Synthesis, Characterisation and Cytotoxicity of Gold Nanowires for Ultra-Sensitive Biosensor Development

Nurul Akmal Che Lah
Universiti Malaysia Pahang Fakulti Kejuruteraan Pembuatan  https://orcid.org/0000-0001-8530-1061

Robert Gray
University College London Medical School

Sonia Trigueros (✉ sonia.trigueros@zoo.ox.ac.uk )
https://orcid.org/0000-0001-5621-7303

Research

Keywords: nanobiosensor, biomarkers, antimicrobial, cytotoxicity, gold nanowires

DOI: https://doi.org/10.21203/rs.3.rs-49469/v1

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Abstract

With the long-term goal of developing an ultra-sensitive microcantilever-based nanobiosensor for versatile biomarker detection, new controlled bioreceptor-analytes systems are being explored to overcome the disadvantages of conventional ones. Gold nanowires (AuNWs) have been used as a probe to overcome the tolerance problem that occurs in response to changes in environmental conditions. However, the cytotoxicity of AuNWs is still unclear. Here, we examined the cytotoxicity of AuNWs systems using both commercial and as-synthesised AuNWs. In vitro experiments show that commercial AuNWs with an average quoted length of 6 µm are highly toxic against Gram-negative Escherichia coli (E. coli) at 50 µg/ml. However, this toxicity is due to the presence of CTAB surfactant no by the nanostructure. Conversely, the as-synthesised AuNWs with an average length of 9.5 µm show non-cytotoxicity even at the maximum viable concentration (330 µg/ml). These findings may lead to the development of potentially life-saving cytotoxicity -free nanobiosensors for an early diagnostic of potential diseases.

1. Introduction

A wide array of biomarkers secreted by cancerous cells can be found in blood samples [1–3]. With current emerging proteomics technologies, it is straightforward to obtain reliable sets of disease-specific protein biomarkers [4–6]. Early identification of disease blood protein signatures, is becoming the most promising strategy for effective cancer prevention. Nevertheless, some protein biomarkers, such as those secreted by cell death in tumours at a very early growth period, are produced only at ultra-low concentrations [7–10]. Engineered nanomaterials-based biosensors with high sensitivity levels have been the focus of improved biomarker detection technology [11–14]. Low reproducibility highlights the difficulty in detecting low concentrations of disease protein biomarkers. With concentration, the scales are six to seven orders of magnitude lower than the plasma protein (e.g. the reference range of albumin protein in blood is approximately 35–50 g/L) [15–17].

Nanomaterial-based microcantilever (MC) biosensors have good potential in advanced biosensor technology as they offer a high surface-to-volume ratio [27–29], so they are able to mediate faster and with a higher kinetic electron transfer [30, 31]. The size of nanomaterials can also facilitate effective interaction with targeted biomolecules. Furthermore, since the dimension of nanomaterials is comparable to the Debye length [32–35], their specific surface properties affect the kinetics and thermodynamics of electron transfer significantly [36, 37]. Offering potential for electronic detection of surface functionalisation. Gold (Au) based one-dimensional nanomaterials are a candidate for achieving an ultrasensitive MC biosensor device [38–40]. Specifically for flexible chemoresistive sensors, Au nanowires (AuNWs) are attractive because they possess minimal cross-sectional area which can increase the flood-current along the axial current direction, resulting in higher conductance changes compared to the typical zero-dimensional nanomaterials [41–44]. The chemoreceptive characteristics of AuNWs are key considerations in designing an MC nanobiosensor. Chemoreceptive and also conductometric characteristics are both important for biosensor transduction mechanism. A change in the conductance
that takes place upon binding with a targeted biomarker can be detected, meaning AuNWs have great potential as ultrasensitive MC nanobiosensors [45–47].

There have been many concerns about the potential cytotoxicity of nanomaterials in general, which may arise upon medical application. It has been proved that several quantum materials consisting of heavy metals may release ions that may cause cytotoxic effects [48, 49]. Although, AuNWs are not considered toxic in most cases [50], there is certain ambiguity about AuNWs toxicity impact regarding variability and threshold for specific cell types.

