Review

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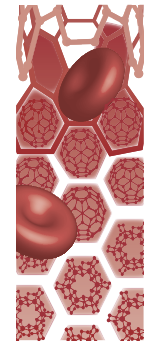
Nanomedicine



Immobilized nanoneedle-like structures for intracellular delivery, biosensing and cellular surgery

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The rapid advancements of nanotechnology over the recent years have reformed the methods used for treating human diseases. Nanostructures including nanoneedles, nanorods, nanowires, nanofibers and nanotubes have exhibited their potential roles in drug delivery, biosensing, cancer therapy, regenerative medicine and intracellular surgery. These high aspect ratio structures enhance targeted drug delivery with spatiotemporal control while also demonstrating their role as an efficient intracellular biosensor with minimal invasiveness. This review discusses the history and emergence of these nanostructures and their fabrication methods. This review also provides an overview of the different applications of nanoneedle systems, further highlighting the importance of greater investigation into these nanostructures for future medicine.

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Nanoneedle-like structures are structures with a high aspect ratio and one or more external dimensions in the size range 1–100 nm [1]. There are several types of nanoneedle-like structures which have been fabricated and applied in nanomedicine, including nanoneedles, nanorods, nanowires, nanofibers and nanotubes (Figure 1). A common feature of these nanoneedle-like structures is that they all have a high aspect ratio. These nanostructures have been used for many applications, for example, delivery of theragnostic agents into cells, cancer therapy, regenerative medicine, biosensing and intracellular surgery (Table 1).

Nanoneedles are needle-shaped structures in the nanoscale range [17]. Nanorods have a smaller aspect ratio in comparison to nanoneedles and can be fabricated using as metal and nonmetallic materials. Nanorods are widely used in electronic and mechanical devices, as their shape anisotropy can enhance the internal electrical field [18]. Nanofibers are cylindrical fibers that can be fabricated by electrospinning, a method also used to fabricate nanowires [19]. Nanowires are fabricated from semiconducting materials which are sensitive to bioelectrical signals [20]. Finally, nanotubes are hollow nanostructures with thermal and electrical conductivity properties [21].

For these nanoneedle-like structures, there are two ways of applying them: mobile (free flow) and immobilized (substrate fixed). Nanoneedle-like structures as a subset of nanoparticles remain largely unexplored, likely due to the difficulty in fabricating and manipulating the shape of these nanostructures. Most applications investigated are in the immobilized form. As a result, the main body of this review is on immobilized nanoneedle-like structures.

Mobile nanoneedle-like structures

Mobile nanoneedle-like structures can be loaded with theragnostic agents, to be used as particulate drug delivery systems. In an example, gold nanorods with fluorescent probes on their surface have been used to deliver probes into HeLa cells [22]. A similar study by Castro-Smirnov *et al.* showed the stable transfer of DNA in mammalian cells using mobile nanofibers [23]. Wu *et al.* also demonstrated drug delivery applications by fabricating high drug loading nanoneedles containing 10-hydroxycamptothecin. These nanoneedles exhibited greater delivery efficiency in

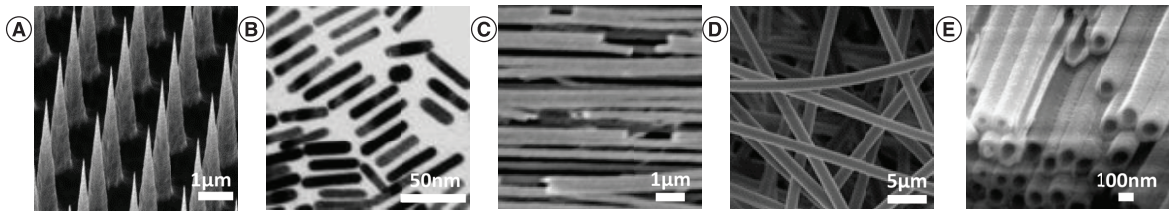

Figure 1. Nanoneedle-like structures. (A) Nanoneedles, **(B)** nanorods, **(C)** nanowires, **(D)** nanofibers, **(E)** nanotube.
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Table 1. Types and properties of nanostructures.

Type	Characteristics	Materials used	Function	Ref.
Nanoneedles	Hollow, solid, dissolving needles	Silicon, boron nitrate	Intracellular delivery, biosensor	[7,8]
Nanorods	Solid needle-like structure	Carbon, zinc oxide, gold, diamond, silicon, boron nitrate	Light emitting diodes, laser diodes	[9,10]
Nanowires	Sensing wires	Titanium dioxide, copper, gold, zinc oxide, silicon,	Coated with antibodies, cancer diagnosis	[11,12]
Nanofibers	Polymer filament	Polymers, polymer blends	Cancer diagnosis, tissue engineering, biosensor	[13,14]
Nanotubes	Cylindrical tubes	Carbon atoms	Drug delivery, electronics	[15,16]

