Chapter 1

Introduction

1.1 Origin of proposal:

Nanotechnology is a fairly new field, but the existence of NPs dates back to ancient times. The ancient counterpart, *Bhasma* is a part of an ethno medicine branch 'Rasayana Shastra', which deals with metal compounds in the size of nano dimensions. It is known that manufacturing methods of *Bhasma* are in tune with nanotechnology of modern era and they are similar to the nano crystalline materials, in terms of physicochemical properties except that *Bhasma* is prepared in presence of various plant products **(Palkhiwala and Bakshi 239)**. In accordance with size as a parameter, currently nanomaterials (NMs) are defined as materials having at least one dimension ranging from 1 to 100nm and those that are intentionally manufactured to a nano scale size are referred to as engineered nanomaterials (ENMs). Recently the European Commission (EC) redefined NMs as "any natural, incidental or manufactured material, containing particles in an unbound state or as an aggregate or as agglomerate and where for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm to 100 nm" **(Bleeker et al. 120)**. Reduction of materials to nano scale dimensions, increases the surface area to volume ratio making surface dependent properties more dominant in addition to properties related to quantum mechanics which result in novel and enhanced electrical, optical and mechanical properties **(Oberdorster et al. 835) (Nel 622).** The alterations of properties at the nanoscale are responsible for the popularity and widespread applications of NMs.

Nanotechnology is a part of our daily life where people involved in manufacturing process are exposed at their work places while consumers are exposed through several market products that contain ENMs. The burgeoning use of this technology is thus impacting human health on a daily basis. Promising characteristics of nanotechnology are due to the unique properties of the ENMs; like small size, higher surface area, large surface to volume ratio, increased chemical and biological reactivity, excellent electrical conductivity, high tensile strength and their ability to form highly resistant, durable, reactive and self cleaning surfaces **(Savolainen et al. 93) (Song et al. 848).**

ENMs categorized into different groups such as carbon based materials, metal based materials and organic dendrimers, are studied in depth for various applications such as diagnostics, therapeutics, cosmetics, electronics, automobiles, construction, etc. The increased use of ENMs has led to safety concern leading to a growing interest in evaluation of their toxicity. Metal-based NMs are one of the most important and commonly present NMs in consumer products. As of March 2011, the presence of Metal and Metal oxide based NMs dominated the NM based consumer products **(Ng et al. 354).** Out of the many ENMs, Titanium dioxide (TiO_2) and Zinc oxide (ZnO) are the popular and most commonly used in consumer products. TiO_2 nanoparticles (NPs) that are widely used due to their brightness and high refractive index are one of the earliest industrially produced and highly manufactured NPs in the world **(Bakare et al. 1) (Lai and Warheit 184).**

The worldwide yearly production of TiO_2 and ZnO NP is estimated to be 3000 and 550 tons per year respectively **(Piccinno et al. 5).** TiO_2 NPs are added in sunscreens, paints, coatings, plastics, pharmaceuticals, food products, cosmetics and toothpaste amongst many other products (**Skocaj et al. 232) (Shi et al. 1)**. ZnO, the other most commonly used NM is used as a photo catalyst, in cosmetics, sunscreens, foot care, ointments, over-the-counter topical products, pigments and coatings for ultraviolet (UV) protection, as fungicide in paints, electronic devices, catalysts and in food packaging **(Vandebriel and Wim 1) (Sahu 1).**

The widespread applications of NPs in consumer products and other industries have thus raised a safety concern. Hence human health effects must be known and toxicity studies are extremely necessary.

