The Effect of Zirconium Oxychloride on *in-vitro* Exposure on Human Erythrocytes and Rate of Haemolysis

A dissertation Submitted to Jawaharlal Nehru University; in partial fulfillment of the requirements for the award of the degree of

Master of Philosophy

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CERTIFICATE

This is to certify that the research work embodied in this dissertation entitled "***The Effect of Zirconium Oxychloride on in-vitro Exposure on human Erythrocytes and Rate of Haemolysis***" has been carried out in this school for the partial fulfillment of the award of the degree of Master of Philosophy (M. Phil). This research work is original and has not been submitted in part or full for any other Degree or Diploma in any other university.

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Abbreviations Used

AT	Anti thrombin
DMSO	Dimethyl Sulphoxide
2D-PAGE	Two-dimensional poly acryl amide gel electrophoresis
EDTA	Ethylene Diamine tetra Acetic acid
HA	Hydroxyapatite
HRP	Horseredish Peroxidase
ISO	International Standards Organization
IARC	International Agency for Research on Cancer
IOVs	Inside-out vesicles
LD_{50}	Lethal Dose 50.
MHD	Minimal Hæmolytic Dose
MHT	Minimum Hæmolytic Time
OSC	Oxygen Storage Capacity.
PDF	Powder Diffraction File
PMMA	Polymethylmethacrylate
PZT	Lead Zirconium Titanate
RGR	Relative Proliferation Rate
RBC	Red Blood Corpuscles
TEG	Thromboelastography
XRD	X-ray Diffraction.
YSZ	Yttrium Stabilized Zirconia
ZOO	Zirconium Oxychloride Octahydrate
ZPP	Zirconium Potassium Perchlorate

Synonyms: Zirconium Oxychloride, Basic Zirconium Chloride; Chlorozirconyl; Dichlorooxozirconium; Zirconium oxide chloride; Zirconium, dichlorooxo-;Zirconium oxydichloride; Zirconyl chloride.

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CHAPTER 1.

1. Introduction

1.1 In vivo Human Responses

1.1.1 Animal Responses

1.2 In-vitro Biocompatibility

1.2.1 Hæmocompatibility.

1.2.2. The nature of the erythrocyte membrane.

1.3 Aims and objectives

1.3.1 Study Methods

1. Introduction

Zirconium (Arabic *zarkûn* from Persian *zargûn* meaning "gold like") was discovered in 1789 by Martin Heinrich Klaproth and isolated in 1824 by Jöns Jakob Berzelius. The mineral was not known to contain a new element until Klaproth analyzed a '*jargon*' in the Indian Ocean. He named the new element Zirkonertz (zirconia). Isolated first by Berzelius pure zirconium wasn't prepared until 1914, and in 1925, the first industrial process for the commercial production of pure ductile metallic zirconium was discovered. The Prevalence of Zirconia's in India is being explored for increasing product development. 18,000 MT and now even more, of Zirconium mineral concentrates in 1996, are being extensively increased by programmes like Deep Continental Studies.

Mining of titanium and zirconium along the Agulhas current which is the anticyclonic Indian Ocean gyre, and other mining-related activities in general, have adverse impacts and disturb sand dune systems, wetlands and estuaries. The pervasive occurrence of zircon has become more important since the discovery of radiometric dating. Detrital zircons encapsulate a more representative record of igneous events diagnosed by oxygen isotopes, which are strongly fractionated by rock-hydrosphere interactions, and crust generation in part of Gondwana was limited to major pulses at 1.9 and 3.3 G.yr ago, when the zircons crystallized (Kemp). Definitive High and Low Zirconium concentrations, characterize the Cardamom massif. It has been found in old Gabbros samples among the Jusra igneous complexes in the North East (Heaman), The Deccan granolithic baddelyite formations of the Dharwad craton, and even implicated along the Aravalis.

Chemical characteristics of Zirconium

Natural zirconium is a mixture of five stable isotopes: zirconium-90 (51.46 percent), zirconium-91 (11.23 percent), zirconium-92 (17.11 percent), zirconium-94 (17.40 percent) and zirconium-96 (2.80percent). Two allotropes exist: below 862° C (1,584° F) a hexagonal close-packed structure, above that temperature a body-centred cubic.

Table1: Shows the abundance of zirconium.

Abundance	**ppb by weight**	**ppb by atoms**
Universe	50	0.7
Sun	40	0.5
Meteorite (carbonaceous)	6700	1600
Crustal rocks	130000	30000
Sea water	0.026	0.0018
Stream	3	0.03
Human	50	3

(Ref.: P.A. Cox in "The Elements": Their Origin, Abundance, and Distribution, Oxford University Press, Oxford, UK, 1989).

$ZrSiO_4$, ZrO_2 SiO_2 and ZrO_2 sands with Hafnium (0.5-2%) need to be made available in *pure forms*, from coarse grains, crystals and gems, glazing materials used in ceramics etc. to high grade neutron reflectors, nano-materials, and thin films; for Global Market products. Highly transparent to neutrons, it became important in the 1940s in nuclear-energy applications. Other uses are in alloys, fireworks, and flashbulbs and as a scavenger for oxygen and other gases. Its compounds, in most of which it has valence 4, are important industrial materials. Zirconia (the oxide) is used in piezoelectric crystals, high-frequency induction coils, colored glazes and glasses, heat-resistant fibers, and preparations to cure the rash of poison ivy. The high valency of Zr (group valency 4 and a coordination number of 8) makes it possible, and common, for the zirconium atom to bond simultaneously with a variety of different atoms or radicals and for zirconium compounds to polynize and to form adducts with other compounds (W. B.

BLUMENTHAL, Zr Chemistry, 1964, p. 1-16). The physiologic and therapeutic properties of Zr shows that it may be regarded as nontoxic and certain favorable physiologic responses may be anticipated.

The toxicity level of zirconium, inorganic tin and tantalum has been found to be very low and that of niobium and palladium relatively low, zirconium has not yet been associated with a specific metabolic function, despite the retention in relatively high quantities in biological systems. Cytocompatibility, mechanical properties and corrosion resistance are needed to be evaluated in more precise terms. (Atsuo Ito et al.1995).