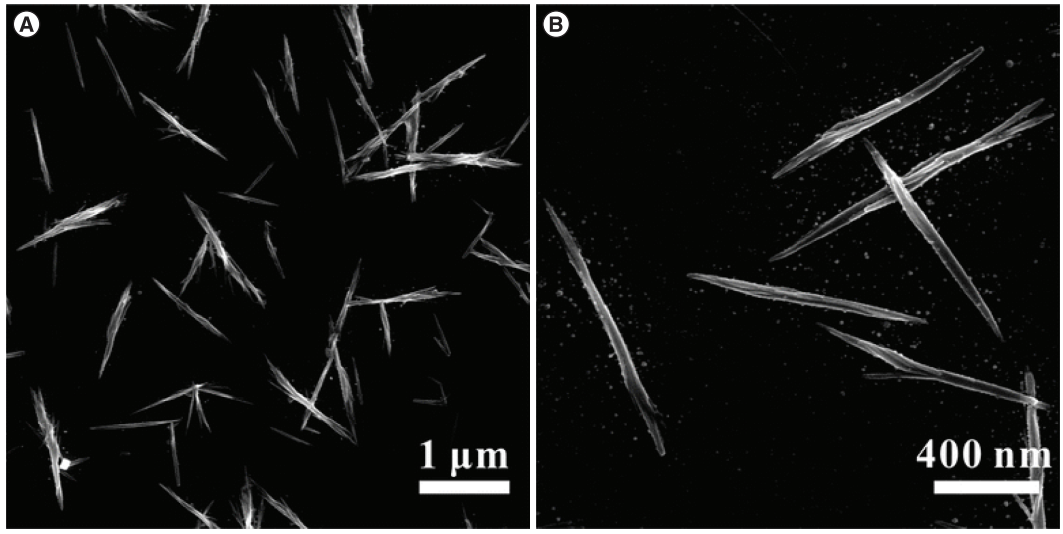

Figure 2. Scanning electron microscope micrographs of mobile nanoneedles carrying two drugs.
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comparison to structures with smaller aspect ratios [24]. Similarly, Yang *et al.* fabricated nanoneedles encapsulating two different drugs, which provided sustained drug release over 380 h (Figure 2) [25].

Nanotubes are another type of mobile nanostructure that are used to deliver drugs. Heister *et al.* designed an anticancer nanotube by noncovalently binding the anticancer agent doxorubicin onto the nanotube sidewalls to be delivered to colon cancer cells [26]. Comprehensive reviews have been published by Janas and Rat *et al.* covering the wide scope topic of carbon nanotubes and their applications [27,28].

Studies have illustrated that these free flow form nanostructures induce minimal cytotoxicity. Abariute *et al.* incubated human lung adenocarcinoma cells on mobile nanowires and found no significant difference in cell viability,

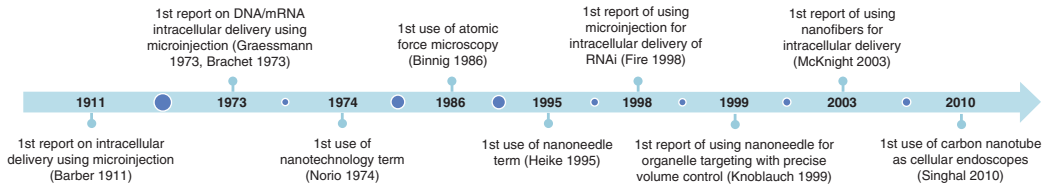

Figure 3. Historical development of nanoneedle-like structures. Timeline exhibiting the study and development of nanoneedle-like structures.

proliferative ability or reactive oxidative species content, indicating that internalizing mobile nanowires does not induce cell cytotoxicity. Confocal images also showed that the nanowires were successfully internalized by the cells [29].

Immobilized nanoneedle-like structures

On the other hand, nanostructures can form a scaffold (e.g., nanofiber meshes), or be attached to a substrate, such as a pad or a patch. For example, nanoneedle-like structure arrays can be fabricated using photolithographic patterning and deep reactive ion etching, where silicon pillars aligned on a silicon wafer are reduced to a needle structure via octafluorocyclobutane polymerization, deep reactive ion etching and isotropic etching [30]. The structure is then immersed in potassium hydroxide solution, where the needles are further reduced into a nanoscale needle-like structure. The nanoneedle-like structure array can be applied onto cell membranes to deliver biomolecules or act intracellularly. Recently, Chiappini reviewed immobilized nanoneedles employed as biosensors to detect intracellular processes and biomolecules, focused on biosensing and properties of nanoneedles which influence membrane disruption [31].

Due to the rapid development of this important field, we review these immobilized nanostructures and their applications in intracellular delivery, biosensing, cancer therapy, regenerative medicine and intracellular surgery. All nanostructures listed in Table 1 are defined as nanoneedle-like structures in this review.

The research articles in this review were identified by using the following keywords in PubMed: nanoneedle-like structures, nanoneedles, nanorods, nanofibers, nanowires and nanotubes. The research articles chosen were all in English and with full text. All selected articles were then screened and irrelevant articles were excluded. A few articles of relevance were also identified through the references and included in this review.

Historical development

History of nanotechnology can be dated back to the fourth century AD with the famous example of Lycurgus cup or ancient dichroic glass made up of silver–gold nanoparticles (50–100 nm) dispersed in a glass matrix [32]. However, the term nanotechnology was first coined by Norio in a 1974 conference [32]. It was not until the discovery of scanning tunneling microscope [33] in 1981 by Gerd Binnig & Heinrich Rohrer and atomic force microscopy (AFM) [34] in 1986 by Gerd Binnig, Calvin Quate and Kristoph Geber, using needle tip at micro/nano scale, can single atom be observed and manipulated.

The nanoneedle term was first mentioned in 1995 by Heike when he used nanoneedle array formed on silicon wafer for direct imaging of scanning tunneling microscope apex tip [35]. However, the idea of using sharp object to deliver cargo inside cells was dated back 1911 when Barber [36] first reported the inoculation of bacteria and other substances into living cells using custom-made micropipette (Figure 3).