Therefore, there is an urgent need to present new data that can assist in developing a mass-sensitive MC-based AuNWs biosensor. Important primary parameters that are involved in cytotoxicity assessments of AuNWs include the aspect ratio of nanowires; Surface functionalisation method; Cell type; Administration of dosage and application protocols.

In this work, we describe the synthesis of bio-friendly AuNWs for MC biosensor applications. The objective of this article is twofold. The first, is to ascertain the maximum dose of as–synthesised AuNWs, which is safe to avoid initial cytotoxicity whilst increasing the sensitivity of the nanowires array in comparison with commercial AuNWs. Bacteria cells are the most sensitive cells to detect toxicity arising from either free or nanostructurated metallic ions [51]. At the same time, bacterial detection will an application for ultra-sensitive nanosensors. For toxicity detection, we use a set of antimicrobial approaches. The transduction mechanism of nanosensors depends on the change in conductivity due to the binding of the protein of interest. AuNWs play a vital role in the transduction mechanism. Hence it is essential to maximize the concentration of AuNWs to achieve maximum sensitivity. The investigation of transduction mechanism is not within the scope of this study. The second objective is to achieve assembling nanowires on the top surface of the Microscopy Probe. Any biomarker should be able to be detected using this platform.

2. Materials And Methods

2.1 Ultra-thin AuNWs Preparation

Oleylamine, OA (technical grade 70%, Merck Sigma-Aldrich, United Kingdom). TIPS (98%, Merck Sigma – Aldrich, United Kingdom, HCl, Merck Sigma-Aldrich, United Kingdom) and hexane (anhydrous, 95%, Merck Sigma-Aldrich, United Kingdom). Commercial AuNWs (dispersion in H2O, contains CTAB as a stabiliser) used was purchased from Merck Sigma-Aldrich, United Kingdom.

2.2 Microbial Cell Culture Preparation

In this article we used commercial *Escherichia Coli (E.coli) DH5-Alpha strain (DH5α)*, an engineered non-pathogenic strain of used for routine subcloning procedures. It is quick and easy to grow and has been used extensively as a lab microbial model system. (ThermoFisher cat number 18263618). E. coli cells were grown in Luria-Bertani medium (LB), rich medium for cell growth. The cells were initially grown from
an original culture sample, kept at -80 °C. Cells were inoculated onto an LB agar plate and incubated at 37 °C for 24 hours. At this point, a number of individual colonies had grown, each from a single cell. To reduce variation across the bacteria under study a single colony was selected and incubated in LB to produce a working cell solution. This was used as the master cell culture for all the bacteria cell experiments. Optical cell density measurements were acquired using a Perkin Elmer Lambda Bio + spectrophotometer (Germany). The spectrophotometric method measured the optical density at 600 nm based on an American Public Health Association (APHA) standard. The optical density (OD) of 0 is set for pure LB, and the spectrophotometer is calibrated so that the OD equal to 1.00 corresponds to a cell density of 109 ± 5 x 108/ml. A linear relationship between OD and cell density is achieved over this range.

2.3 Cytotoxicity Validation methods

2.3.1 Disk diffusion assay

Cytotoxicity test is carried out through the agar disk diffusion assay method which has been used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. The standard measurement is based on the Clinical Laboratory Standard Institute (CLSI) and manufacturers' standard for each sample. The AuNWs colloidal solution (300 µg/ml) was suspended in distilled water and this portion deposited on the sterile paper disk that is 6 mm in diameter with 4 replicate disk per sample. The disk was placed on Gram-negative Escherichia coli (E. coli) DH5α strain. Controls were prepared using pure hexane and/or distilled water.

E.coli DH5α were inoculated at a concentration of 107 to 108 cells/ml at stationary phase strain onto Luria-Bertani (LB) agar. The LB agar plates were then inoculated and incubated for 24 hours at 37 °C to record the cytotoxicity effect. Overnight incubation made the cells cover almost 70% of the growing surface, except for the circles of growth surrounding disc which remained clear. The diameters of these circles are then measured and used as measurements of the inhibitory effects of the sample.