1.2 Definition of the problem:

Nanotechnology, an amalgamation of various scientific disciplines is the latest in the series of technologies that have been developed due to diligence of many scientists and researchers from all over the world. Despite the fact that it brings together different fields to maximize the strengths and utilities of each, it is an independent field (**Drezek and Tour 168).** Drezek et al. explained the reason behind the uncertainty of making rational decisions in nanotechnology. In his opinion, researchers and manufacturers are becoming overburdened with calls for agreement to unclear safety regulations, leaving regulatory authorities exasperated because refusal is an unavoidable outcome **(Drezek and Tour 168).** Moreover, since nanotechnology is an interdisciplinary field, insufficient information can lead to inaccuracy, which would generate incomplete data regarding the effects of NMs. The growth of nanotechnology is a global phenomenon and therefore a global approach is required to evaluate the possible health hazards and environmental risks. In accordance with a report published recently, there has been a substantial time lag between the emergence of products containing NMs, the generation of environmental health and safety (EHS) data and their subsequent use by regulatory agencies **(Linkov et al. 204)** (Fig 1.1)**.**

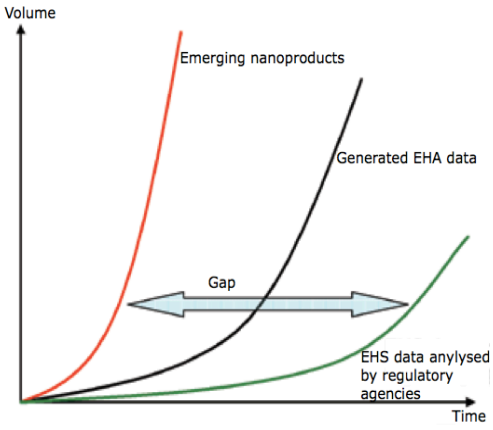

Figure 1.1: Representation of the emergence of nanotechnology products in comparison with generated EHS data
(adapted from Linkov et al. 206)

International standards are important to set the foundations for risk management programmes that safeguard human health and environment **(Murashov and Howard 635).** Rising concern about potential risks and the need to manage at an early stage of expansion has led to publications and reports on safety of manufactured ENMs by the Organization for Economic Cooperation and Development (OECD) and the World Health Organization (WHO). Realizing the importance of ENMs related safety concern, the Nanomission of Govt. of India constituted a Nano regulatory task and issued guidelines for safe handling of NMs in research and industries on the basis of published reports by regulatory bodies like International Standards Organization (ISO), Organization for Economic Cooperation and Development (OECD), The National Institute for Occupational Safety and Health (NIOSH) and Occupational Safety and Health Administration (OSHA). The guideline addresses the exposure risks and control measures to be followed while working with NMs, thereby emphasizing the need on regulations and safety assessment **(Centre for Knowledge Management of Nano science & Technology 1).** The apprehension regarding the applicability of hazard assessment is due to the unclear modes of toxicity of NMs and the inability to predict biological interactions through the current hazard evaluation methods. This issue has been addressed by the OECD, which launched a programme to ensure that the approaches for hazard, exposure, and risk assessment were appropriate for evaluation of NMs

(Boverhof et al. 146). It is thus essential that government and private sectors join hands and participate in establishing standards based on sound science (**Murashov and Howard 635).**

Nanotechnology deals with different characteristics of NPs attributed to their unique physicochemical properties, therefore even though an arduous approach is required for risk management, the increasing human exposure to NPs demands sophisticated risk assessment and regulation strategies. Therefore, nanotechnology related regulation should no more be rhetoric or misled by obfuscating the consumers. It is time, the bureaucratic inertia of the governments and regulatory bodies adapt to new regulations, giving importance to emerging risks of NPs **(Maynard et al. 577).**

Despite many studies indicating potential toxicity of NMs, governments and other agencies still require reports and reviews on their effects on human health **(Biasotto and Baun 409).** Hansen et al., emphasized in his report the need by governments for data as a replacement for making an effort, suggesting the risk and hazard assessment of NPs to be paralyzed by analysis. More scientific information is definitely needed, nonetheless, actions based on the present knowledge need to be taken so that industries can manufacture and market products based on nanotechnology that are as safe as possible **(Hansen et al. 3)**. With the increased use of NPs in various fields, there has been a shift in focus to understand the potential of NP induced genotoxicity **(Singh et al. 3892)**. Magdolenova et al. conducted a literature review regarding toxicity of NPs, from 2000 to 2012 of which less than 3% deal with genotoxicity studies. A recent study showed that till 2012, there was a distinct increase in the annual number of publications for *in vitro* and *in vivo* ENM genotoxicity studies, followed by a plateau **(Magdolenova et al. 234)**.