In 1973, Brachet showed that microinjection could be used to deliver hemoglobin mRNA into oocytes and embryos to study cytological structure of cells and hemoglobin synthesis [37]. This has laid the groundwork for intracellular delivery of RNA and DNA. In 1998, Fire and Muller reported intracellular delivery of RNAi, which won them a Nobel prize in 2006 [38]. Knoblauch *et al.* reported fine control of volume injection from femtoliter to attoliter using nanoneedle coupled with heat-induced expansion of an alloy [39]. This was the first report on subcellular delivery or organelle targeting using nanoneedle with precise volume control. In 2003, McKnight and co-workers demonstrated the first use of vertically aligned carbon nanofibers to delivery plasmid DNA inside viable cells for controlled biochemical manipulation [40]. Nanoneedles with conventional configuration of conical geometry may damage cells during penetration and fail to handle continuous fluid injection. To overcome this challenge, Singhal and coworkers have demonstrated an excellent cellular endoscope using carbon nanotubes

Table 2. The different types of nanoneedles, their structures and their potential applications.

Types of nanoneedles	Structure	Use	Ref.
Solid nanoneedles	Load theragnostic agents via physisorption	Penetrate cell membrane to access cytosols	[42]
Coated nanoneedles	Coated with antibodies, proteins, enzymes	Mechanical detection of cytoskeletal components	[7,43]
Biodegradable nanoneedles	Produced using biodegradable materials, for example, silicon	Slowly degrade over time for sustained drug release	[44]
Hollow nanoneedles	Hollow needle structure containing liquid formulation	Inject theragnostic agents into cells	[7,42]

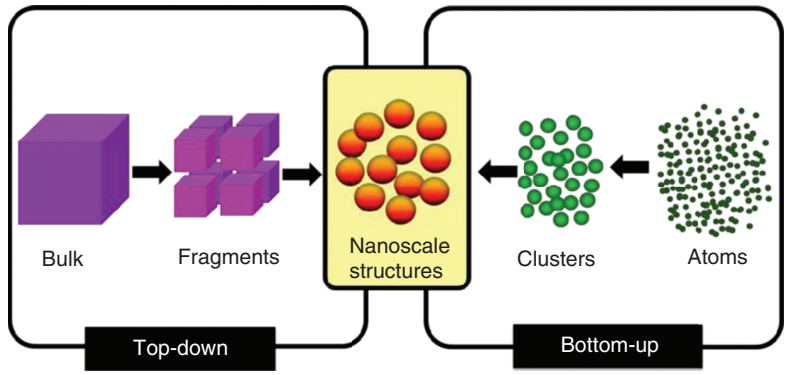

Figure 4. Nanofabrication methods. Top–down and bottom–up methods employed in nanofabrication. Reproduced with permission from [48], licensed with CC BY 3.0.

which allow minimally invasive intracellular probing, transporting fluids, performing optical and electrochemical diagnostics at the single organelle level [41].

Methods of administering theragnostic agents using nanoneedles

There are several types of nanoneedle-like structures that are all utilized for different purposes (Table 2). Solid nanoneedles are made from materials such as silicon, polymers and metals. Using strategies such as physisorption, drugs mixed in a polymeric solution can be loaded into the needles [7]. Hollow nanoneedles resemble conventional hypodermic needles as their structure consists of a hollow portion filled with liquid formulation. This channel allows drugs to flow through from the reservoir to site of action [42]. Dissolving nanoneedles are commonly polymeric where the drug is incorporated into the needle-forming materials. This type of nanoneedle-like structure allows for a controlled, sustained drug release [24]. Nanoneedles have also become a useful tool in detecting a cell's intracellular activity. Nanoneedles coated with antibodies, proteins or enzymes can recognize and interact with cytoskeletal components [43].

Nanoneedle-like structure fabrication

To manufacture these nanostructures, different fabrication processes can be employed, using either the top–down or bottom–up strategy (Figure 4). Top-down strategy exploits lithographic tools (physical top–down) or chemical-based processes (chemical top–down) to manufacture long range order structures through the controlled removal of materials from large solid structures [45]. Physical top–down creates detailed nanoscopic features of nanomaterials by utilizing electron beam lithography, focused ion beam or advanced optical lithography [46] whereas chemical top-down involves the application of heat or acid-base reactions such as templated etching, selective dealloying and anisotropic dissolution [45]. By etching and patterning, a larger silicon wafer structure can be deconstructed to carve out features on several nanostructures. This gives rise to the significant advantage which is the potential for mass-production of structures. However, due to limited capability and resolution of this method, the size of features and structures that can be constructed are limited [47].

On the other hand, bottom–up fabrication is a low-cost, additive process which involves assembling small, basic atoms or molecules through strong covalent bonds to build the final nanostructure (Figure 4). This method does not involve the use of expensive physical or chemical approaches and allows users flexibility to design their own

Table 3. Types of fabrication methods and their advantages and limitations.

Fabrication method	Advantages	Limitations	Ref.
Chemical etching	Cost-efficient, simplicity, versatile	Poor repeatability Chemical contamination	[51,52]
Lithography	Can be used on large surface areas of photoresist	Limitation to possible designs	[53]
Mechanical exfoliation	Low contamination, can be stacked on 2D materials	Low process yields	[54]
Chemical vapor deposition	Accurate control, low defects, high purity and crystal quality	High production cost	[55]
Physical vapor deposition	Range of matrix composition can be used	Expensive equipment, simple geometries only	[56,57]
Self-assembly	Low cost, better controllability, produce multiple designs	Poor repeatability, requires complex materials	[58]
Electrospinning	Low cost, high production rate	Poor pore size control	[59,60]

nanostructure [49]. Overall, bottom–up is an inexpensive, flexible method that is most suitable for short-range order structures.