2.3.2 Colony Forming Unit Counting Method (CFU)

Methods for in vitro evaluation of antimicrobial activity, seek to assess the total number of E.Coli cells in culture media. [52]. Colony forming units (CFU) of E. coli DH5α were counted after plating triplicate serial dilutions of LB cultures on LB-Agar plates and incubating overnight at 37 °C. Plates containing between 2 and 200 individually identifiable colonies were counted. The relative number of colonies on a plate compared to the control gives a value which is a measurement of the toxicity.

2.4 Nanostructures Characterisation Methods

2.4.1 Characterisation of as synthesized and commercial AuNWs

AuNWs were resuspended and sonicated in ethanol medium. And let it dry on an aluminium substrate at room temperature. FESEM imaging was conducted by a JEOL JSM 7500 F microscope equipped with a
cold field emission gun at an acceleration voltage of 15 kV. Secondary electrons and backscattered electrons signals respectively were collected by an in-lens detector and in-chamber multi-quadrant annular retractable solid-state detector. Element analysis was carried out using Zeiss Neon 40EsB (Carl Zeiss NTS GmbH, Oberkochen, Germany) at 20 kV equipped with EDX (Inca software, Oxford Instruments). Image analysis on FESEM data was carried out using ImageJ 1.50i [44–46]. AuNWs density was determined using the plugin ‘analyse particles’ function of ImageJ on a total of 20 SEM images of AuNWs samples. The aggregated size of AuNWs was calculated from a total of 2000 randomly picked aggregated AuNWs from FESEM images.

2.4.2 Assembly on cantilever

AuNWs were assembled onto a cantilever surface by AuNWs-ethanol droplets evaporation. Aluminium-coated cantilevers (Concentric GmbH-Switzerland) of (400 um long, 1 um thick and 100 um wide) were used in the process. 20 µl of the colloidal solution was sonicated on an Ultrasonic Baths for 15 min. A 5 µl drop of the solution was added onto the cantilever surface. After letting the drop air dry for 24 h, AuNWs were adhered to the surface. The AuNWs assembly was imaged using FESEM.

3. Results

3.1 Synthesis of Ultra-thin AuNWs

AuNWs were synthesised based on the method described in the literature [53]. The synthesis reaction involved mixing 100 µl oleylamine (OA) and 150 µl triisopropylsilane (TIPS) with about 3–5 mg gold (III) chloride solution, (HAuCl4 99.9% trace metals basis, 30 wt.% in dilute HCl,) in 2.5 ml final volume of hexane. Nanowires produced, according to the published method, have diameters of about 2 nm, lengths of a few µm and self-assembled on deposition into organised structures. In this work, the resulting AuNWs were 3 nm in diameter with a standard deviation of 0.8%. The relative concentration of the AuNWs obtained was > 50 µg/ml. A thin layer of AuNWs arrays were formed through drop coating of the AuNW solutions on silicon SEM discs (Fig. 1(A)).

3.2 Characterisation of Synthesised Ultra-Thin AuNWs and Commercial AuNWs

Samples of As-synthesised AuNWs obtained were characterised for their physicochemical properties by standard techniques. Figure 1(B) show morphology, distribution and purity of synthesised AuNWs verified by FESEM analysis with EDX. The size distribution of parallel AuNWs bundles was difficult to measure. Higher magnification of the assembled ultra-thin AuNWs was unachievable due to their highly sensitive towards the electron beam which resulted in melted nanowires within a few seconds of exposure. However, we expected that the ultra-thin AuNWs possess diameters of approximately smaller than 3 nm with an aspect ratio (length-to-width ratio) above 1000 nm. In this case, the AuNWs tended to form a stack of parallel bundles which then self-assembled into 2-D network structures over macroscopic distances through spontaneous directional aggregation that occurs during solvent evaporation. The
directional aggregation is typically formed via oriented attachment in which AuNWs are permitted to fuse together as the chemical potential between each chain is different [54]. Therefore, the smoothing extension process to interconnect the nanoparticles of the nanowires takes place through diffusion. The use of TIPS in the synthetic reaction at room temperature controls the acceleration of the process. AuNWs exhibit higher stability in polar solvent which is favourable for the subsequent immobilisation of biomolecules.

