Furthermore, the aspect ratio achieved thus far is quite limited, and the large needle size and requirements for saponification limit the potential applications of these needles and negatively impact biocompatibility.

8.3 Drug loading of nanoneedles and release patterns

The strategies for loading nanoneedles with drugs are mostly determined by the nature of the nanoneedles. Solid, porous and hollow needles are amenable to different loading mechanisms, resulting into markedly different release patterns.

8.3.1 Solid nanoneedles

Solid silicon nanoneedles, which have been fabricated by VLS or FIB, are loaded with drugs by physisorption on their surface (Shalek et al., 2010; Han et al.,
2008). Electrostatic interaction is conventionally used to improve physisorption, by ensuring the needles’ surface charge is opposite to that of the molecules being adsorbed. Common strategies include initial oxidation of silicon to provide a hydrophilic and negatively charged surface at physiological pH (Tasciotti et al., 2008). Surface functionalisation with an amine-terminated silane (the most common being 3-(aminopropyl)triethoxysilane, APTES) provides positively charged surfaces, which electrostatically favour adsorption of nucleic acids and most proteins, and are thus favoured over negatively charged ones (Shalek et al., 2010; Tasciotti et al., 2008). Further, the instability of the silane layer towards hydrolysis can potentially favour desorption of molecules from the surface and their release into cells, as suggested by the marked improvement in delivery efficiency when employing APTES compared with polyethylene imine (Shalek et al., 2010; Kim et al., 2007). Chemisorption of molecules on the walls of nanoneedles has been attempted, but although they were successfully loaded, they did not effectively mediate delivery (Han et al., 2005b; Obataya et al., 2005a; McKnight et al., 2004).

This loading strategy has successfully delivered a wide variety of bioactive compounds, including some, like nucleic acids, that do not easily transverse the cell and nuclear membranes. The surface physisorption loading readily exposes the drug to solution, causing it to rapidly release it away from the needles, limiting the timeframe for successful delivery by nanoneedle application (Han et al., 2008). To overcome this issue, a large excess of dissolved drug is spotted on the nanoneedles and often left to dry, in order to form thick coating layers that prevent immediate desorption of all the drug. Nonetheless, AFM-operated nanoneedles loaded in this fashion have a maximum of 3 minutes in solution before they become unable to deliver the drug intracellularly, as a result of its diffusion away from the needle. Similarly for nanoneedle arrays, it was never demonstrated that the drug delivery occurs from the nanoneedles into the intracellular compartment. The localisation of the delivered moieties in the perinuclear region suggests trafficking through the endolysosomal system instead of intracellular presentation (Shalek et al., 2010). Alternative explanations for the observed delivery include cell poration by nanoneedles in a fashion similar to electroporation, or uptake caused by the significant concentration in solution close to cells. As delivery of nucleic acids, known for the complexity of their delivery, can be achieved by appropriately coated flat surfaces, the needles array need not play a different role from the flat surfaces in this instance, and thus may not actively insert the drug payload into cells.

### 8.3.2 Porous nanoneedles

Porous nanoneedles possess certain advantages with respect to their solid counterparts. Their high surface area and their pore volume provide a large reservoir for the loading of drugs, improving payload density by several orders of
magnitude over solid structures (Salonen et al., 2005). The ability of porous structures to harvest molecules from solution allows attainment of loading concentrations several orders of magnitude higher than the equilibrium in solution. Moreover, alongside the electrostatic loading method, porous structures can be loaded with drugs from melt powders, to achieve higher loading concentrations and loading in the amorphous phase (Ambrogi et al., 2010; Riikonen et al., 2009). Further, it is possible to cap the pore openings and protect the payload from the external environment, modulate its release and prevent its premature release. Capping the pores with agarose prevents the degradation of protein payloads by proteases without influencing the release profile (De Rosa et al., 2011). Pore openings capped with environmentally responsive molecular valves that open at low pH prevent extracellular leakage of the payload, maximizing intracellular delivery (Xue et al., 2011).

Differently from solid nanoneedles, mesoporous needles are well positioned for loading nanoparticles that can penetrate and accumulate within the porous structure, from which they are slowly released (Tasciotti et al., 2008). Several different classes of nanoparticles were loaded successfully within pSi enhancing their therapeutic properties. Liposomes loaded with siRNA were loaded into pSi to extend their efficacy at silencing target genes for over 21 days (Tanaka et al., 2010), while pSi-loaded Gd nanoparticles enhanced their MRI contrast potential (Ananta et al., 2010) and Au nanoshells improved their photothermal effects (Shen et al., 2012).

