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How to execute nano research in dentistry- a narrative review

Abstract

Nanotechnology can lend several multipronged theragnostic applications which could revolutionize dental practice. The objective of this review is to provide the elementary and applied knowledge of nano-particles that would complement the dentist to conduct nano-particles based dental materials research and innovation. The basic knowledge of applied and elementary sciences like cytotoxicity, microbial tests, microhardness and strength, tribological assays, characterization of the shape, size, and distribution of the developed materials at a molecular scale is required. Understanding some ancillary tools, equipment and sites for material procurement would complement the journey to the desired results. This paper will give an insight into the collaborations required when one ventures into this arena. A comprehension of the various alliances would ease a researcher in exploring the material sciences world for developments of adhesives, E-chains, wires, and appliances that are smarter, and biologically and mechanically more efficient.

Keywords: nanotechnology, nanoparticles, dentistry, tests, nanometrology, analytical chemistry

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Panchali Batra,[1] Jahirul Ahmed Mazumder,[2] Sanjay Miglani,[3] Sakshi Katyal,[4] Arisha Izhar,[5] Muskan Jakhal[6]

[1]Professor, Department of Orthodontics, Faculty of Dentistry, Jamia Millia Islamia, India
[2]Post Doctorate Fellow, Department of Chemical Engineering, Khalifa University of Science and Technology, UAE
[3]Professor, Department of Conservative Dentistry & Endodontics, Faculty of Dentistry, Jamia Millia Islamia, India
[4]Senior Resident, Department of Dentistry, All India Institute of Medical Sciences, India
[5]Specialist Orthodontist, Bellevue Medical Center, UAE
[6]BDS Student, Faculty of Dentistry, Jamia Millia Islamia, India

Correspondence: Panchali Batra, Professor, Department of Orthodontics, Faculty of Dentistry, Jamia Millia Islamia, New Delhi, India, Tel +91-9999908022

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Introduction

Nanotechnology has come a long way to find its application in almost every field of science and technology.[1–3] Nanotechnology is the science and technology that concerns the structures at nano scale.[4] The confederation of nanotechnology with the field of dentistry has given rise to new stream called Nano-dentistry. It is the science and technology of maintaining near-perfect oral health through the use of nanomaterials including tissue engineering and nanorobotics.[5] Despite commendable scientific advancements in the field of nanotechnology and its palpable contributions in the field of medicine, there remains a paucity of research on applications of this technology in the field of dentistry.

However, to conduct any research; basic knowledge of applied sciences would be beneficial. Any material development would require elementary tests like cytotoxicity tests, microbial tests, microhardness, strength tests, tribological assays, and characterization of the shape, size, and distribution of the developed materials at a molecular scale. Moreover, the development of nanomaterials would require an understanding of some ancillary tools, equipment, and sites for material procurement.[6]

The purpose of this article is to guide a young researcher who is keen to experiment in this area of material sciences. The following manuscript would answer the most common question that would come to mind when commencing a nano-research:

Which nanoparticle to use?

The choice of nanoparticles depends primarily on the properties that are desired. If the goal is to fabricate an antimicrobial material/coating, one could select from Nanoparticles that have good antimicrobial propertiesand thus should have enhanced reactivity properties and appropriately small size like Silver (Ag), iron oxide (Fe_3O_4), titanium oxide (TiO_2), copper oxide (CuO), and zinc oxide (ZnO).[7] For targeted drug delivery, nanoparticles that are highly biocompatible and biodegradable, are generally used like biopolymeric nanoparticles (Chitosan, alginate, cellulose, Polymeric micelles etc.) and inorganic nanoparticles such as metallic nanoparticles, quantum dots etc.[8] If the objective is to fabricate a lubricant that could reduce friction,nickel-phosphorus and tungsten disulfide, carbon nitride, etc can be used.[9]

The next question is whether to use commercial or indigenously synthesized nanoparticles. If you are planning to develop a material that can be escalated to a commercial scale, it will be beneficial to use commercially available nanoparticles which are developed in bulk and their properties are tested before releasing them in the market. The process of synthesis of nanoparticles significantly affects the unique physicochemical properties as well as the biosafety of the nanoparticles, thus one needs to be careful in indigenous synthesis.[10] When nanoparticles are synthesized by biological means for example using plants or fungi,they are developed in small quantities, also even if an investigator is using the same methodology, the second time, there are chances that slight variation in properties are present of the newly developed nanoparticles.Another issue with green synthesis is that the procedure is dependent on the availability of reducer (plant bacteria or fungi) and its availability cannot always be guaranteed in ample always. Furthermore, synthesizing, purifying followed by characterization of nanoparticles in the laboratory, is not economical requires large resources, and is often a cumbersome process. The advantage of indigenoussynthesis is that processes like tunneling the shape, size, and property of nanoparticles are comparatively easy as compared to commercial ones. Moreover, in the case of synthesis, we will be well aware of the type of reducing agents or chemicals that we are using for the process for their biocompatibility and toxicity assessment.