Despite inconsistent reports in literature, there has been compelling evidence showing genotoxicity of NPs, as several positive test results have been reported **(Landsiedel et al. 245)**. It is noteworthy that even though several investigators have tried to improve the relevance of guidelines for genotoxicity studies of NMs, no prescribed guidelines on standard battery of genotoxicity tests have yet been proposed **(Doak et al. 105) (Wang et al. 13213)**.

Moreover, there is still inadequate information on the characterization of engineered physico-chemical properties of NMs **(Gonzales et al. 265)**. Even though there have been models proposed for genotoxicity testing of ENMs, it is presently uncertain how well can standard genotoxicity tests designed for conventional chemicals be used to assess the genotoxicity of ENMs **(Wang et al. 13213) (Azqueta and Dusinska 1)**. This uncertainty of ENMs and its resulting carcinogenicity needs to be addressed. Moreover, the requirement of nano-specific modifications also needs to be understood and necessary steps need be taken.

"All substances are poisons—there is none which is not a poison. The right dose differentiates a poison from a remedy."- Paracelsus (1493–1541) **(Sharma and Sharma 407)**. Any NP administered at a high dose will induce "toxicity. The pertinent question is therefore not whether engineered NPs can be toxic but whether they are toxic at the concentrations mankind is exposed to intentionally or unintentionally. Risk of NPs is thus a function of the hazard and the degree of exposure **(Wiechers and Musee 411)**. Dose is defined in nanotoxicology as the nominal dose i.e., the amount of NP introduced into the culture media, but there exists a possibility to measure the deposited, cell associated or intracellular dose. Cellular doses are obtained by understanding the specific behavior of NPs in biological media (cell culture media) **(Lison et al. 1-2)**. As reported for soluble compounds, the dose that cells are exposed to is frequently assumed to be proportional the concentration of NPs in suspension **(Teeguarden et al. 305) (Hirsch et al. 7325)**.

However, NPs exhibit a wide range of diffusion coefficients and sedimentation velocities depending on their particle size and density, altering the applied dose **(Teeguarden et al. 304)(Hirsch et al. 7325)**. Expression of dose in nanotoxicology (selection of a dose metric) remains a challenge because of lack of agreement on the most appropriate approach, indicating the fact that under different conditions, different approaches are needed **(Lison et al. 3)**. The possible dosing approaches include mass, particle number and surface area (**Hussain et al. 10)**. Lack of consistency in dose metrics and very few publications reporting conversion factors generates a need for more understanding in this area for comparison between studies **(Singh et al. 3909)**. The present work aims at characterizing the NPs with respect to their physicochemical and particokinetic properties expressing the concentration in

terms of particle number per volume i.e., micro molar per volume (μM) **(Wittmaack 189)**. The concentrations can also be reported using gravimetric doses (μg/ml culture media, μg/cm^2 culture well) indicating mass per volume, but studies suggest this dose should be combined with NP number per volume concentration to provide sufficient information to interpret toxicity data of different studies **(Lison et al. 3) (Rivera et al. 745) (Teeguarden et al. 32)**. Moreover, few reports also propose that dose should be normalized by total surface area of cells or tissue (cm^2/ml culture media) though counter reports state that it is not appropriate for NP exerting their toxicity through release of soluble ions **(Hussain et al. 10) (Lison et al. 3)**. In the present study we have reported the conversion factors, thereby explaining the concentration expressed as number per volume (μM) also as mass per volume (μg/ml). A critical issue in nanotoxicity is of dose selection (concentration range) and the incubation time. Many studies use very high exposures that generate a genotoxic response, but bear little similarity to true exposure levels that may be experienced by humans **(Doak et al. 5)**. Therefore it is suggested that doses used in genotoxicity tests should be comparable to possible human exposures and since human exposure occurs at low dose levels, we decided to study the toxicity at comparable doses for NPs **(Greim and Norppa 422)**. Since short-term genotoxicity studies are believed to play an important role in predicting the *in vivo* carcinogenicity of potentially harmful chemicals, we incubated the NPs in culture media for 24 hours **(Dorato et al. 360)**.