The primary objective of this work is to produce ultra-thin AuNWs with high aspect ratio. Also analyse quantitative and qualitative properties of the as-synthesised AuNWs by EDX analysis. Figure 1(C) shows the nanoscale characterization of the chemical composition of obtained nanowire samples. For as-synthesised AuNWs, the amount of Au is at the level of 98% with the remaining concentration of Si (substrate). The presence of high purity Au resulted from homogeneous nucleation of metallic Au as spherical clusters and hence determined the growth of 2-D nanowires. Demonstrating that in the presence of TIPS, Au seeds have a rapid kinetics in the formation of nanowires. EDX point measurements were carried out for an accurate estimation of Au amount presence in each sample.

Figure 1

(A) As-synthesised AuNWs reaction formation. The reaction product has a dark violet colour solution after 24 h. (B) FESEM and (C) EDX analyses of AuNWs. EDX spectrum recorded from the area of AuNWs presence.

During the synthesis reaction, the potential of Au + reduction to Au is higher in the presence of TIPS. The primary limiting factor for constant electron transfer rate would be the TIPS reagent. [55]. During the synthesis reaction, in the absence of heat, a colour change occurs from a light orange to red followed by dark violet during the period of reaction as shown in Fig. 1(C). For a 24 h reaction, the resultant colloid colour is dark violet which indicates extended filament-like structures. The ultra-thin self-assembled networks of individual crystalline AuNWs with a gap distance of ~ 2 nm can be of use to trap the analyte molecules and automatically locate them within the gap of closely packed parallel AuNWs making them as suitable substrate candidates for SERS studies due to the presence of closely packed ‘hotspot’ [56].

Representative FESEM images of commercial AuNWs suspended in CTAB aqueous solution are shown in Fig. 2. It can be seen that the nanowires are less than 35 µm in length (Fig. 2(A)) and build up a cross network which aggregated randomly (Fig. 2 (B and C)). The diameter of the nanowires is not uniform and has a size ranging from ~ 50 nm to ~ 200 nm. The network formed did not show any self-assembling properties. Commercial AuNWs are randomly connected to each other. The cloudy region encircling AuNWs is due to the capping action of CTAB as the solvent evaporates leaving the residue behind. The CTAB monolayer micelle helps to stabilise Au nanowires. This stabilization effect, while making them soluble, can be an inhibitor of subsequent self-assembling process.

Bottom-up synthesis of AuNWs which is easy and highly effective method for rapid synthesis of single-crystal ultrathin (below 4 nm ) AuNWs. This method do not require any mechanical stirring, it is
performed at room temperature. This method can also be applicable for the synthesis of other metal nanowires as long as the chemical combination is suitable and correct. The as-synthesised ultrathin AuNWs have a tendency to self-assemble into 2-D nanowire networks and closely packed forming parallel structures when viewed under the SEM. The nanowire networks could serve as a well-defined surface-enhanced SERS-active system. The ultrathin nanowires assembly could allow trapping of molecules for molecular recognition and signal amplification.

Although, the thinner AuNWs the better to increase the nanosensor sensitivity, commercial AuNWs are ideal for the MC deposition. Therefore, more studies need to be undertaken to elucidate the synthesis of thinner and stable AuNWs with both high defect characteristic and intrinsic quality to allow better operation and function at a higher temperature.

Correspondingly, the proof-of-concept on the cytotoxicity of the nanosensors-based nanowires requires long acquisition times. Some nanowires synthesis protocols, such as chemical reduction method can introduce toxic materials from the capping agent used to stop the reaction. Also, the metal itself can produce toxicity, being unfavourable for \textit{in-vivo} applications. For this reason, we examined the potential cytotoxicity of both types of AuNWs.