Another factor that should be considered is whether the study is going to be funded or non-funded**.** The cost of the study includes the cost of purchasing or synthesizing the nanoparticles, which could be variable depending on the size, type, and amount of the nanoparticles

that are required. For example, gold nanoparticles would cost much more as compared to Zinc oxide nanoparticles for the same size and amount. Apart from this, the cost of the various laboratory tests required to check for the properties and biocompatibility of new material should be included and kept in mind while planning.

From where to procure the nanoparticles?

Procurement of nanoparticles can be done from many companies that are involved in the fabrication of engineered nanoparticles directly from the websites of these companies. A few of them are Nanoshel,[11] Sigma-Aldrich,[12] nanocomposix,[13] MKnano,[14] Sisco research laboratories[15] (Table 1).

Table 1 Source and website of organizations from where nanomaterials can be procured

Sl. No.	Source	Website
	Sigma Aldrich	https://www.sigmaaldrich.com/
	Nanocomposix	https://nanocomposix.com/
	Nanocs	http://www.nanocs.com/
	Nanopartz	https://www.nanopartz.com/
	Nanografi	https://nanografi.com/
	American Elements	https://www.americanelements.com/
	Nanostructured & Amorphous Materials Inc.	https://www.nanoamor.com/
	Strem Chemicals	https://www.strem.com/
	Nanocyl	https://www.nanocyl.com/
	SkySpring Nanomaterials	https://ssnano.com/
	Nanoramic	https://www.nanoramic.com/
	Nanothinx S.A.	https://www3.ubu.es/nanogentools/consortium-2/nanothinx-s-a/
	Nanoshel	https://www.nanoshel.com/
	US Research Nanomaterials	https://www.us-nano.com/
	Nanorh	https://www.nanorh.com/

Characterization of nanoparticles (NPs)[16]

In the case of indigenously fabricated NPs, their size, shape, and surface characteristicsdetermine their properties and hence must be characterized. Several characterization techniques are performed to reveal the physicochemical properties of the particles such as scanning electron microscopy (SEM), X-ray diffraction (XRD), infrared spectroscopy (IR), transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), and Brunauer–Emmett–Teller (BET) and particle size analyses.[17]

Ultraviolet (UV)-visible spectroscopy of NPs

It is important to check whether the NPs formed are the desired element or not. Hence the formation and purity of the sample is confirmed with ultraviolet (UV)-visible spectroscopy, in which a strong absorbance peak is observed corresponding to the element formed.[18] Ultraviolet (UV)-visible spectroscopy's principle is based on the absorption of ultraviolet light or visible light by chemical compounds, which results in the production of distinct spectra.[19] Spectroscopy is based on the interaction between light and matter. Every Nanoparticle has a unique optical property that is sensitive to the size, shape, concentration, agglomeration state, and refractive index of the nanoparticles, which makes UV-Vis a valuable tool for identifying and studying nanomaterials.[20]

Transmission and scanning electron microscopy

It is possible to use various procedures for estimating the size of the nano-materials such as TEM, XRD, and SEM, although the zeta potential/ dynamic light scattering (DLS) may be applied for finding the sizes of extremely small nano-materials.[21] Electron microscopy works by bombarding the sample with electrons and analyzing the resulting transmission (TEM) or scattering effects (SEM). TEM produces a 2D image whereas, SEM produces a 3D image, but the resolution of images is better with TEM.[22] TEM should be preferred for observing the size and shape of the nanoparticle, whereas for surface characteristics of the sample, SEM is the preferred method. Both these technique helps in identifying crystallinity and lattice structure.