Extent of dispersion of NPs in media can affect their bioavailability and physicochemical properties. The physicochemical properties of dispersed molecules and the media in which they are dispersed determine the intermolecular force driven reactions by Dipole-Dipole, Dipole induced dipole and London dispersion forces. Intermolecular forces catalyze the interaction or dispersion between two molecules, and contribute to density, viscosity, surface tension, Friccohesity, activation energy, and molecular radii, which was studied by the Borosil Mansingh Survismeter. We aim to understand the genotoxicity potential of NPs in nano form in culture media, therefore study of dispersion of NPs is essentially important. The culture media in which NPs are dispersed affect agglomeration state and thus stability of NPs. Therefore in order to assess the agglomeration state and stability of NPs in media, Dynamic Light Scattering (DLS) and Zeta potential analysis were performed.

Interestingly, most studies that have reported positive results for genotoxicity in literature used high doses of NPs **(Iavicoli et al. 501)**. Careful selection of appropriate and relevant concentration is a prerequisite for toxicological assessment of NPs. Studies done at higher doses than the realistic exposure, can lead to misinterpretation because the mechanistic pathways operating at a low realistic dose are expected to be different from those operating at high doses **(Elsaesser and Howard 131) (Oberdorster et al. 2005, 828)**.

No chemical is considered to be safe unless it has been proven otherwise. The question that needs to be addressed is whether NMs are different from other chemicals, and whether they should be treated in a different way. Some argue that NMs are smaller version of their bulk counter parts and therefore should be treated with the same caution. While others recognize and recommend that since physical and chemical properties of materials are different, they should be considered as a new chemical entity, justifying the need to assess toxicity of NPs. For ensuring safety to health and environment, protocols for safety assessment and criteria for evaluation need to be made for ENMs. Any new NM, despite its impressive applications should be treated with caution as a new chemical by itself without comparing it with the safety properties of its bulk counterpart. A number of metal compounds having particulate forms are known to be genotoxic and carcinogenic for humans, and the probability that their nano forms could be more genotoxic and carcinogenic creates concern, which is not sufficiently addressed.

1.3 Rationale:

Since the physicochemical characteristics of NPs play a key role in determining their vast potential for various applications, their consequences need to be comprehended. Chemical carcinogenesis is a chain of events that include crucial chromosome damage, gene mutations and transformation of cells that lead to development of cancer subsequent to exposure **(Ellinger-Ziegelbauer et al. 36)**.

A mechanistic association between DNA damage and cancer development has already been known **(Niida and Makoto 3)**. Assessment of genotoxicity is used as a surrogate of carcinogenicity due to the limitations of carcinogenicity assays **(Ellinger-Ziegelbauer et al. 37)**. Moreover, to reduce, refine and replace the use of animal testing in toxicology, *in vitro* test systems are a valuable alternative for understanding toxicity **(Boverhof and Raymond 957)**.

In spite of significant attention given to *in vitro* systems for NP risk assessment, not much attention has been given to a critical evaluation of NP stability, especially in particle dispersion kinetics and dosimetry. Contrary to soluble chemicals, NPs can sediment, diffuse and agglomerate according to their size and surface characteristics, affecting the dose at cellular level **(Teeguarden et al. 300)**. Moreover when suspended in culture media, NPs can flocculate, agglomerate or interact with serum components of the media altering the hydrodynamic diameter of NPs, subsequently altering nano-bio interactions. Size, size distribution, and overall dispersion stability of NPs is dependent on the dispersion conditions and the dispersant used **(Pal et al. 871)**. Therefore there is a necessity to understand the effect of dispersion medium on the particles in addition to ensuring a stable monodispersed suspension, in which NPs remain in 'nano' form, affecting their bioavailability prior to their dose response assessment. The stability of NPs depends on the coating, or presence of stabilizing agent, and can be assessed by measuring zeta potential **(Hristozov et al. 76)**.