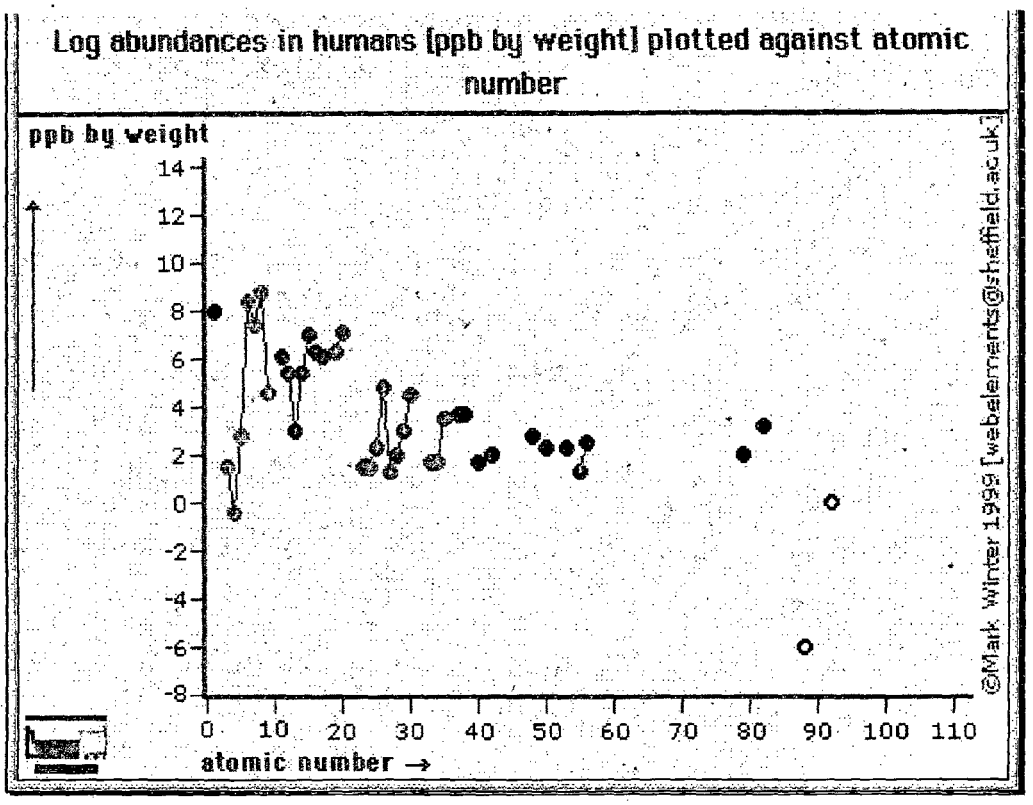
Uses of Zirconium Compounds

- Foundry sand, Refractory material (Zirconium Silicate), lab crucibles, opacifizer (zirconium and zirconium silicate), tile glazing and enamel.
- In blood-contacting devices, i.e. cardiac valves or cardiac assist devices.
- Artificial cell, Bioencapsulation for use in bioartificial organs drug delivery.(Thomas Ming)
- Zirconia and Zirconia bricks in lining of blast furnaces.
- Zircon as gemstone.
- Hafnium free zirconium having low area of cross section for neutron capture used in nuclear power generation.
- Manufacturing of cast iron, steel and surgical appliances.
- Powdred zirconium metal is used as a "getter" (of O_2 and N_2) in thermoionic tubes to absorb the last traces of air.
- Alloy of zirconium and columbium is superconducting magnet with a field of 6.7.T.
- Zirconium diboride and Zirconium carbide used in cutting tools for metals.
- Zirconium tetrachloride is used as water repellents for textiles and leather industry (as a tanning agent).
- Water-soluble salts used in body deodorants cosmetics and topical ointments e.g., zirconium lactate and zirconium oxide.
- For topical treatments of Rhus (Poision ivy).

- Zirconia (ZrO_2) used in Oxygen sensor as a component in lead zirconium titanate (PZT) Piezoelectrics. As capacitor materials.
- Zirconia (ZrO_2) films are durable hard, low thermal conductivity, high refractive index, and low optical loss.
- Hardening of platinum and ruthenium, clinically as a radio-opaque compound for X-rays of the gut.
- Zirconium Oxychloride is used as a chemical precipitant for phosphate to control eutrophication. Zirconium Oxychloride precipitates phosphate and limits algal growth at fairly low concentrations (100 ppm) at a pH range of 2-11. At this concentration, $ZrOCl_2$ does not seem to be harmful either to the tested fish or algae. (Kumar, H.D. Rai, L.C. 1978).

1.1 In Vivo Human Responses to Zirconium Exposure

Fig. 1. Shows log abundance of elements in humans (ppb by weight). Light weight elements such as C, H, and O. are most abundant and elements with higher atomic no. are less common in living systems. Elements with atomic number beyond 55 are present in negligible amount.



http://www.webelements.com/webelements/elements/text/Zr/biol.html

Zirconium compounds are though encountered relatively rarely by most people is gaining ground in the manufacture and uses of its compounds in many products. The metal dust should be treated as a major fire and explosion hazard. Human abundance has been calculated as ppb by weight: 50 and atoms (C = 1000000)]: 3. Biokinetic data for zirconium directly in healthy human subjects about the kinetics of intestinal absorption and of blood plasma clearance has been reported. Daily oral intake of total zirconium (through food and water) by man has been estimated as 4 mg/day. Most of it is excreted in the faeces (4 mg) and hardly any in the urine (0.15 mg). Levels in tissues are generally below10 μg/g (wet weight). Highest amounts were found in fat (19 μg/g) and gallbladder (14 μg/g). Levels in erythrocytes amounted to ca. 6 μg/g. It binds to albumin and globulin protein.

In coal miners, the presence of zirconium has been demonstrated in the lungs, pulmonary lymph nodes, blood, and urine. Biological half-lives of zirconium in human lung of ca. 70 and 225 days have been reported. Zirconium Oxychloride induced chromosomal aberrations in human leukocytes *in-vitro and* chromosomal aberrations and polyploidy in bone marrow of orally exposed male mice *in vivo.* But no data is available on the reproduction toxicology of zirconium. Retention is initially in soft tissues and then slowly in the bone. The metal is able to cross the blood brain-barrier and is deposited in the brain and the placental barrier to enter milk. The toxic effects induced by very high concentrations are nonspecific in nature. Zirconium tetrachloride hydrolyses to zirconium Oxychloride at physiological pH. There are some experimental reports of allergic reactions appearing as epithelioid granulomas at the site of applying deodorants containing both water soluble as insoluble zirconium compounds have also been published (Mon97, Ske93, Gre 99).