Although both top–down and bottom-up strategies involve different methods, usually a combination of both methods is employed in nanofabrication. The most common nanofabrication methods are etching, photolithography, exfoliation, chemical and physical vapor deposition and self-assembly (Table 3) [50].

The chemical etching process is a top-down method where a chemically active plasma containing positively and negatively charged ions reacts with the material to carve out details. Tetramethyl ammonium hydroxide can be used as an etching mask as it is very selective to silicon oxide, thus is a commonly used anisotropic etchant of silicon [61]. This technique is a commonly used nanoneedle fabrication method as it can obtain sharp, accurate angles as small as 2.9° [62]. For lithography, patterns outlined on a wafer coated with photoresist layer are exposed to ultraviolet rays, resulting in photoresist polymerization and the formation of the nanostructure shape [63]. Exfoliation is a top-down method which involves the fabrication of structures through the expansion of materials using heat [64]. Chemical vapor deposition is a bottom-up technique which involves reacting precursors, such as a gas or vapor, on preselected substrates at high temperatures [55]. However, physical vapor deposition involves the vaporization of liquid or solid molecules, which are then transported through a vacuum and deposited onto a substrate through condensation to improve the substrate's surface properties [57]. The common bottom-up method is self-assembly which exploits certain polymers to construct 2D or 3D nanostructures [65].

Nanofibers and nanowires are often fabricated using electrospinning. Electrospinning is an efficient, low-cost and high production rate method utilized to fabricate nanofibers in different assemblies. It employs an electrohydrodynamic phenomena and high voltage supply to synthesize extremely thin fibers [59]. These fibrous materials are featured with controllable fiber diameters, high porosity and large surface area. The following reviews explore the process of electrospinning in greater depth [66–68].

Recently, the advancements in 3D printing technology have revolutionized engineering by significantly reducing the cost and time required to print medical devices. Two-photon polymerization, that is, two-photon absorption induced solidification of photoresists, has enabled 3D printing to produce nanoscale objects, also known as nanoprinting [69]. Nanoprinting has been used to make complex nanostructures with high resolution. Companies such as Nanoscribe have launched their 3D printer, namely, Photonic Professional GT2, which employs a laser lithography system to fabricate complex nanostructures [70]. It is foreseeable that nanoneedle-like structures may be designed and printed by using 3D nanoprinting technology, for personalized medicine and other applications.

Applications of nanoneedle-like structures

Delivering biomolecules into living cells

One of the main obstacles that limits biomolecule delivery is the cell phospholipid bilayer permeability. The high aspect ratio of nanoneedle-like structures gives rise to their pivotal role in biomolecular delivery, allowing them to penetrate cell membranes. To determine a cell's capability of recovering post-injection, DU145 cells were stained with proprium iodide, a fluorescent agent commonly used to evaluate cell viability [8]. Although there were several individual non-viable cells, the rest of the cells were counted as viable. Furthermore, there was no significant difference between cell viability immediately post-puncture and 24 h later, indicating that repairing of the cell membrane is rapid.

Pinese *et al.* conducted a study to determine the different siRNA delivery efficiencies between two methods: conventional bolus delivery and nanofiber scaffold-mediated delivery [71]. The siRNA targeted collagen type 1 expression, where the nanofiber scaffold-mediated siRNA delivery achieved greater downregulation of collagen both *in vitro* (human fibroblasts) and *in vivo* (rats) in comparison to bolus delivery. Yum *et al.* demonstrated the intracellular delivery of fluorescent quantum dots into HeLa cells [72]. A single fixed nanotube was coated with a thin layer of Au which increased the mechanical strength of the needle and acted as an adhesive for the quantum dots to attach via disulfide bonds. The cargo release was regulated by exploiting the cell's ability to maintain redox equilibrium inside the cytoplasm, where the disulfide bonds were reduced into thiol groups. Once the needle was injected into the cytoplasm, the quantum dots were released due to the cleavage of disulfide bonds (Figure 5Ai).

Another study transfected rat hippocampal neuron with plasmid DNAs encoding fluorescent proteins using fixed silicon nanowires after a 24-h incubation period [73]. Over 95% of cells were found to have fluorescently labelled biomolecules in all cell types, highlighting the efficient delivery of exogenous materials into the cytosol using fixed nanowires (Figure 5Aii & iii). Biomolecule delivery using nanostructures allows for direct delivery to specific sites and in the case of drugs, would enhance the therapeutic effect while minimizing potential side effects.

Studies have explored what factors influence the needle penetration efficiency. First, operating temperature influences membrane fluidity and thus, needle penetration [43]. Immunostaining and western blotting analysis demonstrated that lower temperatures, such as 4°C, increased the chances for successful penetration as the cell membrane structure remained unaffected at 4°C. Also, inappropriate approaching velocity of the nanoneedle can lead to nanoneedle insertion failure. The suitable approaching velocity was determined to be between 3 and 10 μm/s, as higher velocities led to more successful insertions. However, these factors are dependent on cell type, nanoneedle shape and penetration location. For example, nanoneedles with diameters of 200 and 800 nm had a penetration rate of 70–90% and 20–60%, respectively [77].