Porous silicon also modulates the solubility of the payload, both by limiting its diffusion from within the pores and by progressively dissolving and desorbing payload (Salonen et al., 2005). Modulating solubility allows controlling the release rate of the payload, enables sustained release, mitigating the limitations of solid nanoneedles that rapidly release their payload, and capturing the advantages of hollow nanoneedles, without the need for a stable transmembrane opening that exposes the inside of the cell to the outer environment. Although porous nanoneedles can sustain drug delivery, their reservoir of drug is limited to what can be loaded within their small volume, whereas hollow nanoneedles can feed off an arbitrarily large external reservoir, acting as conduits.

8.3.3 Hollow nanoneedles

Hollow needles put in communication a drug reservoir with the cell cytosol (Peer et al., 2012; VanDersarl et al., 2012). In this fashion the needles are not loaded with drugs in the conventional interpretation of the term, but simply act as a conduit for drug delivery. This type of nanoneedle is the most strictly analogous to conventional needles and microneedles that act as conduits to carry drugs from large reservoirs where they are present in solution at high concentrations. Whereas needles and microneedles tend to be used to actively inject drugs into the target, nanoneedles for the most part rely on passive diffusion through nanochannels.
The passive diffusion delivery reduces the needles’ cytotoxicity, as part of the toxicity associated with microinjection is caused by intracellular pressure buildup following injection (Zhang and Yu, 2008). By relying on diffusion the delivery is slower, but nanoneedles are parallelised into arrays feeding off a common reservoir, which grant a higher throughput than manually operated microneedles. However, similar to what occurs in microneedles, those nanochannels could be easily blocked by proteinaceous material depositing across their opening, preventing their use over long periods of time. In this regard, it is as yet unknown whether hollow nanoneedles can sustain intracellular delivery for longer than porous ones, which are limited by the size of their drug reservoir.

8.4 Drug delivery using nanoneedles

Typically, two strategies allow nanoneedles to deliver drugs to the cell cytosol: either the cells internalise the needles, or a force is applied to the needles in order to cross the cell membrane.

8.4.1 Internalisation

Internalisation is the strategy most often employed with arrays of nanoneedles supported on a substrate (Peer et al., 2012; Shalek et al., 2010). Whether solid, porous or hollow, cells seeded over substrates decorated over nanoneedles can internalise a wide range of bioactive payloads, which are able to alter cell phenotype as expected. Small molecules, nucleic acids, proteins and nanoparticles can all be localised in the cytosol following growth on appropriately loaded nanoneedle substrates. DNA and siRNA delivered to cells correctly alter the gene expression pattern of target cells. This delivery strategy requires cells to play an active role and can be sensitive to cell type and environmental conditions (i.e. temperature, inhibitors, medium). The density of needles is an important parameter that affects their ability to penetrate cells (Qi et al., 2009). Cells grow on top of needles with a density of several needles per \( \mu \text{m}^2 \), whereas they grow at the bottom of needles at densities lower than 1 needle/\( \mu \text{m}^2 \). It is as yet unproven whether this strategy can mediate \textit{in vivo} delivery, as it relies on cell growth on a needle decorated substrate, which is not a process known to occur \textit{in vivo}.

8.4.2 Forcible insertion

The AFM-operated needle instead relies on forcible insertion to present the payload intracellularly. A force between 0.5 and 2 nN is required to insert a needle of less than 200 nm diameter across the cell membrane, and there is indirect evidence that penetration across the nuclear membrane is also possible (Han et al., 2008; Obataya et al., 2005b). As the needle diameter increases, its insertion requires a stronger force and results in bulging of the membrane, with associated
cytotoxicity (detailed in the following section). DNA plasmids were successfully delivered by AFM-operated needles and expressed target genes (Han et al., 2008). Two examples exist where supported nanoneedles were forcibly inserted into cells, in one instance using the force originated by a centrifuge, in another simply relying on the substrate weight (McKnight et al., 2004). In both instances the estimated force applied was greater than that required by the AFM experiments and needles successfully localised within the cell cytosol. The capillary interfacing was successful in delivering the drug; efficacy could not be evaluated in the centrifugation strategy, as the DNA plasmid payload was covalently attached to the needles and failed to express the target gene.

The forcible insertion strategy closely resembles the usual mechanism of delivery through needles, at a smaller scale. This delivery strategy does not depend on the interaction of the needles with cells and is clearly more amenable to in vivo applications than internalisation. Additionally, the reduced diameter of nanoneedles improves their cytocompatibility, as will be clear in the next section.