3.3 Cytotoxicity Evaluation of As-Synthesised and Commercial AuNWs

To study the potential cytotoxicity of synthesised and commercial AuNWs, in this work we used a commercial bacteria Escherichia Coli (E.coli) DH5-Alpha strain (DH5\textalpha{}), an engineered non-pathogenic bacterial strain of used extensively as a lab Cytotoxicity microbial model system.

In the work we used several test, first use the disk diffusion method (Fig. 3). This method is performed to detect cytotoxicity by inducing a gradient of concentration around a disk loaded with AuNWs. The gradient of AuNWs halts the growth of bacteria, and creates an inhibition area around the disk. The ratio of the area (measure in mm) is directly proportional to the sample toxicity (White marks). Non-toxic samples will not form any inhibition area (red circles) (Fig. 3(A)).

For further validation of toxicity detection of our nanostructures, we performed CFU counting experiments on commercial and as-synthesized AuNWs. Pure Hexane, H\textsubscript{2}O and ionic gold at 3 different concentrations were use as negative controls (Fig. 3B). Cell-Viability assessed by CFU remained at 100% on control samples. For 10 µg/ml Au ion, the CFU counting measured a viability of 72%. At 30 µg/ml Au ion, approximates value were almost similar to the other one giving rise to 69% for CFU counting. Cells incubated with 100 µg/ml of commercial AuNWs and both 2 µg/ml and < 300 µg/ml of as-synthesised AuNWs, respectively, cell viability were 0% for all in CFU counting. Pure hexane also showed a cell viability of 0%. Assessment at 96 hours was likely to be optimal for AuNWs strain. Indeed, the effect of AuNWs and high dosage of Au-based ion seemed strong antibacterial agent in this assay. This corroborated the result that AuNWs work markedly better, meaning presents less bacteria toxicity than Au ions as happened to other metallic materials [57, 58].
Our results suggest, that nano wire-structured Au do not induce any antibacterial activity or toxicity effect on bacterial cells. Therefore, the toxicity effect observed in our experiments using commercial AuNWs could be induced by the presence of the commercial capping agent, in this case CTAB. CTAB or Cetrimonium bromide, is a quaternary ammonium surfactant. It is one of the components of the topical antiseptic cetrimide. The cetrimonium cation is indeed an effective antiseptic agent against bacteria and fungi. To clarify the inhibitory antibacterial activity observed is induced by the CTAB layer, commercial AuNWs were purified from CTAB capping layer and re-dispersed in 2 mM sodium bicarbonate (NaHCO₃). Cells grown in LB media were then observed AuNWs in CTAB and NaHCO₃ with an approximate volume of 250 µl for both. Water served as the standard control. The cell-nanowire-SB solutions were approximately 5 µg/ml, incubated for 90 minutes and was serially diluted to 105 times of the initial concentration with the set to dispense 100 µl onto plates. CFU showed about 130% and 4% of capture efficiency for AuNWs in NaHCO₃ and CTAB solutions Fig. 3(C). The low CFU in the CTAB solution were found to be threefold lower than in NaHCO₃, indicating that the toxicological effect of CTAB is quite high which significantly displayed susceptible inhibit growth of E.coli cells. NaHCO₃ had lowest inhibitory effect.

3.3 Assembly of AuNWs on Cantilever

Integrating AuNWs onto a cantilever is crucial for the exploration of a multifunctional MC biosensor. To functionalise the cantilever, AuNWs were attached to an aluminum coated AFM cantilever surface. The surface of the cantilever was covered with AuNWs solution, and the nanowires on the surface repel each other and assemble. In this case, for the functionalization process we used commercial AuNWs and the evaporation process described in material and methods. The resulting attachment between the surface of the cantilever and AuNWs is shown in Fig. 4. This assembly is adequate for MC biosensor functionalization viability. Here, we demonstrated a direct depositing of AuNWs and its binding tendency onto the cantilever.

4. Discussion

The undoubted benefit of the MC surface Au nano-structuration is the expansion of the cantilever limited sensitive range. It is well known that one of the utmost drawbacks of micro or nanoscale sensors is their low dynamic range. A higher-performance functional surface of AuNWs could increase the binding capacity, and therefore, increasing the sensor detection to a broader range of molecule type and concentrations.