Since characterization of NPs is imperative before toxicity screening, we performed Dynamic Light scattering (DLS) and Zeta potential analysis to assess the size and charge present on NPs when dispersed in RPMI cell culture media, used for *in vitro* experiment. DLS measures hydrodynamic diameter under conditions having a resemblance to the exposure conditions, and therefore helps in perceiving the stability of NP suspension with respect to time and medium **(Dhawan and Sharma 592)**. In order to address the issue of NP particokinetics, we assessed the physico chemical properties of NPs dispersed in water and culture media by Borosil Mansingh Survismeter. Our findings address the need to analyze the bioavailability of NPs in their 'nano' form in culture media, prior to nano toxicological testing.

Assessment of genotoxic effects of NPs is of utmost importance because it can cause alteration of genetic material. Genotoxic and non-genotoxic carcinogens are differentiated on the basis of risk and relevance of positive results of carcinogenicity studies **(Bolt and Degen 3)**. As far as the single hit, single target model is considered, a single base change leading to a mutation is known **(Kirsch-Volders et al. 64)**. Thus risk associated with exposure to DNA damaging compounds is estimated using linear extrapolation methods, which suggests that even low exposure levels are associated with a risk of developing cancer **(Ellinger-Ziegelbauer et al. 37)**. Moreover, if a genetic event occurs in germ cells, a genetic disease or reproductive toxicity may result, with a possibility of passing the risk for both, in the next generation. Thus genotoxicity testing is an essential part of the safety assessment of NPs **(Doak et al. 104)**. The mechanism of genotoxicity is generally by direct DNA binding capacity in addition to indirect DNA damage by ROS generation **(Singh et al. 2009, 3892)**. In order to understand the interaction of DNA and NPs, DNA binding studies were also performed. The assessment of genotoxicity of NPs was done in short term human peripheral blood lymphocyte culture. The 24 hour short term culture period was used because it is equivalent to 1.5 cell cycles, a condition favorable for assessment of clastogenicity in lymphocytes, which was done using two endpoints Chromosomal aberration assay and Comet assay **(Henderson et al. 163)**.

Since NPs are small in size, they can easily penetrate into cells and interact with cellular organelles and macromolecules such as DNA, RNA, and proteins. DNA being the central element of risk assessment for any chemical compound that human may be routinely exposed to, we aimed to assess genotoxicity of the widely used TiO_2 and ZnO NPs. Genotoxicity studies are crucial for assessing the safety of NPs since their interactions may lead to other responses such as mutagenesis and carcinogenesis. These studies led to the development of Nano genotoxicology, a field where research is done exclusively on the possible deleterious effects of NPs on DNA **(Singh et al. 3892)**.

NP induced genotoxicity can be attributed to the various factors such as direct interaction of NP with the genetic material and indirect DNA damage due to ROS generation, along with release of toxic ions **(Kumar and Dhawan 1887)**. The other factors responsible for induction of genotoxicity are interaction of NPs with cytoplasmic/nuclear proteins, binding with mitotic spindle or its components, increased oxidative stress, disturbance of cell cycle check points, generation of ROS at NPs surface or interaction of NPs with cellular components **(Kumar and Dhawan 1887)**. It is very important now to get information regarding possible genotoxic potential and mechanism of TiO_2 and ZnO NPs, using standard *in vitro* genotoxicity assays. The present work supports the possibly carcinogenic to humans (class 2B) classification given to TiO_2 by the International Agency for Research on Cancer (IARC), and demands for more attention on the genotoxicity potential of ZnO NPs since ZnO, has been given the Generally recognized as safe (GRAS) status by Food and Drug Administration (FDA) **(IARC vol. 93) (www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/SCOGS/ucm261041.htm)**.

We propose that NPs may gain direct access to the DNA after entering the nucleus during mitosis when the nuclear membrane breaks down leading to direct interaction of NPs with DNA causing breakage and similar other aberrations **(Frohlich 5583)**. As per previous studies, binding of NP to DNA could change the normal conformation and the local electrical properties of DNA molecules, in addition to causing local denaturation and compaction of DNA thereby interfering adversely with the genetic functions of DNA **(Li et al. 9664 - 9665)**.

Our hypothesis of direct interaction of NPs with the DNA is supported by the DNA binding experiments conducted in this study followed by *in vitro* cytogenetic endpoints, the Chromosome aberration assay and Comet assay.