Alongside the advantages of nanoneedles, investigations completed *in vitro* have demonstrated that cytotoxicity can take place. Membrane bulging occurs when intracellular fluid leaks due to the needle penetrating the cell membrane. Cytotoxicity was found to be proportional to insertion force, where membrane bulging is more likely to occur at higher insertion forces [42]. Significant membrane bulging can lead to cell dysfunction or even death. Potential technical difficulties can also be encountered when delivering molecules using hollow nanoneedles, such as nanotubes. These types of needles require high insertion forces, but can also easily become clogged [78]. Increasing the needle diameter can prevent clogging, however, diameter morphology is negatively proportional to cell viability. Shalek *et al.* demonstrated that cell death took place after a day when grown on 400 nm diameter substrate-fixed nanowires, whereas cells survived for 5 days when grown on 30 nm diameter substrate-fixed nanowires [79]. Therefore, these studies highlight the importance of optimizing the nanoneedle design according to the experiment to reduce induced cytotoxicity. In order to study the safety of nanoinjection *in vivo*, real-time bioluminescent imaging post-injection of luminol exhibited no local acute inflammation in the muscle or skin 5 and 24 h after injection [44]. Histological analysis of the muscle structure also showed no significant difference from the control.

Currently, the mechanism of biomolecule delivery using nanoneedle-like structures is not fully understood. To determine whether a nanoneedle-like structure can deliver biomolecules, the structure must be able to deliver different types of biomolecules efficiently in several cell types while inducing minimal cytotoxicity [73]. Delivery is believed to take place through two possible mechanisms: intracellular penetration and membrane deformation [80].

Intracellular penetration involves the delivery of biomolecules following the physical insertion of the needle tip into the cell. When the needle comes into contact with the cell membrane, it generates highly localized stress, eventually causing the membrane to rupture [81]. Three different forces are involved in the penetration process, namely, an initial increase, a sudden decrease and a constant force [82]. The force initially increases when the needle first encounters the cell membrane, then there is a sudden decrease once the needle penetrates the cell membrane and this force must remain constant once in the cell.

Whether the needle successfully penetrates the membrane or not depends on puncture force and needle-tip dimensions. A needle-tip of 1–100 nm in diameter and 1–20 μm long, required an insertion force between 0.5 and 2 nN to penetrate into the cell cytosol [76,82,83]. Xie *et al.* reported that 50 nm radius nanowires required a puncture force in the nanonewton range, a force much greater than gravitational force [81]. Cells seeded on nanowires deformed by spreading and wrapping themselves around the substrates by the 12-h mark but failed to penetrate the membrane. It was concluded that an externally applied force is required to pierce the membrane as there must be enough tension within the lipid bilayer.