The fraction absorbed into the systemic circulation is $(2.5\pm0.1)\times10^{-3}$. The value of 2×10^{-3} recommended for ingestion of inorganic Zr by workers (ICRP (1979) and ICRP (1994)), is 4 times lower than that recommended for members of the population (ICRP (1989) and ICRP (1993). The higher value for the population was set to allow for the possible greater bioavailability of Zr incorporated into food components and other exposures (Veronese).

1.1.1 Animal Responses:

In several animal species, zirconium was demonstrated to be deposited and retained primarily in the lungs and pulmonary lymph nodes following exposure by inhalation to its oxide and Oxychloride. Concentrations in these tissues were of similar order of magnitude but varied largely among species. Concentrations in kidney, liver, and femur were only a few tenths of a per cent of those in the lung. Twenty-four weeks post-exposure, there were still significant amounts in the lungs and lymph nodes of rats (other species not examined).

Benign chondromas developed locally in the ear cartilage in some mice receiving Zirconium Oxychloride and mice receiving sodium zirconium lactate. Contact sensitisation capacity of zirconium tetrachloride was evaluated in the guinea

pig maximisation test (GPMT) as well as in the adjuvant and patch test (APT) and in a sensitive mouse lymph node assay (SLNA). Zirconium tetrachloride upon exposure is converted into Zirconium Oxychloride upon being dissolved — increased mortality rates in rats and guinea pigs, but not in rabbits, cats, or dogs. The cause of death, although not well established, was stated to be an intercurrent respiratory infection. Post-mortem examination showed varying congestion, oedema, and haemorrhage in the lungs of one-half of the exposed animals, but similar changes were seen in the control animals. Apart from borderline reductions in hæmoglobin content and erythrocyte count in dogs, no other changes were found. When a single dose of 20 µg of zirconium Oxychloride (octahydrate)(vehiculum: aqueous 0.9% sodium chloride solution) was injected into the dorsaof the ear lobes of female ICR and CBA/J mice (n=10/strain), all treated mice showed ecchondromas (outgrowths from the cartilaginous plate) at post-mortem examinations after 2 and 5 months, respectively. These chondromas were not seen in a separate group of 10 female CBA/J mice sacrificed 2 weeks after the treatment (She73).

1.2 In-vitro Biocompatibility

Biocompatibility is based on four tenets and principles, which are the following—

i) The ability of a material to perform with an appropriate host response in a specific application. (Williams, 1999).

ii) A collective biological response of an organism by advocating a large battery of in-vitro tests that is used in accordance with ISO 10993; and with the quality of not having toxic or injurious effects on biological systems. Zirconium exhibit little blood haemolysis or systemic toxicity in rabbit, mouse, and guinea pig animal models. $ZrOCl_2$ – intra-peritoneal - Rat - LD - 400 mg/KG. Inhalation of ZrCl mist at a level of 6 mg/Zr/m3 was perceptibly toxic after two months. In an earlier study the rat erythrocyte membrane response causing hæmolysis we found $Zr.OCl_2.8H_2O$ causing a 50% hæmolysis in-vitro which occurred at the very implausible high dose of 37.87 ppm (514.61mg/m^3) in the first 6 minutes. Also the biocompatibility and safety of a novel orthopedics

materials-graded zirconia (ZrO_2)-hydroxyapatite (HA) composite used in novel orthopedics materials was graded for acute toxicity in small mammals and cytotoxicity which, included the in-vitro hemolytic rate, and was found to be 1.66%, which, accords well with the standard, that hemolytic rate should be lower than 5% specified in ISO (Y.H.Yun et al. 1996).

iii) Toxicity studies of various Zirconium salts have been listed in Material Safety Data sheets, from Occupational Exposures and can range from totally nontoxic to reported LD50 in animals. Porous films, and powders, have low skin sensitivity and are present in extensively used cosmetic formulations, but can still cause dermatitis, conjunctivitis, lung granulomas and some target organ toxicity which, have been scantily reported; and,

iv) Dorland Medical defines biocompatibility as the absence of host response and does not include any desired or positive interactions between the host tissue and the biomaterials. This inability to elicit a host response has been the major advantage of Zr- to be used in human tissue engineering products and drug delivery. The biocompatibility of a scaffold or matrix for a tissue-engineering products refers to the ability to perform as a substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signaling systems, in order to optimize tissue regeneration, without eliciting any undesirable effects in living cells, or inducing any undesirable local or systemic responses in the eventual host.

Bioactivity of material emphasizes the ability of direct contact between substrate and cells as well as through soluble ions released by these materials during their resorption. (Chengtie Wu et al. 2005). Importance of zirconium in bio-environment, the cells and tissues influence the attachment to a biomaterial at micro and macro scale. The response of a biomaterial to its environment is the surface chemical activity. Implants materials like Ti have good biocompatibity but inferior wear resistance whereas Stainless steel and its alloys have better

mechanical properties than Ti alloys but have inferior biocompatibity. While Bioceramics made from zirconium have good fracture toughness, Bioceramics like zirconia have good fracture toughness, bending strength, excellent biocompatibility and have been coated on Titanium alloys.

1.2.1 Hæmocompatibiliy

Hæmocompatibiliy is a first order barrier for study of human consumption patterns and the effect of such exposures. There are two commonly used tests to asses the biocompatibility hæmolysis and thromboelastography (TEG) (to assess the evolution of blood clotting parameters).