Dynamic light spectroscopy(DLS)/ zeta potential size analyzer[23]

If one is incorporating or coating material with nanoparticles one needs tocheckforits size, charge (zeta potential), molecular weight, and stability at different temperatures and PH.DLS is a technique that can be used to determine the size distribution profile of small particles in suspension or polymers in solution. The polydispersity index in DLS also provides us with information on particle agglomeration and stability. Zeta potential indicates the stability of nanoparticles and their ability to adhere to cell membranes. Brunauer–Emmett–Teller (BET) is the best technique to determine the surface area of NP materials. This technique is based on the adsorption and desorption principle and the Brunauer–Emmett–Teller (BET) theorem. Normally nitrogen gas is used for this purpose.One should also check for the uniform distribution of the nanoparticles and for that polydispersity index (PDI) can be used.

X-ray diffractometer- XRD is one of the most important characterization techniques to reveal the structural properties of NPs like **composition, crystal structure, and crystalline grain size of nanoparticles especially of powdered nanoparticles.**[24] It gives enough information about the crystallinity and phase of NPs. It also provides rough idea about the particle size through Debye Scherer formula.[25] This technique works well in both single and multiphase NPs identification. Nevertheless, in the case of smaller NPs having size less than hundreds of atoms, the acquisition and correct measurement of structural and other parameters may be difficult. Moreover, NPs having more amorphous characteristics with varied inter-atomic lengths can influence the XRD diffractogram. In

that case, proper comparison of the diffractograms of bimetallic NPs with those of the corresponding monometallic NPs and their physical mixtures is required to obtain accurate information. Comparison of computer-simulated structural model of bimetallic NPs with observed XRD spectra is the best way to get good contrast.

X-ray photoelectron spectroscopy- XPS is considered to be the most sensitive technique and it is widely used to determine the exact elemental ratio and exact bonding nature of the elements in NP materials.[26] It is a surface-sensitive technique and can be used in-depth profiling studies to know the overall composition and the compositional variation with depth. XPS is based on the basic spectroscopic principles and a typical XPS spectrum is composed of the number of electrons on the Y-axis plot versus the binding energy (eV) of the electrons on the X-axis. Each element has its own fingerprint binding energy value and thus gives a specific set of XPS peaks.[27]

Fourier transform infrared spectroscopy

Vibrational characterization of nanoparticles is normally studied via FT-IR and Raman spectroscopies. These measurements are usually performed to identify and classify probable biomolecule functional groups that are attached to the nanoparticles. These techniques are most developed and feasible as compared to other elemental analytical methods.[28]

From where to procure the bacteria/microbes to be tested?

There are 647 culture collections in 70 countries registered with the WDCM (World Data Centre for Microorganisms) database as of 4 September 2013. There are around 11 culture collections registered with WDCM from Africa, 153 from America, 220 from Asia, 22 from Oceania, and 220 from Europe (www.wfcc.info). Some important cultural collections in the world along with their holdings can be obtained from the World Data Centre for Microorganisms.[29]

(Source http://www.wfcc.info/ccinfo/statistics)

Culture collections in India[29]

Although 27 culture collections in India are registered with world data centre for microrganisms but only a few provides regular services to the scientific community. Major among them are Microbial Culture Collection (MCC, Pune), Microbial Type Culture Collection (IMTECH, Chandigarh), National Fungal Culture Collection of India (Pune), National Collection of Industrial Microorganisms (Pune), agriculturally important National Bureau of Microorganisms and National Collection of Dairy Cultures (Karnal), etc. (Source http://www.wfcc.info/ccinfo/collection/col_by_country/i/91). Both MCC and MTCC are recognized as International Depositary Authority (IDAs).

For purposes of research, diagnosis, and education, bacterial samples are essential. Although there are several ways to store bacteria, the best strategy depends on the bacteria being stored, the goal of the experiment, and the survival of the cells. Bacteria may generally be stored viably for longer periods of time when storage temperatures drop. Cryoprotectants, however, are necessary to lessen cell damage brought on by the freezing process once the temperature has dropped below the freezing point.

The bacterial strain determines the precise period that a culture will stay alive in a given storage environment. Even though cell death during storage is unavoidable, it should be reduced as much as possible, even at the cost of usability. Regularly used (daily or weekly) bacterial cultures can be kept on agar plates or in stab cultures in a conventional refrigerator at 4°C. However, for optimal bacterial viability, more long-term preservation techniques should be taken into consideration if cultures won't be employed for more than a few weeks.

How to check for antimicrobial activity of a material?