8.5 Toxicity of nanoneedles

To date the toxicity of nanoneedles has only been investigated in vitro, and is limited to cytotoxicity studies. AFM-operated needles of different diameter showed that cytotoxicity decreases with decreasing diameter, and is proportional to the insertion force and the degree of membrane bulging (Yum et al., 2010). Membrane bulging is thought to correlate with intracellular fluid leaking on needle insertion. When minimal bulging occurs for minimal insertion forces, none to minimal leaking occurs and the cells are likely to survive the nanoneedle penetration. Conversely, if the membrane is bulged by several microns, a significant portion of intracellular fluid is thought to leak out when the needle finally pierces the cell, resulting in likely cell death, or major disruption to cell function. A nanoneedle diameter of around or less than 200 nm has little impact on cell viability and proliferation following needle insertion, whereas diameters larger than 400 nm results in the death of the large majority of cells that underwent interfacing with nanoneedles. The forcible insertion of arrays of nanoneedles with diameter smaller than 200 nm also do not induce significant cell death, affect cells’ proliferation or their metabolism in cell culture (McKnight et al., 2004).

A similar trend occurs when cells internalise nanoneedles, with needles up to 200 to 300 nm in diameter having little effect on cell viability while still being capable of drug delivery. The cells grown on nanoneedles survive for several days on the silicon substrate. Further cells grown on arrays of nanowires with diameters of 30, 90 and 400 nm show that cell longevity is directly dependent on the diameter, with cell death occurring within a day for the 400 nm diameter wires and cells lasting for 5 days when 30 nm nanowires were used. Cells internalising nanoneedles express all 300 immune response genes investigated at levels
comparable with those of untreated cells (Shalek et al., 2012). Their metabolic activity, proliferation and viability, as measured in multiple studies, with multiple types of needles, and by multiple assays appear unaffected by the presence of the needles, if needle diameter is below the 200 to 300 nm threshold. The density of the needles also does not affect their viability, while it determines the vertical location of cell growth as mentioned above. These data indicate that forcibly inserted nanoneedles can be optimised to cause such minimal disruption to the cell integrity that they do not elicit toxicity, while cells that are grown on arrays of nanoneedles can perceive them as a standard cell culture substrate.

8.6 Overview of nanoneedle applications

8.6.1 Atomic force microscope (AFM)-operated nanoneedles

AFM actuation is the first strategy implemented for the use of silicon nanoneedles for drug delivery (Fig. 8.2). The force measurements with an AFM instrument can be used to study cellular events in individual cells with great sensitivity (Lamontagne et al., 2008). Among the applications is the injection at the nanoscale of specific molecular entities in individual living cells. Solid silicon nanoneedles optimised for harmless cell penetration (Obataya et al., 2005b) are developed with 6 μm length and 200 nm width by FIB from Si AFM tips (Han et al., 2005a). Subsequent treatment of the surface of the newly formed nanoneedles with

![Image](a) (b) (c)

8.2 The atomic force microscope (AFM)-operated nanoneedle induces gene expression in vitro. AFM-operated nanoneedles can load fluorescently labelled DNA plasmids by drying a drop of plasmid solution on their surface (a); they cross the cell membrane and potentially the nuclear membrane displaying their payload in the cytosolic and nuclear environments as shown by the cross-sectional view obtained by confocal microscopy of fluorescently labelled needles interfaced with cells genetically engineered to attain a fluorescently labelled membrane (b). The AFM nanoneedle can deliver a GFP plasmid that is efficiently expressed by cells (c). Images reproduced with permission from Han et al., 2008.
3-mercaptopropyltrimethoxysilane (MPTS) followed by incubation with N-(6-maleimidocaproyloxy)succinimide (EMCS) provides the means for further functionalisation. DNA is bound to the needle by first soaking the succinimidyl nanoneedle in an avidin solution and then incubating with a solution of biotinylated green fluorescent protein (GFP) DNA fragment. Force–distance measurements indicate that the nanoneedles can penetrate human embryonic kidney cells with a reproducible profile that outlines the different stages of the needle penetration. The penetration force profile does depend on DNA immobilisation on the nanoneedle’s surface, suggesting that the DNA molecules and cell components do not interact. Furthermore, calculations of friction force applied to one molecule of DNA in the penetration process indicate that the DNA does not detach from the surface of the nanoneedle during insertion. The cells manipulated by AFM can proliferate after repeated penetrations with a DNA-functionalised nanoneedle (Han et al., 2005a).