1.4 Aims and objectives of the study

The aim of this study was to investigate the *in vitro* genotoxicity of TiO_2 and ZnO NPs using short term cultured human peripheral blood lymphocytes through cytogenetic endpoints, Chromosomal aberration assay and Comet assay.

In order to achieve this aim and elucidate the possible mechanism behind genotoxicity, we proposed to study the stability and dispersion of NPs by characterization of physicochemical properties and particokinetic analysis, followed by DNA binding studies and NP induced genotoxicity. This study emphasizes on the importance of physicochemical parameters for dispersion of NPs besides DNA damage induced at specific concentrations of NPs using *in vitro* lymphocyte cultures.

1. Characterization:
    - X-ray diffraction was performed to characterize the NPs in powdered form in terms of crystallinity, composition and size.
    - UV absorption spectroscopy was performed to determine the optical properties of NPs.
2. Particokinetic and Physicochemical assessment:
    - Borosil Mansingh Survismeter (BMS) assessed physicochemical parameters such as density, viscosity, surface tension, activation energy, molecular radii and Friccohesity based on intermolecular forces in water and RPMI-1640 complete growth culture media. Since molecular distribution of NPs is one of the contributing factors for agglomeration, Borosil Mansingh Survismeter was

used to study the physicochemical properties of dispersed NP molecules.

- Physicochemical aspects of dispersion of NPs are important for dose response studies in toxicity assessment. Therefore, to understand the dosimetry and kinetics of NPs we studied its suspension dynamics in water and cell culture media, thereby focusing on particokinetics of NPs.
- In order to understand bioavailability of NPs in 'nano' form, when added to culture media, stability and dispersion studies were performed using Dynamic Light Scattering and Zeta Potential analysis.

3. DNA binding studies for interactions between NPs and DNA.
    - To gain insight into the potential genetic damage exerted by the NPs, in addition to understanding the extent and strength of binding, mode of binding, effect on DNA conformation, and whether the binding is thermodynamically favorable, DNA binding studies were carried out.
    - Electronic absorption spectroscopy and Fluorescence emission spectroscopy were performed by titration of human genomic DNA with increasing concentrations of TiO_2 and ZnO NPs.
    - The detection of type of DNA binding is important for understanding the mechanism of genotoxicity potential. Thus, from this study, we could decipher whether the NPs were binding to human genomic DNA, which helped in understanding their genotoxicity.
4. Assessment of genotoxicity potential at low dose NP exposure for 24 hours.
    - *In vitro* short-term human peripheral blood lymphocyte culture was performed to evaluate NP-induced genotoxicity at 25μM, 75μM and 125 μM using Chromosomal aberration assay and Single cell gel electrophoresis (Comet) assay. The criterion for dose selection was based on earlier study reports and the dispersion of NPs in RPMI-1640 media.
    - We tried to determine the clastogenic effects of NPs that could be detected at the metaphase stage of cell division by Chromosomal aberration assay, while Comet assay in cultured cells was performed to investigate the DNA damage during interphase. Since very few studies have been done using human peripheral blood lymphocyte culture at such low doses for 24 hours, our study

provides an insight regarding the effect caused by low dose human exposure of NPs to DNA.

1.5 Outline of thesis:

The study addressed the *in vitro* genotoxicity potential of TiO_2 and ZnO NPs on human peripheral blood cultures. The thesis consists of 6 main chapters. This chapter introduces the topic, providing background information about applications of nanotechnology, safety concerns and the regulatory status currently in addition to rationale with the aims and objectives. The contents of remaining chapters are as follows: Chapter 2 contextualizes the study of TiO_2 and ZnO NPs in literature. Chapter 3 is an account on the materials and methods used to address the objectives. In Chapter 4, the observed results are explained and discussed in detail in sections titled as Characterization and Particokinetic study, DNA binding study, Assessment of genotoxicity in terms of Chromosomal aberrations and Comet assay, followed by discussion. Chapter 5 summarizes the complete work with conclusion of study, followed by references in Chapter 6.