Whole blood and RBCs have been found to be good indicators of interactions with most innocuous and even to simple crystalline silica (quartz DQ12, Min-U-Sil5) and Quartz DQ12 and Min-U-Sil5. The polymorphic forms of Quartz/silica (silicon dioxide; SiO_2) and its electrostatic factors interact with the cell membranes of many mammalian cells, including red blood cells (RBCs), causing haemolysis (Gerashchenko). The adsorptive interaction of colloidal quartz with erythrocyte membrane alters the morphology and lysis of the cells. Quartz has been classified as a carcinogen by the International Agency for Research on Cancer (IARC) in 1997. The hæmolytic action of quartz is suppressed by preincubation of mineral powder in a solution of polyvinyl-pyridine-N-oxide, a compound that shows an antisilicogenic effect in animal experiments. Hæmolysis increases with surface area of dust particles and decreases with particle size. Toxicity indicators to ubiquitous crystal-induced hæmolysis can be reduced/prevented in whole blood by the presence of metal binding proteins forming into metalloprotiens and by free radical scavenger, antioxidant mechanisms and the coating of the silica surface by proteins, antibodies and complement (Hadnagy).

The red blood cell membrane is perhaps the most complex natural membrane, prone to deformability in-vitro by changes in osmotic pressure, by injury, change in surface charge (Light and Wei 1977), and metabolic and enzymatic changes in the erythrocytic membrane and within the red cell. The

average red cell in humans lives 100–120 days; there are some 5.2 million red cells per cubic millimeter (mm^3) of blood in the adult human.

Animal models are essential in providing phenomenological information on biological reactions but in-vitro haemolysis; however represent ideal systems for studying cell behavior with the material, thus avoiding the complications and interferences encountered in-vivo. Thus hæemolysis is a simple model for the damage of the membranes of the cells and sub cellular particles. Most current knowledge about the biochemical constituents of plasma membranes originates in studies of red blood cells. The chief advantage of these cells for experimental purposes is that they may be obtained easily in large amounts and that they have no typical membrane structure, other than the plasma membrane itself, to interfere with study of that structure. RBCs offer an early and extensively studied, convenient and informative model for examining the surface properties of silica.

The oxide layers present on the surface layers of Zr material are known to specifically promote the interaction of contact phase proteins, Factor XII and HMWK (Yun Y.H. 1996). Extracorporeal systems for blood detoxification as cross linked with zirconium oxide powders were used for the removal of Paraquat, and inorganic phosphate from hyperphosphatemic animals. It is also a model for direct contact removal of LDL-cholesterol from the blood of familial hypercholesterolemic patients by hemoperfusion and avoids the use of plasmapheresis (Sideman).

1.2.2. **The Nature of Erythrocyte Membrane**

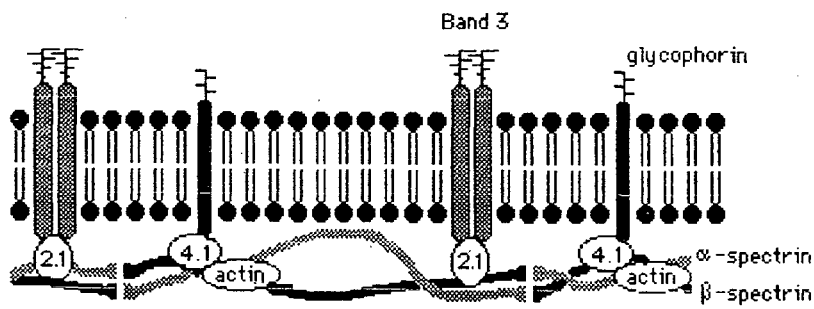


Fig: 2. The RBC membrane is a phospholipid bilayer with varying amounts of membrane cholesterol. A number of transmembrane (band 3 and several glycophorins) and membrane support [actin; ankryn (band2.1); band4.1; spectrin] proteins are present.
The actin - spectrin - 4.1 complexes help maintain the structural strength and stability of the RBC membrane. The spectrin - ankryn - band 3 complexes stabilize the phospholipid bilayer.

Normal RBC membrane is remarkable in durability (preservation of permeability barrier function) and also it need to remain deformable for the benefit of micro vascular blood flow. Erythrocytes are freely permeable to water and vary greatly in their permeability to solutes, so they are especially suitable for studying osmotic and permeability properties of cells. The properties are of course interdependent, for a solute to which the cell is freely permeable exerts no osmotic effect, where as one to which the cell is completely impermeable elicits the maximum osmotic response. A whole spectrum of related behavior lies between these two extremes. The mechanical stability of erythrocytic membrane is a good indicator of the effect of various in-vitro insults levied on it by various compounds for the screening of cytotoxicity and is dependent on their physical and structural properties. Thus erythrocyte is a best indicator model which can correlate toxic effects of chemicals and cellular function with overall physiology of organisms.

There are a variety of ways of investigating the osmotic and permeability behavior of erythrocytes but, provided that it is acceptable to study only the terminal stage of cell swelling, the hæmolysis method has the advantage of simplicity. The erythrocyte hæmolytic activity assay model is a well established test for determining membrane chemical interaction, hæmolysis is a characteristic end point in erythrocyte swelling; it occurs at the critical volume and is marked by release of hemoglobin from the cell. Hæmolysis is best evaluated using an in-vitro method, which can show the effect of increasing concentration and to be sigmoidally related to the logarithm of contact time, as was studied for various surfactants (Mohan et al., 1992; Krzyzaniak and Yalkowsky, 1998). In itself the erythrocytic membrane is a dynamic structure that can dictate significant changes in its interaction, best illustrated with detergents (Aki and Yamamoto, 1991) and well characterized drug induced hemolysis The circulation

ensures that any blood sample is statistically representative of the total red cell population and, because hæmolysis depends on critical volume, the course of Hæmolysis relative to time or solute concentration change reflects the distribution of terminally swollen cells in the sample population. It doesn't reflect the pathologic change but it is important to investigate the chronic and acute exposure at work place. The hæmoglobin released is easily and quantitatively measured by a simple analytical method, so that the mean cell response can be determined with precision. The Kinetics of such time dependence can be determined quite easily and accurately by first –order rate constants, which are obtained by calculating the slopes and intercepts with various agents (Pazos-sanou and Mata –Segreda, 1985).

Sickle Cell Disease and Hemolysis:

Sickle cell disease, one of the most common genetic disorder worldwide. "Sickling" is the phenomenon whereby the abnormal sickle hemoglobin undergoes polymerization when deoxygenated, giving rise to the typical sickled RBC shape. The abnormal RBC properties in sickle cell disease also include abnormal ion transport and decreased water content.