The first question that would come to mind is against which bacteria should the antibacterial activity be tested? This is entirely based on the experimental needs. Since dentists work in oral microflora, one needs to know which bacteria are predominant in that microflora. If one is planning a disinfectant for the endodontic space, the bacteria of research could be facultative anaerobes or aerobes or some resistant species such as Enterococcus faecalis which is one of the main microorganisms associated with endodontic failures. If the research question is to study the antimicrobial activity of material against caries, gram-positive Streptococcus mutans (ATCC 25175, designated as Sm), an aerobic microbe involved in tooth decay could be of interest. For periodontal diseases Aggregatibacteractinomycetemcomitans (ATCC 33384, designated as Aa), an anaerobic microbe related to acute periodontitis could be studied.

Tests to check for antimicrobial efficacy

The third important aspect is which tests should be conducted to verify the antimicrobial efficacy of the material. Many tests can be used to check for the antimicrobial efficacy of the material. A few most commonly used ones are the disc agar diffusion (DAD) test, MIC, agar diffusion test-paper discs, and live dead essay with flow cytometry.

Disk agar diffusion (DAD) test- This test is also known Kirby-Bauer test. The bacterial cultures are grown on Mueller-Hinton (MH2) agar plates.[31] Bacteria samples from one- or two-over-night grown colonies are suspended in a test tube containing nutrient broth. A sterilized cotton swab is dipped in the resulting suspension, and a layer of bacteria is applied on the agar plates. Engineered nanoparticle-laden filter discs are applied on top of the bacterial cultures.[32] The side containing the particles is placed in such a manner that it faces downwards to ensure direct interaction with the agar and the bacteria, and the samples are incubated at 37 °C for 48 h. The inhibition zones around the filters are measured every 12 h according to the Kirby-Bauer test protocol (Bauer et al. 1966).[33]

a) MIC (minimum inhibition concentration)

The lowest concentration of a bacteriostatic antimicrobial ingredient or agent is known as the Minimum Inhibitory Concentration (MIC) (prevents the visible growth of bacteria). MICs measure the impact of decreasing antibiotic/antiseptic concentrations over a certain period on the inhibition of microbial population development and are used to assess the antimicrobial activity of diverse drugs. Since the concentration of the drug needed to produce the desired effect is typically hundreds to thousands of times lower than the concentration found in the finished dosage form, these evaluations can be very helpful during the R&D phase of a product to determine the appropriate concentrations required in the final product. To establish the MIC endpoint, different concentrations of the substances are injected with cultivated bacteria, and the results are assessed using agar dilution or broth dilution (macro or micro). Typically, organisms that contribute to an infectious process that justifies antimicrobial treatment are used in susceptibility testing.

b) Live dead assay with flow cytometry

For intracellular immunophenotyping, the capacity to stain live cells with a viability dye and maintain that staining pattern after fixation is essential. Dead cells being removed from the data enables more accurate cell population separation and identification. After fixation and/or permeabilization, the Invitrogen LIVE/DEAD Fixable Viability Dyes are fixable viability dyes that aid in precise cell viability measurement in samples.[34] The basis for Fixable Viability Stain Kits is the interaction of cellular proteins and a fluorescent reactive dye (amines). Since these dyes cannot pass through the membranes of living cells, only the proteins on the cell surface may interact with them, producing faint staining. More stronger staining can be achieved by the reactive dye penetrating the damaged membranes of dead cells and staining both the interior and outside amines. One of the most effective methods for quickly analyzing individual cells in a mixture is flow cytometry. In microbiology, flow cytometry enables the quick and accurate detection of one or more bacteria as well as the ability to gather data on the dispersion of those microbes inside cell populations. The use of flow cytometry may also result in a quicker method for determining the viability of microorganisms while also improving knowledge of all bacterial cells in a given population. Dead cells can significantly affect the dynamic range of detection and make it challenging to tell apart truly positive signals when using the flow cytometer as a method. Therefore, it is strongly advised that the samples have some type of viability stain.

Which tests to conduct to check for cytotoxicity on cell lines?

For any material to be used in humans, its cytotoxicity on cell lines needs to be checked. To be used in the oral cavity the materials cytotoxicity should be checked on oral cell lines such as Human periodontal ligament fibroblast cells (HPLF), Human gingival Fibroblast cells (HGF), and Human Pulp cells. The cell lines can be obtained from NCS Pune (National Centre for cell science), in India. Apart from these there are many internationally recognized organizations which deals with various cell lines, few of them are:

Established in 1998, CLS is an independent cell repository that focuses on the development and preservation of cell lines. American Type Cell Culture (ATCC) offers the largest and most varied collections of biological materials, including not just microbe but also cell products. The European Collection of Authenticated Cell Cultures (ECACC) is one of four Culture Collections of the UK Health Security Agency. They supply authenticated and quality-controlled cell lines, nucleic acids and induced Pluripotent Stem Cells (iPSCs).