The silicon AFM-based nanoneedle system can also mediate intracellular presentation proteins, allowing insertion of two different His-tagged, fluorescently labelled proteins into HeLa cells. The nanoneedle surface is chemically modified with nitrilotriacetic acid (NTA) groups, and then chelated with NiCl₂ to conjugate poly-histidine-modified proteins. The protein–nanoneedle hybrid inserted into HeLa cells shows constant fluorescence intensity at the surface of the device while kept inside the cell, to indicate a stable conjugation of the protein to the needle (Obataya et al., 2005a).

Although neither of these examples demonstrate drug delivery, they indicate an avenue to use silicon nanoneedles for manipulation of living cells and intracellular access, without inflicting critical cell damage. Indeed an AFM nanoneedle can successfully transport electrostatically bound GFP plasmid DNA into cells. The transfection efficiency of over 50% is sufficient to prove molecular delivery through AFM-operated nanoneedles (Han et al., 2005a). Similarly AFM nanoneedles mediate efficient (above 70%) transfection of GFP plasmid DNA into the nucleus of mesenchymal stem cells, known hard-to-transfect cells using microinjection because of their flat shape (Han et al., 2008). When the plasmid DNA is only non-specifically bound to the surface of the nanoneedle, the system releases its load inside the target cell but also in the surrounding media. If the cell penetration is not rapid enough, the delivery fails.

Using a nanoneedle actuated by AFM surmounts the limitation of whole-cell population studies of typical cell biology methods by being able to manipulate single cells (Lamontagne et al., 2008) with minimal invasiveness. Moreover, these nanoneedles can also access specific regions (e.g. nuclei) inside living cells and deliver to target areas, a feature not available with conventional delivery methods. However, it is a time-consuming technique because of the need to manipulate each cell individually, limited by the availability of the nanoneedles, and requires a highly specialised setup, trained operators and costly consumables.
8.6.2 Vapour–liquid–solid nanoneedles for universal intracellular delivery

Large arrays of nanoneedles, employed in parallel, allow for high throughput delivery to multiple cells (Fig. 8.3). Nanowire arrays fabricated by VLS can act as a substrate for mouse embryonic stem cells and human embryonic kidney cells (Kim et al., 2007). Electrostatic forces can immobilise DNA encoding GFP on a nanowire array for gene delivery in the HEK293 cell line. The fluorescence emitted by GFP allows the estimation of the transfection efficiency to the cells, with less than 1% of cells expressing the protein. The limited transfection efficiency observed is attributed to the chemistry used to retain the DNA and this could be improved with other conjugations.

Similarly, an array fabricated by VLS can mediate the delivery of virtually any type of molecule desired into both immortalised and primary cells (Shalek et al., 2010). Molecules adsorb on the surface by electrostatic interaction through the simple modification of the surface with an aminosilane (3-aminopropyltrimethoxysilane) to attain non-covalent and non-specific binding of the molecules. The incubation time affects the position of HeLa cells with respect to the needles: whereas after 15 minutes the cells sit on top of the silicon nanowires, after 1 hour most of the cells are completely penetrated, irrespective of the nanowire density (always lower than 1 needle/μm²) or the molecule
immobilised on the nanowire surface. The impaled cells retain normal metabolism and growth, although the initial growth rate is slightly reduced when compared with glass coverslips. Further, the integrity of the cell membrane appears to be conserved as rat hippocampal neuron cells can retain the intracellular ionic concentrations required to fire action potentials.

Delivery and co-delivery of various biomolecules (fluorescently tagged or encoding fluorescent proteins) are possible in HeLa cells and primary cells. Expression of fluorescent proteins occurs when plasmid DNA is administered through the silicon nanowires or following delivery of fluorescently labelled biomolecules (siRNA, DNA, proteins, peptides). Further, molecules co-deposited on the surface of the nanowires can be co-delivered. This system is compatible with microarray technology because of the direct mediation of biomolecule delivery by the surfaces of the nanowires. Also, by arraying biomolecules on a nanowire surface, parallel live-cell screening of diverse biological effectors is possible.