It arises from a point mutation of hemoglobin. Glutamic acid replaces valine at the sixth position of the beta globin chain of sickle hemoglobin, leading to an unstable protein with the well-known vulnerability to polymerization upon deoxygenation. Sickle cell disease is characterized by hæmolysis (increased destruction of erythrocytes), abnormal sickle-shaped erythrocytes, and vaso-occlusive and bone pain. Painful crises stems from tissue ischemia and infarct, due to decreased blood flow. Some sickled cells can revert back to their original spherical shape upon reoxygenation. Some cells, however, remain irreversibly sickled, probably due to permanent membrane deformation. Membrane transport abnormalities also occur in sickle erythrocyte (sickle RBC), such as increases in the calcium-dependent loss of potassium (Gardos phenomenon), and loss of water from the erythrocyte. Disruption of the RBC membrane can result in the release of hemoglobin from within the RBC. Normally, RBC remains intact and resists hemolysis by intricate regulation of ion and water exchange across the RBC

membrane. Alterations in the osmolarity of the fluid surrounding the RBC, for example, results in water uptake by the RBC to maintain equal osmolarity within the RBC. These RBC regulatory mechanisms can be overwhelmed, however, so that an extremely hypotonic solution will cause RBC to swell and take up water to the point of rupture - a phenomenon known as osmotic lysis.

Detrimental Effects of Hemolysis: The detrimental effects of hemolysis in the body include both the worsening of anemia and the release of free hemoglobin and other RBC contents. Renal excretion of the free hemoglobin can then lead to accumulation of hemoglobin in the renal tubules and progress to tubular necrosis. Simultaneously, release of potassium and phosphate can lead to electrical problems in cardiac and skeletal muscle, as well as brain. Sickle RBCs shows greater hemolysis than does normal RBC (approximately 3% vs. 1%). Basically, sickle RBC is more sensitive than normal RBC to hemolysis in every hemolytic condition, and this is consistent with the body of knowledge that sickle cell anemia is a hemolytic disease.

General Information from Material Safety Data Sheets.

Table 2: General information on common Zirconium Salts: The table includes general compilation of toxicity studies, health and safety limits & health rating from 1(less toxic) to 3 (more toxic). (Ref. Material Safety Data Sheet.)

S No.	Zirconium Salt description	Toxicity studies	Health and Safety limits & Health rating	Remarks	Reference
1.	Zirconium (Zr) Zr tetra fluoride ZrF_4 Zr H_2 Zr $(NO_3)_4.5H_2O$ Zr Nitride ZrN Zirconium Oxide ZrO_2	Almost nil No Carcinogenicity Abrasive irritant	OSHA /PEL & ACGIH/TLV 5mg/m^3 STEL 10 mg/m^3 Health Rating 1-2 Irritation of skin epidermal mucous membranes respiratory tract and git. Lung Granulomas on chronic exposures	Spongy to volatile. Generally insoluble in water. TLVs are influenced by the total respirable dust fraction	ESPI Corp Inc. High Purity Metal Specialists. http://www.espi-metals.com/index.htm
2.	$ZrSiO_4$; Zirconium Silicate; Zircon; Zr 4+ salt; Zirconium Silicon Oxide; Hyaciuth; Silicic acid.	Decomposes to Zr_xO_y and Si_xO_y Suspected Carcinogenicity.	Health Rating 3 Nuisance dust with sneeze and cough. Abrasive irritant Dermatitis, Skin and Pulmonary and granulomas. Fibrosis of lung tissue.	Metal Silicate Mildly Alkaline Reacts with H2O to form Silane	ESPI Corp Inc. High Purity Metal Specialists. http://www.espi-metals.com/index.htm

TH-14647

	CAS# 10101-52-7,				
3.	Zirconium Tetrachloride Metal Anhydrous Halide $ZrCl_4$, CAS# 10026-11-6	$ZrCl_4$- Oral- Rat LD50 1688mg/Kg ZrCls- Oral- Rat Rat LD50 3500mg/Kg. $ZrOCl_2$- IP Rat 400mg/Kg Inhalation ZrCl mist 6mg/Zr/m^3 in 2 months	NIOSH # 717500 PEL-C 7 mg/m^3 PEL-A 5mg(Zr_3)/m^3 Skin 'sensitizer'; irritation to severe burns. Respiratory distress with chocking burning and coughing. Laryngeal spasm and lung edema with death. Eye irritation with visual impairment and blindness.	Strongly acidic; Hydrolyses in H_2O with release of corrosive hydrogen chloride fumes.	ESPI Corp Inc. High Purity Metal Specialists. http://www.espi-metals.com/index.htm
4.	Zirconium tungstate; Zirconium tungsten Oxide $Zr(WO_4)_2$ CAS#16853-74-0	Zirconium lactate may cause skin granulomas on chronic exposure. Aerosols may cause Lung granulomas.	OSHA PEL 5mg(Zr)/m^3 ACGIH/TLV 5 mg(Zr)/m^3 1 mg(W)/m^3 \ Health hazard rating -2	Decomposition products Zr_x O_y, W_xO_y, Zr, W.	ESPI Corp Inc. High Purity Metal Specialists. http://www.espi-metals.com/index.htm

1.3 Aims and Objectives of the Study.

In this study we have attempted to assess the rate of hæmolysis with Zirconium Oxychloride Octahydrate and also investigation was carried out to find out, the interaction of Zirconium Oxychloride Octahydrate (ZOO) with erythrocyte, and was compared with hemolytic activity of Quartz which is chemically silicon dioxide (Analytical grade). The rate of hemolysis, its correlation with time as an independent variable, was derived for the activation energy of the interaction between that of Zirconium Oxychloride and Quartz.

The effect of temperature on *in-vitro* hæmolysis is related to the fragility, expense and sensitivity to temperature changes. Zirconium is known to improve the stability of membrane structures based on the thermal stabilities of some of the intercalated proteins like hemoglobin and can be monitored in powder XRD For our study we have used X-Ray diffraction for characterization of the Zirconium Oxychloride with the erythrocyte and its hemolytic potential at two temperatures i.e. at 4°C, 40°C. The XRD at 80°C was also done.