Minimising the thickness and width of the sensing element is also essential to retain an excellent signal efficiency from a strain-sensing component of MC sensor. The thickness of the AuNWs is in the range of 80–100 nm and width of 2.47 mm, which rules out the employment of sensors made of conventional self-sensing elements including Si and PZT [59]. The small diameter with a much longer length of AuNWs permits the fabrication of strain sensors on small MCs without significantly affecting their dynamic properties. Also, the achievable wider sensor length is crucial in maintaining low resistance and a very
high sensitive detection bandwidth. Altogether, the fabrication example in this study, highlights the potential multifunctional design of AuNWs-based MC probes. Also take into account that bulk fabrication of the MC probe at the larger scale could be possible.

5. Conclusions

As discussed, de novo synthesis of AuNWs and nano-assembly for MC biosensor has tailored ultra-thin nanowires that formed irregular chain-like oriented nanowires. These ultra-thin AuNWs are around ten times smaller than the conventional ones but are unstable at relatively high temperatures, melting within a few seconds of heat exposure. Our results indicate that the longer and thin commercial AuNWs are stable at high temperatures and the toxicity observed is due to the antiseptic CTAB used as capping agent and not due to the material at the nanoscale. AuNWs capped with NaHCO₃ show low cytotoxicity making them the optimal material for integrating and implanting in MC biosensor devices including high-throughput applications.

Declarations

Author Contributions:

ST. conceived and designed the experiments; ST, RG and N.C.L performed the experiments. ST, RG and N.C.L wrote the manuscript.

Acknowledgments:

All individuals listed as authors have contributed substantially to the experimental and discussion of the reported work. ST and RG were supported by EPSRC IAA D4D00620. S.T. also acknowledges support from the Oxford Martin School. N.C.L was supported by University Malaysia Pahang RDU1703152 and RDU190360.

Conflicts of Interest:

“The authors declare no conflict of interest.”

References


16. Oran PE, Trenchevska O, Nedelkov D, Borges CR, Schaab MR, Rehder DS, Jarvis JW, Sherma ND, Shen L, Krastins B, Lopez MF, Schwenke DC, Reaven PD, Nelson RW. Parallel Workflow for High-


57. El Kurdi R, Patra D. Amplification of resonance Rayleigh scattering of gold nanoparticles by tweaking into nanowires: Bio-sensing of α-tocopherol by enhanced resonance Rayleigh scattering of curcumin


Figures

Figure 1

(A) As-synthesised AuNWs reaction formation. The reaction product has a dark violet colour solution after 24 h. (B) FESEM and (C) EDX analyses of AuNWs. EDX spectrum recorded from the area of AuNWs presence.
Figure 2

(A) FESEM images of sonicated commercial AuNWs with CTAB as a capping agent. (B), (C) and (E) individual Au nanowires. (F) AuNWs average length of 6.5 µm.
Figure 3

(A) AuNWs antimicrobial activity by disk diffusion assay. Disc diffusion assay results with E.coli cells (100µl) on LB agar overlay. White lines mark the diameter of the white zone where the growth of bacteria is prevented by the AuNWs (inhibition area). Disk diffusion measurements obtained using several concentration of ionic gold and nanowires. The bars represent one standard error. (B) AuNWs antimicrobial CFU assay. Control H2O, 10, 30 µg/ml and 100 µg/ml of ionic gold, 2 µg/ml of commercial and < 330µg/ml as-synthesised AuNWs suspended in CTAB and hexane, respectively. (C) Antimicrobial CFU assay of AuNWs capped with CTAB and NaHCO3 after 24 hours of plate-incubation. In this experiment H2O is used as control.
Figure 4

FESEM micrograph of commercial AuNWs deposited on the aluminum coated AFM cantilever surface. The formation of the scaffold is due to the coffee-ring effect during the evaporation process. AuNWs are deposited along the solid-liquid contact line.