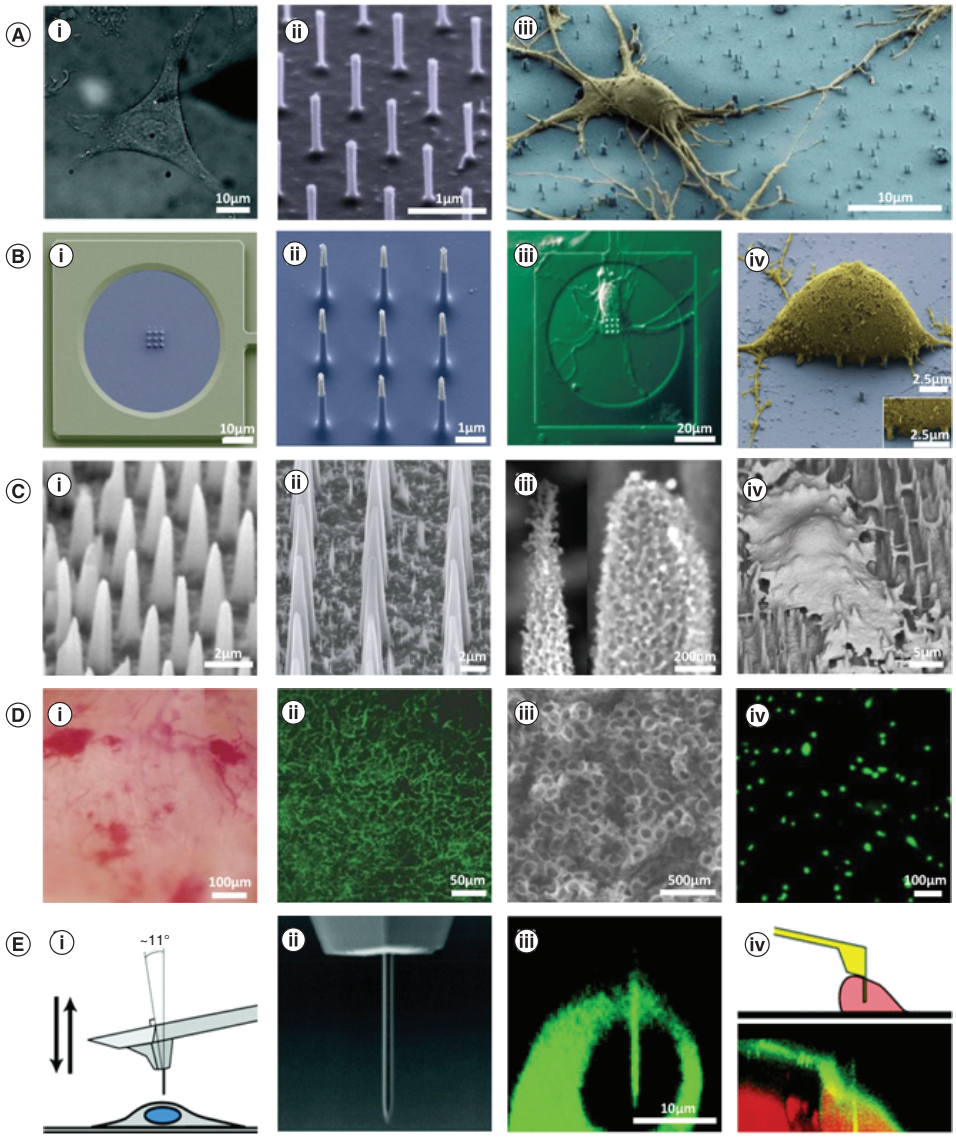

Figure 5. Applications of nanoneedle-like structures. (A) Intracellular delivery of fluorescent quantum dots into HeLa cells: **(i)** Micrograph of nanoneedle penetrating a HeLa cell, **(ii)** SEM micrograph of vertical silicon nanowires fabricated using etching, **(iii)** SEM micrograph of rat hippocampal neuron seeded on etched silicon nanowires for plasmid DNA transfection, **(B)** Rat cortical neuron cultured on a vertical nanowire electrode array (VNEA) pad. **(i)** SEM micrograph of a VNEA pad; **(ii)** SEM micrograph of nine silicon nanowires on VNEA pad; **(iii)** DIC micrograph of a rat cortical neuron cultured on a VNEA pad and; **(iv)** SEM micrograph of a rat cortical neuron cultured on a VNEA pad, **(C) (i–iii)** SEM micrograph of Cy3-labelled GAPDH-siRNA and GFP-expressing DNA plasmid retaining porous silicon nanoneedles developed to transfect HeLa cells and; **(iv)** SEM micrograph of HeLa cells seeded over nanoneedles. **(D)** Nano-injected mice muscle exhibiting greatest **(i)** vascularization using intravital bright-field and; **(ii)** hVEGF165 expression using confocal microscopy images. **(iii)** SEM micrograph of 15.14% TiCu nanotubes and; **(iv)** Fluorescence image showing NO in endothelial cells. **(E)** Nanoneedle-incorporated atomic force microscopy cantilever system: **(i)** Schematic diagram of AFM nanoneedle over a cell; **(ii)** SEM micrograph of nanoneedle; **(iii)** Cross-section confocal micrographs of nanoneedle successfully penetrating a HEK293 cell and; **(iv)** Reconstructed 3D image from confocal slices of AFM nanoneedle penetrating HEK293 cell.
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Park *et al.* demonstrated how a 0.5 N puncture force exhibited a significantly higher biomolecule delivery efficiency in comparison to 0.1 N puncture force [8]. However, larger puncture forces decreased cell viability. Puncture time was also investigated, and it was shown that longer puncture time did not increase delivery efficiency but reduced cell viability. Other factors such as membrane stiffness, nanoneedle-like structure geometry and membrane-substrate contact geometry are factors that can also influence delivery efficiency and thus must be investigated. For example, lower temperatures can alter the membrane fluidity and improve needle penetration [43]. Thus, it is a challenge to determine how nanoneedle-like structures impale the membrane due to the sensitive nature of cells.

For deformed cell membranes where no intracellular penetration happens, cellular uptake relies on biomolecules diffusing across the cell membrane and into the cytosol [84]. Several cells cultured over nanoneedle-like structure tips have been found to allow molecules to simply diffuse through membrane pores [8]. However, upon further investigation, it is believed that other cells received cargo through clathrin or caveolae-mediated endocytosis [85]. Nanoneedle-like structure tips pressing against the membrane causes membrane curvature and in turn, modulates the composition of proteins and lipids that regulate the membrane [86]. This process can induce membrane budding and deliver cargo into the cell [87]. However, further research must be done to elucidate the mechanism of how nanoneedle-like structures induce endocytosis and successfully deliver biomolecules into the cell. A shortcoming of this internalization process is that delivery efficiency is immensely dependent on factors such as pore size, biomolecule properties and the intracellular environment. Nanoneedles loaded with macromolecules have a lower delivery efficiency in comparison to structures loaded with smaller molecules due to the inverse relationship between molecular radius and diffusion rate [88]. Pore size also limits the types of biomolecules that can undergo diffusion [89].

Biosensing

Nanoneedles are excellent biosensors and are able to sense the intracellular environment due to their high aspect ratio [90]. The nanosized sensing area can detect minute changes at the molecular level and thus gives rise to its high transducer sensitivity. Nanosensors can also measure processes in real-time and thus collect real-time quantitative data. To successfully detect biological changes within a cell, properties such as the nanoneedle geometry (diameter, length and density), along with the type of cell it is interacting with should be investigated. These factors impact on the ability of the nanoneedle to obtain real-time quantitative data on the cellular environment [91].