1.3.1 Study Methods

Hemolysis (Cell fragility studies and kinetic studies) is a well established experimental toxicity study with immense and direct applicability in Human erythrocytes. This study was designed to evaluate and confirm the true toxicity of a popularly low/non toxic material like Zirconium. The hemolytic potential is a good indicator system with the rate of hemolysis amenable to logical analysis and deriving the percentage hæmolysis/min.

The differences with the incubation at different temperature for 24 hours in the presence of hemoglobin a major protein may be reflected in the XRD patterns and intensities. Zirconium compounds like γ-zirconium phosphate have been shown to prepare matrixes of immobilized hemoglobin can help understand in-vitro biomembranes and also as a fundamental step to the understanding of ex vivo layered materials. (Geng 2004).

Chapter 2.

2.1 Summary of literature review.

2.2 Excerpts of Some Relevant Studies.

Literature Review

2.1 Summary of literature review.

Red Blood Cells offer a convenient and informative model for toxicity studies and examining the properties (biocompatibility) of materials. The research work related to zirconium and its compounds is related to its biocompatibility (blood compatibility, cyto-compatibility, osteo-integrating properties and evaluation of its acute toxicity). Since zirconium finds its wide uses in biomaterials, evaluation of its relation to immediate environment is of immense importance, authors try to find out its blood compatibility, its thrombogenicity, complement activating properties, platelet counts, thrombin-antithrombin (AT),. At shear stress below the hæmolytic threshold the deformed RBCs showed enhanced K^+ efflux (counter balanced by Na^+ influx) and findings help to explain the abnormal monovalent cation leak under Oxygen stress condition. Hæmolysis for zirconia (ZrO2)-hydroxyapatite (HA) composite were found to be 1.66 %.(Zhongguo Xiu Fu et al, 2006). Whole blood and 1% erythrocyte suspensions were treated with crystalline silica (quartz DQ12, Min-U-Sil5) at concentrations of 0.5, 1, 2, and 5 mg/ml. At 1% of erythrocyte suspension reaching nearly total hæmolysis (> 80%) at the highest tested concentration of 5 mg/ml (Hadnagy W. et al 2003). The hæmolytic effect of quartz and inflammatory reactions are reduced upon coating with alumina and zirconium salts is shown by Kriegsciset. It was concluded that ZrO2-HA composite bioceramics has good biocompatibility and is suitable for orthopedic biomaterials. Blood is directly incubated with test materials *in-vitro* (*in-vitro* chamber model) and the above information's are continuously updating. A due attention was made to hæmolysis because it is a relatively simple experiment and it can be done for any material.

Zirconium Oxychloride is reported to cause chromosomal aberration in the form of sister chromatid exchange, chromatid breaks, dicentrics and rearrangements studied by Giemsa staining technique. $CaZrO_3$ formation is reported to cause destabilization of HA-zirconia composite, and causes porosity increase in composite material which is known to affect its immediate environment of blood and serum. Zirconium may affect the general physiology of the body. Atmospheric concentration of (radionuclide ^{95}Zr (half-life 64 days) and its daughter nuclide ^{95}Nb (half-life 35 days), both β^- and γ emitters) Zr increases significantly after nuclear accidents. By intestinal uptake or by inhalation it is known to accumulate in the soft tissues of the body. Biokinetic data are still in premature phase. The decrease in Ca^{2+} transport activity during aging may accentuate the age-related decline in several erythrocytic properties. Zirconium activates the intrinsic pathway of coagulation and is opsonized with complement factor 3(C3).Zirconium and titanium in human whole blood is determined by digestion utilizing ICP-MS coupled to an ultrasonic nebulizer (USN) and desolvating membrane. X-Ray Diffraction technique is used to characterize the existing phase of zirconia composites. Scanning Electron Microscopy (SEM) is used in investigating the surface properties. Simulated body fluid (SBF) has been used for *in-vitro* studies to study the ionic exchanges and concentration is determined by above techniques. It mimics physiological conditions outside body in *in-vitro* studies.

Hæmoglobin can be immobilized on zirconium dioxide nanoparticles Hb. retains its bioactivity and native structure, Song Liu et.al. (2003).Ellipsometry/antibody technique is used to detect kallikrein formation by the surface of spontaneously oxidized zirconium in citrated plasma upon adsorption by 10 min. incubation and measured using colorimetric assay. Zirconium oxide (Beads of 1 mm diameter cross linked with agarose containing zirconium oxide powders) is used to remove inorganic phosphate from hyperphosphatemic animals and paraquat (insecticide). Cross linking provides high mechanical strength, heat stability, prolonged shelf life, good blood flow characteristics, and prevents the release of fine particles into the

blood. Zirconium is known for its very low hæmolytic properties.$CaZrO_3$ can be used as high-temperature thermistor material. Electrical response of calcium zirconate was found to be sensitive to methane and was practically unaffected by humidity and carbon monoxide. This property can be harnessed to sense hydrocarbon. Undoped calcium zirconate ($CaZrO_3$) (p-type semiconductor in air). Doping with oxides (Al_2O_3, Y_2O_3 and MgO, or with a small excess of ZrO_2 or CaO), it becomes predominantly an oxygen-ion conductor. (Erkin Gonenli et al. (1998)). Zirconium-Cerium is known for its oxygen storage capacity (OSC). Due to possible Valence change of the Ce. Ion from 3^+ to 4^+ or from 4^+ to 3^+, it should have the ability to store more oxygen. A 1:1 mixtures of apo-Fbp and Zr^{4+} (Zr^{4+} is an oxophilic ion) lead to the assembly of trizirconium clusters in periplasmic space which may control iron trafficking may be used in –mineral interface, virulence and the design of novel antibiotics. The control of cross-linking kinetics in sol-gel systems is creating a strong incentive to use "green" cross-linkers such as the key point in several industrial processes such as ceramic zirconium complexes (zirconium citrate1,2), zirconium lactate other oxopolymers.

2.2 Excerpts of Some Relevant Studies.

Haemolysis initiated *in-vitro* by ceramic powders ($Al2O_3$, $ZrO_2/Y2O_3$, AlN, B4C, BN, SiC, Si3 N4, TiB_2, TiN, TiC), graphite and diamond was studied. The haemolysis was found to be almost zero for all powders, except AlN which showed slight haemolysis and TiB2 which had high hæmolytic potential. The chemical composition of the powders was studied by X-ray microprobe, ICP-AES & ICP-MS. Dion I. et al. 1993.