8.6.3 Hollow nanoneedles for sustained delivery

Hollow nanoneedles are synthesised both from alumina and silicon dioxide, and employed for repeated delivery of biomolecules from an effectively infinite reservoir (Peer et al., 2012; VanDersarl et al., 2012) (Fig. 8.4). The silicon dioxide needles are realised by first forming a 10 μm silicon membrane through KOH etch of a silicon on insulator wafer, which also defines the drug reservoir. Pores with

![Image](image_url)

8.4 The hollow nanoneedle induces gene expression in vitro. Hollow nanoneedles act as a conduit between a reservoir of fluorescent dextran and the cell culture environment enabling multiple exposure of cells to bioactive agents, as shown by the highly fluorescent spots associated with the needles (a); they allow interfaced cells to grow successfully as evidenced through scanning electron microscope (SEM) micrographs (b). The hollow nanoneedles can deliver red fluorescent protein expressing plasmids to cells cultured over them and in the neighbouring area with the adjuvant effect of surfactants in solution (c). Images reproduced with permission from Peer et al., 2012.
500 nm diameter and 5 μm pitch are patterned by e-beam lithography and etched through the membrane by deep reactive ion etch. Thermal oxide is grown on the whole system to a thickness of 100 nm. The top oxide surface is removed by reactive ion etching and the hollow needles formed by thinning the silicon membrane through deep silicon etching.

This fabrication strategy requires multiple deposition, growth and etch steps on a thin membrane, which is very fragile, and easily destroyed by interfacial stresses. Additionally, the overall thickness of the membrane is limited by the depth of the pores that can be etched by deep reactive ion etch (DRIE), which in turn is determined by the pore diameter. The DRIE step fundamentally limits the aspect ratio of the needles, and is inefficient at forming nanopores. Smaller pore diameter would mean more shallow etches, and in turn shorter needles. Even for these very large nanoneedles with 500 nm diameter, their length is limited to approximately 3 μm in order to preserve the integrity of the 10 μm membrane.

These nanoneedles can transport fluorescently labelled dextran and DNA plasmids across the membrane during a 2-hour period. Two human cell lines (HEK 293 and NIH3T3) can be seeded over the needles and grow for up to 48 hours, without noticeable effect on cell proliferation. Cells tend to grow on the upper section of the needles without being able to reach the bottom substrate. It is unclear whether the needles are able to cross the cell membrane. Loading a solution containing fluorescently tagged dextran mixed with the permeation enhancer saponin at concentrations of 3 and 4 μg/mL allows repeated delivery of dextran molecules following multiple sessions of 10 minutes’ incubation. Similarly, the hollow nanoneedles deliver DNA plasmids for the expression of red fluorescent proteins, also in the presence of saponin. The delivery is not limited to the cells growing on the needles, but extended to neighbouring cells grown on flat surfaces.

Although the platform appears interesting, and can regulate sustained diffusion across two reservoirs, the need for saponin to mediate the delivery shows that the needles alone cannot penetrate cells and mediate intracellular delivery. To this extent, the advantage of this platform over a porous membrane is unclear.

8.6.4 Biodegradable porous silicon nanoneedles

Combining MACE with conventional microfabrication allows formation of arrays of vertically aligned nanoneedles (Fig. 8.5). The simplest form of needles, as vertically aligned porous nanowires, results from MACE of a silicon substrate covered with a noble metal mesh from electroless deposition. Ordered and shape-defined nanoneedles result by first depositing a thin film of low stress silicon nitride over a silicon substrate (Chiappini et al., 2010). The nitride is then patterned with a large-scale array of nano-sized dots with desired pitch, and metal is selectively deposited in the field. The silicon then undergoes MACE to form high aspect ratio pillars. The pillars can then be shaped into conical needles by reactive ion etch, to form porous silicon nanoneedles.
Controlling MACE parameters allows for control of the porosity and pore size of the needles over a wide range, which in turn influences their biodegradation kinetics, and the loading and release kinetics of molecules and nanoparticles. Biodegradation is faster for higher porosity materials, which results in faster release. Further, nanoparticles can be size excluded through pore size, allowing for selective nanoparticle harvesting from solution. MACE also allows for forming needles with segments of different porosity along their axis, enabling differential loading of nanoparticles (Chiappini et al., 2010). Quantum dots of 3.3 nm and 6.3 nm can be segregated into different segments of the same nanoneedle, allowing orthogonal loading of multiple nanoparticles. Also, the porous structure allows loading of multiple drugs simultaneously.

Cells grown on MACE nanoneedles maintain their normal viability and metabolism, and appear to internalise them. Molecules loaded on the nanoneedles are effectively delivered to the cell, and nucleic acid delivery can either induce (DNA) or silence gene expression (siRNA). The porous needles degrade over a few days, leaving behind a smooth substrate that resembles and behaves similarly to a standard cell culture substrate.