Asif *et al.* fixed glucose oxidase-coated zinc oxide nanorods onto the tip of a glass capillary. These nanorods were then inserted and detected the intracellular glucose concentrations of human adipocytes and frog oocytes [92]. Similarly, Boo *et al.* manufactured a nanoneedle biosensor fixed onto a nanotube to monitor the effect of dopamine and glutamate levels on the development of neurological disorders, such as Parkinson's and epilepsy [93]. The nanoneedles served as an electrochemical nanosensor and collected real-time data at the neurotransmitter levels. Robinson *et al.* also developed a 4 μm vertical arrays with 150 nm diameter and 3 μm long substrate-fixed silicon nanowires using top-down nanofabrication to record intracellular activity, stimulate rat cortical neuronal activity and illustrate synaptic connections (Figure 5B). The nanowire consisted of a silicon core and sputter-deposited metal tip which facilitated electrical access into the inside of the cell. This core was wrapped by a glass shell to prevent current leakage. Using electrochemistry at the nanowire tips, the device could simultaneously measure and control the cell membrane potential [74].

Cancer therapy

The high loading efficiency of nanostructures can be applied to gene and cancer therapy. Gene therapy is commonly used in cancer treatment by targeting cell RNA biomarkers or delivering a gene via a vector. A significant challenge of gene therapy was found to be successful vector delivery into the cell [94]. To circumvent this problem, nanoneedles can be used to directly deliver cargo straight into the cytosol.

A study conducted by Chiappini *et al.* used Cy3-labelled GAPDH-siRNA and green fluorescent protein-expressing DNA plasmid retaining substrate-fixed nanoneedles on the cervical cancer cell line, HeLa cells, and monitored the release of cargo over 12–18 h (Figure 5C). The siRNA delivery to cells was successful 30 min post-injection, however was found to be quickly distributed throughout the cytosol with a transfection efficiency greater than 90% [44]. The successful siRNA transfection allowed for gene expression regulation.

Similarly, Shen *et al.* synthesized a self-assembly biodegradable substrate-fixed nanoneedles that delivered siRNA into the cell and impeded on vascular endothelial growth factor (VEGF) expression in mice tumors. There was a noticeable reduction in VEGF levels, a biomarker for lymphoblastic leukemia, which led to a decrease in tumor

size [95]. Both studies emphasized the important role of nanoneedles in cancer diagnosis and its potential use as chemotherapeutics.

Tissue regeneration

Recently nanoneedles have also been applied to regenerative medicine. This structure can provide a minimally invasive intracellular delivery method to help tissue regeneration. Angiogenesis is a crucial step in wound healing and scar-tissue remodeling. Through the controlled intracellular delivery of DNA and siRNA, biodegradable substrate-fixed nanoneedles were used to transfect the *VEGF-165* gene, an angiogenic gene. Chiappini *et al.* compared the difference between the nanoinjection and direct muscle injection of 100 μg of human *VEGF165* plasmid DNA. Although both methods expressed *VEGF165*, mice treated with nanoinjection were found to have a significantly higher expression of human *VEGF165* in comparison to the direct injection mice. The transfection successfully induced neovascularization and mediated an increase in blood perfusion in mice (Figure 5Di, ii [44]).

Zong *et al.* manufactured Cu-containing TiO_2 nanotubes which acted to upregulate nitric oxide (NO) synthesis and VEGF secretion from endothelial cells (Figure 5Diii, iv [75]). Significantly higher NO fluorescence was exhibited in endothelial cells with 4.62 and 15.14% in comparison to 0 and 2.69%, respectively. After 4-h incubation, greater node formation was noted in higher Cu^{2+} concentration nanotubes. Since Cu^{2+} upregulates hypoxia-inducible-factor-1α, a factor known to promote VEGF expression, this stimulated greater nitric oxide synthesis [44]. Therefore, nanoneedle-like structures can enhance early tissue regeneration by stimulating growth factor secretions.

Surgery on single cells

AFM is a useful tool for nanosurgery. AFM is commonly used to image cell surfaces, allowing researchers to further investigate intracellular physiological conditions. The probe utilized in the AFM system can be modified to induce chemical modifications on cell surfaces. Thus, by manipulating the AFM tool, a system involving a cantilever and fixed nanoneedles was developed. This system opened a vast range of possibilities as it was found to be capable of penetrating both the membrane and nucleus [76]. Studies also loaded or coated the needle surface with molecules such as proteins and nucleic acids. Obataya *et al.* demonstrated the penetration of cell using a cantilever-fixed nanoneedle using confocal imaging (Figure 5E).

The applications of nanosurgery are significant as nanosurgery would enable researchers to study single cells with high accuracy and minimal cytotoxicity. However, this has proven to be difficult as a major factor that influences cell cytotoxicity is the load force. Ashrf *et al.* designed and tested a unique solid nanoneedle of 104 and 250 nm diameter on the tip of a flexible AFM cantilever to determine the optimal load force [96]. This structure was utilized on a hypo elastic single spherical cell model using a transverse load force ranging from 10 to 100 mN. It was found that this design required low forces for successful cell manipulation and surgery. Durability is another factor that greatly influences nanoneedle penetration. Han *et al.* determined that a tapered nanoneedle tip provided greater durability than a cylindrical nanoneedle tip. However, simple cylindrical nanoneedles demonstrated a high insertion efficiency of 54–95%, whereas the insertion efficiency of tapered nanoneedles varied from 49 to 92% [97]. Therefore, nanoneedle design can determine whether a nanoneedle will successfully penetrate the cell membrane.

Conclusion

Substrate-fixed nanoneedle-like structures possess a one-dimensional structure which allows them to penetrate the cell membrane with great specificity and deliver drugs and molecules directly into cell cytosols. These fixed nanostructures can be manufactured from biodegradable materials, such as polyglycolide, which degrades and dissolves by itself over time and does not generate a build-up of toxic by-products within the body [44]. Furthermore, adjusting the solubility and design of these materials can induce a controlled, sustained release, thus increasing the length of therapeutic effect. Substrate-fixed nanoneedle-like structures are capable of collecting high resolution quantitative intracellular environment data, making them efficient biosensors [98].