Zirconium Oxychloride was found to be potentially harmful for DNA. Its aquous solution is incubated with human peripheral blood lymphocyte culture (set up from healthy donors of both sexes belonging to age groups of 0-10, 11-20, 21-30, 31-40, 41-50 and 51-60 years) Giemsa staining techniques were applied for the study of sister chromatid exchange. The

endpoints screened were chromosome and chromatid breaks, dicentrics and rearrangements. The frequency of chromosomal aberrant could not be related to age of the donor. However, the frequency of sister chromatid exchanges increased with increase in age of female donor. Ghosh S et al.

Adsorption of heparinized human blood plasma and serum on spontaneously oxidized zirconium were studied using Ellipsometry/antibody technique formation of kallikrein by the surface in citrated plasma were measured using a colorimetric assay after 10 min. incubation. The important finding upon contact with blood plasma, zirconium activates the intrinsic pathway of coagulation and is opsonized with complement factor 3(C3). Tengvall P et al 2001.

Erythrocyte offers a convenient and informative model for examining the surface properties of silica. The polymorphic forms of silica (silicon dioxide; SiO_2) interact with the cell membranes of many mammalian cells, including red blood cells (RBCs), causing hæmolysis. The electrostatic factor is believed to be a major contributor to the silica-cell contact and affect cell surface properties. The surface properties of SiO_2 particles had various effects on the RBCs. After thermal reduction of the surface hydroxyl groups, the membranotoxic effect of silica increased and then decreased. Geraschenko B I. et al 2002.

Hæmolytic activity was studied in human RBC cells by kinetic analysis of the percentage of hemoglobin released. Kinetic studies were performed by incubation and parameters were obtained for comparative studies of the initial velocity (V_i) and the maximum hæmolysis (H_{max}).The kinetics of chrysotile and nemalite were hyperbolic, amosite and crocidolite were close to straight line up to 7 min. for 0.5 mg. of fibers. M. C. Jaur, L. Magne and J. Bigon.

Zirconium and titanium in human blood serum is determined by pressurized digestion utilizing ICP-MS coupled to an ultrasonic nebulizer (USN) and desolvating membrane. For better X-ray contrast ZrO_2 is

incorporated with a volume fraction of 10 to 15%. Thus, the zirconium present in the polymethylmethacrylate (PMMA) matrix can be used as an indicator for the PMMA particulate debris. Kunze J. et al 2000.Titanium and its derivatives are among the most thrombogenic materials and shows compatibility with blood along with its osteo-integrating properties. However little is known about zirconium and its derivatives. Author aims to characterize the thrombogenic and complement-activating properties of zirconium using Polyester chips. Polyester chips were coated with 50- to 100-nm thick layers of zirconium using magnetron sputtering. The metal-coated chips were then incubated in direct contact with whole blood in an *in-vitro* chamber model, and the blood was then analyzed for platelet counts, thrombin-antithrombin (AT), fXIIa-AT, fXIa-AT and fXIIa-C1INH complexes and the complement parameters C3a and sC5b-9.Zirconium shows intermediate thrombogenic properties. Hong J .et al 2005.

Whole blood and 1% erythrocyte suspensions were treated with are crystalline silica (quartz DQ12, Min-U-Sil5) at concentrations of 0.5, 1, 2, and 5 mg/ml. Quartz DQ12 and Min-U-Sil5 revealed a strong dose-dependent hemolytic activity in the 1% erythrocyte suspension reaching nearly total hæmolysis (> 80%) at the highest tested concentration of 5 mg/ml. This effect may be ascribed to surface reactivity by silanol groups. In contrast, using whole blood cultures the tested silica dusts caused no or only minor hemolytic activity (< 4%). The mechanism by which the hemolytic activity is prevented in whole blood cultures can be attributed to a number of factors such as the presence of metal binding proteins and free radical scavenger, antioxidant mechanisms and to coating of the silica surface by proteins, antibodies and complement. In contrast to separated erythrocytes whole blood represents an independent physiological compartment with functions of host defense and regulatory functions against cell damaging effects produced by oxidative stress. Hadnagy W. et al 2003.

The pure HA coating shows the lowest bonding strength; Stress-induced phase transformation can be used to increase the strength of brittle

materials, in which ZrO_2 is often added as a second phase ZrO_2 reacts with CaO in HAC to form $CaZrO_3$ during the fabrication process; meanwhile this reaction causes destabilization of HA to decompose into more a-TCP phase. HA will decompose into b-TCP and CaO and ZrO_2 will further react with CaO to produce a new $CaZrO_3$ phase. Addition of ZrO_2 also causes an increase in porosity of the composite coatings. The bonding strength of HA increases with addition of Y-ZrO_2, and the bonding strength of the TZ3Y reinforced HA increases with increase of TZ3Y content in the range 0 to 10 wt% studied. E. CHANG E et al 1997.

Zirconium compounds used as green cross-linkers are environmentally sound and cause less harm to the environment. The reinforcement of regulations concerning environment protection in most countries is a strong incentive to use "green" cross-linkers for sol-gel processes. Among them, water soluble zirconium complexes are attractive for various applications in the surface coatings, oil production, sol stabilization, and agriculture industries. The control of cross-linking kinetics in sol-gel systems is creating a strong incentive to use "green" cross-linkers, the key point in several industrial processes such as ceramic zirconium complexes (zirconium citrate1,2), zirconium lactate and other oxopolymers. Je´ro^me Rose et al 2003.