Porous needles are versatile, in that they allow co-loading, segregated loading and sustained delivery of multiple molecules, as well as efficient loading of nanoparticles. Porous needles are potentially less toxic than the solid ones in the long term, owing to their biodegradability. The elevated biocompatibility and biodegradability of pSi make porous nanoneedle structures amenable for all applications where silicon remaining in situ poses concerns. All applications where the nanoneedles are directly employed in vivo or where cells are treated in vitro with nanoneedles for further use in vivo fall into this category.

8.5 The porous silicon nanoneedle induces gene expression in vitro. Porous silicon nanoneedles load fluorescently labelled molecules throughout their volume as shown by the uniform intensity through each needle of an array in confocal micrographs (a); and can penetrate the cell cytosol as shown by confocal microscopy of dye-labelled cells and nanoneedles (b). The porous nanoneedles can deliver green fluorescent protein plasmids to cells with high efficiency (c).
Although porous needles may appear more fragile than their solid counterparts, their mechanical properties can be optimised, controlling their porosity. Mechanical properties can range from values close to those of solid silicon to significantly lower values that could better match those of cells or the tissue with which to interface. The dissolution of the needles limits the timeframe of their usability, as the degradation process shortens, dulls and makes the needles more flexible, thus less capable of performing their function. They may thus be at a disadvantage wherever repeated applications from the same needles are required over extended periods of time. Solid or hollow needles would be advantageous in comparison for such applications.

8.7 Conclusion

All of the different nanoneedle-based approaches developed thus far can effectively deliver drugs inside the cell. They combine low cytotoxicity with broad applicability in a manner that surpasses most of the currently available strategies to negotiate the cell membrane. Nanoneedles also do not appear to disrupt or alter any cellular function, an important aspect when trying to investigate the change in cellular phenotype as a consequence of drug delivery.

Nanoneedles deliver many classes of bioactive molecules inside cells, and can deliver them simultaneously. Drug co-delivery, together with cytocompatibility, makes nanoneedles an extremely promising material for intracellular drug delivery in vitro. The cytosolic drug delivery approach also requires lower overall amounts of drugs and minimises unwanted off-target interactions in the intracellular space.

Both when operated by AFM and when used as large supported arrays, nanoneedles can deliver their payload in patterns of choice. Patterned delivery can modulate cellular phenotype of a subset of cells within a culture to form defined structures, and allows for designing co-cultures with arbitrary arrangements of differently behaving cells. The patterned delivery, combined with the minimal disruption to cellular activity, makes nanoneedles an extremely promising approach for high-throughput microarray setups that investigate cell response to cytosolic presentation of drugs. Further, patterned delivery enables complex organotypic cultures that contribute to investigation of cell interaction in tissue structures. The information from such cultures would provide important design guidelines when developing topographical cues in scaffolds for tissue engineering.

The low impact, minimally invasive approach of nanoneedles is well amenable for in vivo applications. Combining stimuli-responsive strategies with nanoneedle delivery would enable targeted intracellular drug delivery, contributing to a novel strategy to the practice of personalised medicine, where the therapeutic index is maximised by both the lower amount of drug required and its selective delivery. Further in vivo applications can include localised patterned delivery of genetic material for the small scale, precision engineering of tissue. In such vision, the
local alterations to cell phenotype will form nano- to microscale functional tissue structures, which are essential for tissue function. However, significant barriers still exist to the efficient use of nanoneedles in vivo, requiring further innovation before their implementation. Presently, nanoneedle arrays rely on cell seeding and internalisation to deliver drugs, and this would not occur in vivo. The major obstacle towards using needles to actively pierce cells is the large amount of cells that detach from the original substrate in this case. Strategies to alleviate this concern are being developed, by increasing the interaction between the substrate and cells, but still rely on cell behaviour that is not observed in vivo.

Nanoneedle length and overall mechanical stability are also not suitable at present for transdermal delivery, presently requiring surgical placement of the needles to the site of interest. Further non-degradable needles would not be easily allowed in vivo, especially because of the known adverse effects of high aspect ratio silica structures. In this regard, porous silicon nanoneedles possess an advantage for in vitro applications as they biodegrade completely into harmless byproducts.

Overall, we are still at the infancy stage in the development of nanoneedle technology for drug delivery, which nonetheless promises to efficiently negotiate the major biological barrier constituted by the cell membrane without altering cell behaviour. Whether these promises will be transformed into technological advances that can see their way into laboratories and the clinic still depends on the ability to overcome what currently appear as strict design limitations of this technology.

8.8 References

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