Although fixed nanoneedle-like structures have been proven to be a practical tool, intensive research and skill are required in the complex manufacturing process. There are also multiple parameters that must be considered when designing nanoneedle-like structures, such as the solubility, density, geometry (length and diameter of needle) and insertion force [96]. All these factors must also be suitable for the cell type and membrane stiffness. The internal environment of the cell also influences the internalization drug delivery as it relies on cells to actively uptake the cargo, thus intracellular environment must be considered when developing the nanoneedle-like structures and

material [98]. Furthermore, the minute dimensions of nanoneedle-like structures only allows a limited number of cells to receive cargo, giving rise to the low throughput limitation of nanoneedle-like structures.

In summary, substrate-fixed nanoneedle-like structures have shown immense potential in the nanomedicine field. The high spatial and temporal precision further highlights the future prospective of using such nanoneedle-like structures to treat diseases in a targeted manner by delivering biomolecules. These structures can also provide a highly sensitive, noninvasive method of studying intracellular processes by acting as biosensors. However, further study on the mechanism of action is required as the precise mechanism of membrane penetration and diffusion is still unclear.

Future perspective

Nanoneedle-like structures have had a significant impact on multiple biomedical fields such as biomolecule delivery, biosensing, cancer therapy and tissue regeneration. These structures continue to show promising prospective in the future as they have considerable potential to revolutionize industries and improve peoples' lives. These high aspect ratio structures can deliver biomolecules into cells and thus can be applied to vaccine therapy. Delivering vaccines through nanoneedle patches is a painless vaccine delivery method that would significantly reduce the manufacturing costs. Furthermore, the high aspect ratio of nanoneedle-like structures allows them to act as biosensors with high temporal and spatial precision. Wearable nanobiosensors can become a noninvasive technique used to detect and monitor biomarkers for medical diagnosis and treatment.

However, several limitations highlight the importance of continuing research on these nanoneedle-like structures. There are still a great number of factors and safety concerns that need to be investigated. Disturbed cellular function was exhibited using confocal microscopy in HeLa cells post-nanowire piercing. Images also showed atypical contours on the cells seeded on vertical nanowires, most likely due to the nanowires preventing the cell membrane from attaching onto the substratum completely. Annexin V binding analysis also suggested that lipid scrambling occurred post-nanoinjection [96]. Although this study suggests that fixed nanowires do not induce significant disturbance, it is crucial to confirm that the nanoneedles do not impede on cellular function to generate accurate measurements. At last, in order to determine the full potential of such nanoneedle-like structures, research must be conducted to establish how these structures act upon cells.

Executive summary

Nanoneedle-like structures
- Definition: structures with high aspect ratio and one or more external dimensions in the size range 1 nm–100 nm.
- Several different types of nanoneedle-like structures: nanoneedle, nanorod, nanowire, nanofiber, and nanotube.
- There are two different methods of applying nanoneedle-like structures: mobile and immobilized.

Historical development of nanoneedle-like structures
- The term 'nanotechnology' was first coined by Norio in a conference in 1974.
- The term 'nanoneedle' was first mentioned by Heike in 1995.

Types of nanoneedles
- Solid nanoneedles are commonly used to penetrate cell membranes to access cytosol.
- Coated nanoneedles are used to detect cytoskeletal components.
- Biodegradable nanoneedles are nanoneedles which slowly degrade over time and can be used for sustained drug release.
- Hollow nanoneedles have been used to inject theragnostic agents into cells.

Nanoneedle fabrication methods
- Chemical etching process is a top-down method where chemically active plasma containing positively and negatively charged ions react with material to carve out details.
- Photolithography is a process involving the exposure of photoresist layers to ultraviolet rays which results in the formation of nanostructures.
- Exfoliation is the method that involves the expansion of materials using heat to create nanostructures.
- Chemical vapor deposition requires the reaction of precursors on preselected substrates at high temperatures to form nanostructures.
- Physical vapor deposition requires vaporization of molecules which are then transported through a vacuum and deposited onto a substrate through condensation to improve the substrate's surface properties.
- Self-assembly exploits polymers to create nanostructures.

Delivering biomolecules into cells
- One-dimensional structure allows nanoneedle-like structures to penetrate cell membranes and deliver biomolecules.

- Insertion force, operating temperature and approaching velocity are factors which must be suitable when inserting needle into cell.
- Membrane bulging occurs when intracellular fluid leaks due to needle penetration.

Biosensing

- High aspect ratio of nanoneedle-like structures allows these structures to detect intracellular environment and collect real-time quantitative data.

Cancer therapy

- Nanoneedle-like structures can be applied to gene and cancer therapy.

Tissue regeneration

- Minimally invasive intracellular delivery of DNA and siRNA using nanoneedles and nanotubes significantly increased neovascularization and tissue regeneration respectively.

Single cell surgery

- Atomic force microscopy system with a fixed nanoneedle at the end of the cantilever can be utilized in single cell surgery.
- Load force, durability and insertion efficiency are factors to be considered when designing nanoneedle-like structures to be used in single cell surgery.

Financial & competing interests disclosure

D.V. Nguyen and L. Kang are the inventors of a microneedle patent licensed to Nusmetics Pte. Ltd, a co-founder and shareholder of Nusmetics Pte. Ltd, respectively. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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