The following tests are conducted to test the cytotoxicity of a material:

i. **Live and dead staining-** The most important criterion for distinguishing between viable and irreversibly damaged cells is membrane integrity. Live cells with intact membranes can exclude DNA-binding dyes that easily penetrate dead or membrane-compromised cells. This principle is routinely applied for microscopic live–dead discrimination (Nebe-von-Caron et al., 2000, Shapiro and Nebe-von-Caron, 2004).

ii. **Lactate production-**Cell death can be assessed with a series of assays, such as lactate dehydrogenase (LDH) leakage, ROS generation, caspase-3 activity, and DNA fragmentation. Assessments of membrane integrity such as lactate dehydrogenase (LDH) release assay, and measurement of metabolic activity such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay have been successful in the toxicity screening. Biomarkers for cytotoxicity and oxidative stress, namely reactive oxygen species (ROS), glutathione (GSH), malondialdehyde (MDA), and lactate dehydrogenase (LDH), can also be measured using 2′,7′- dichlorofluorescindiacetate (DCFH-DA), high performance liquid chromatography (HPLC), and LDH assay. Lactate dehydrogenase (LDH) activity in the cell medium can be determined using a commercial LDH Kit (Pointe Scientific, Inc., Lincoln Park, MI, USA).

Spectroscopy: Although conventional *in vitro* cytotoxicological techniques provide valuable information about the particle toxicity, the importance of gaining high content information in a single assay with the analysis of multiple parameters in a non-invasive and label-free way is still one of the biggest challenges in nanotoxicology. As a vibrational spectroscopic technique, the power of Raman spectroscopy for the analysis of cells, tissues and also nanoparticle localization within cells has been shown previously. Raman spectroscopy has its origin in the inelastic collision of photons with molecules and provides fingerprint information about the specimen under investigation (Wang 2018). Raman spectroscopy can be used to monitor dose and time-dependent changes of cellular constituents of different cell lines upon nanoparticle exposure.[35]

Which tests to conduct to check for uniform distribution of NPs?

It is important to check the uniform distribution of nanoparticles as there is a tendency for these particles to aggregate. Dynamic light spectroscopy (DLS) can be done for this purpose. The details are mentioned in the characterization section.

Which tests to conduct to check for bond strength?

If the material is developed to be bonded on tooth surface, e.g. nano-filled resin, its bond strength can be tested by:

Instron machine- Instron universal testing machine can be used for measuring tensile and shear bond strength. The rectangular specimens are inserted in relative jigs on a universal tensile testing machine and jigs are tightened. The apparatus exerts increasing tensile force at a crosshead speed of 5 mm/minute until the specimen fractures, and the load at the time of fracture is recorded as it appears in the computer monitor connected to the apparatus.

Adhesive remnant index (ARI) scores[36]**-** The shear bond strength of the bracket bonded with nanocomposite can be checked with ARI scores.ARI score is calculated based on the following scoring system under a 10× stereomicroscope (Olympus, Japan):

a) Score zero: Indicated absence of composite remnants on the enamel surface.

b) Score one: Less than 50% of composite remaining on the enamel surface.

c) Score two: More than 50% of composite remaining on the enamel surface.

d) Score three: The entire composite remained on the enamel surface with a clear impression of the bracket base on the remaining composite.

Which tests to conduct to check for micro leakage?

Numerous methods have been described to determine the sealing ability of endodontic restorative materials. Besides dye penetration tests, autoradiography, electrochemical methods, fluid filtration techniques, and bacterial penetration tests have been applied to determine microleakage.[37] Among these methods, dye penetration test is the most popular method for studying microleakage because they are easy to conduct. Dye penetration is observed at the tooth-restoration interface surface *in vitro* is then scored.

Which tests to conduct to check for insolubility of NPs?

When using any material in the oral cavity it is very crucial to determine the insolubility of the nanoparticles in saliva. For this atomic absorption test is used. It is based onabsorption spectroscopy and is one of the most widely used techniques employed for determining the concentrations of nanoparticles in solutions. In this the absorbance signal is detected over a given time interval, typically a few seconds, in which complete atomization of the studied element occurs. Atomic Absorption Spectroscopy (AAS) helps in evaluating nanoparticle dimensions based on the difference in atomization kinetics for the nanoparticles and the solution.[38]

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Conflicts of interest

The authors declare that there are no conflicts of interests.

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