The plasma membrane Ca^{2+} ATPase in erythrocytes is vital for the maintenance of intracellular Ca^{2+} levels. Since the cytoplasmic Ca^{2+} concentration is elevated in older erythrocytes, the properties of the Ca^{2+} transport ATPase were examined during cell aging using inside-out vesicles (IOVs) prepared from density-separated, young and old rat and human erythrocytes. The transport of Ca^{2+} and the coupled hydrolysis of ATP were measured using radio-labeled substrates. The calmodulin-independent Ca^{2+} transport activity (Ey, 38.8 v Y.H.Yun et al. s. Eo, 23.3 n mols/min/mg IOV protein) and the Ca^{2+} dependent ATP phosphohydrolase activity (Ey, 53.5 vs. Eo, 48.8 n mols/min/mg protein) were greater in IOVs prepared from younger (less dense) rat erythrocytes. The calmodulin-independent Ca^{2+} transport

activity in IOVs from human erythrocytes was 12.9 n.mols/min/mg IOV proteins for Ey and 10.7 n.mols/min/mg IOV proteins for Eo. Inside-out vesicles from older (more dense) cells exhibited a lower pumping efficiency as determined by the calculated stoichiometry, molecule of Ca^{2+} transported per molecule of ATP hydrolyzed (rat: Ey, 0.74 vs. Eo, 0.49; human: Ey, 1.22 vs. Eo, 0.77). The enzymatic activity of rat and human Ey IOVs was labile. The Ca^{2+} transport activity in Ey but not Eo IOVs rapidly declined during cold storage (4°C). The decrease in Ca^{2+} transport activity during aging may accentuate the age-related decline in several erythrocytic properties. Seidler N W et al (1991).

The cellular response to a biomaterial is highly affected by the physico-chemical properties of the compound used. The ceramics were characterized regarding physicochemical properties; namely, chemical composition by elementary analyses and specific surface, pore volume and pore size distribution using the BET-method and Hg-porosimetry. The protein-biomaterial interactions of three biomaterials used in hard tissue surgery were studied in-vitro by a dynamic flow system and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to investigate the total protein binding capacity, relative binding capacity for specific proteins and flow-through and desorption patterns, adsorption of proteins from diluted human plasma on hydroxyapatite, alumina and zirconia. The materials were found to adsorb a surprisingly low amount of plasma proteins, leaving more than 70% of the surface free. Further investigation is needed to find the role of the protein on acceptance or rejection of implants. Rosengren A et al, 2002.

Zirconium oxide powders, finds its use in extracorporeal systems for blood detoxification, remove inorganic phosphate from hyperphosphatemic animals (with or without acute renal failure) and paraquat (insecticide). Composite beads of approximately 1 mm diameter made of cross linked agarose and containing Fuller's Earth or zirconium oxide powders. The high

surface area of powder, combined with low resistance to diffusion in the cross linked agarose matrix, is highly advantageous. The cross linking provides high mechanical strength, heat stability, prolonged shelf life, good blood flow characteristics, and prevents the release of fine particles into the blood. These beads show very high capacity (over 5 mg LDL/mL beads) and can be used to replace highly expensive plasmapheresis procedure. Sideman S et.al. 1984.

Zirconium compounds find wide applications in the nuclear power plant and are accompanied by a potential risk of environmental contamination. The radionuclide ^{95}Zr (half-life 64 days) and its daughter nuclide ^{95}Nb (half-life 35 days), both β^- and γ emitters, are important fission products that have been found in significant amounts upon the release of refractory materials in nuclear accidents. It is used as a heat resistant material in internal linings of furnaces as pacifier the data are lacking with respect to safety of the material. A significant concentration of zirconium is found in animal tissues some experiments suggests its intestinal uptake or by inhalation. Its distribution, retention (in the whole body and relevant organ) and possible routes of excretion needs further investigation. It is refractory and radioactive element, therefore it is much important to understand the biokinetics in human blood plasma of zirconium. Due to the potential radiological risk represented by the radionuclide ^{95}Zr and by its daughter ^{95}Nb. Despite the significance of zirconium, few data are available on the actual biokinetics of zirconium in humans. Cantone **M. C. et al 2003.**

Calcium Zirconate-based oxides find its applications as potential sensor/device at elevated temperatures for monitoring oxygen, humidity and hydrogen. Sintered polycrystalline samples were used to characterize carrier types and the concentrations of ionic (proton or oxygen) and electronic charge carriers as a function of temperature, impurity distribution, and oxygen and/or water vapor partial pressures. Pretis et al. reported that undoped calcium zirconate ($CaZrO_3$) is a p-type semiconductor in air. When doped with oxides such as Al_2O_3, Y_2O_3 and MgO or with a small excess of ZrO_2 or CaO, it becomes predominantly an oxygen-ion

conductor. For a sample doped with trivalent cations such as indium, scandium and gallium, it may become predominantly a proton conductor when exposed to a hydrogen-containing atmosphere (steam) at temperatures ranging from 600-1000°C. The protonic conduction, however, tends to diminish at higher temperatures and can be replaced by electronic (hole) conduction, especially in a dry air atmosphere. CaZrO3 has also used as high-temperature thermistor material. The electrical response of calcium zirconate was found to be sensitive to methane, but was practically unaffected by humidity and carbon monoxide. The use of a calcium zirconate-based thermistor is, therefore, limited to atmospheres without methane and/or possibly other hydrocarbon gases. The dramatic response to methane, however, makes $CaZrO_3$ a potential candidate material for hydrocarbon sensing. Erkin Gonenli et al. (1998).

Zirconium-Cerium is known for its oxygen storage capacity (OSC). The mobility of oxygen in Yttrium stabilized Zirconia (YSZ) is high in an electric field but the valencies of both the Zr (+4) and the Yttrium (+3) ions hardly change under any oxygen pressure. This implies that the level of oxygen defects is constant in pure oxides of ZrO_2 and Y_2O_3. Due to possible Valence change of the Ce. Ion from 3^+ to 4^+ or from 4^+ to 3^+, it should have the ability to store more oxygen. Hideo Subukawa et al.

Zhongguo Xiu Fu, Biocompatibility and safety of zirconia (ZrO_2)-hydroxyapatite (HA) composite biomaterials was evaluated and their toxicity manifestation, mortality and the change of weight were recorded. For toxicity measurement' standard curve of proliferation and metabolism, relative proliferation rate (RGR) was calculated. *In-vitro* hemolytic test was divided into 3 groups: extracts, sterile distilled water (positive control) and 0.9% physiological saline. In each of three, 0.2 ml anticoagulant diluted fresh rabbit blood was added. The percentage of haemolysis was tested. In acute toxicity test no mortality is seen within 3 weeks, no symptoms of adverse effects were shown within 3 days. Cytotoxinic test: cytosomes in the positive control group diminished and appeared round, there were pyknotic nucleus, the attached cells agglomerated; the morphology of cells in materials group and negative control

group was normal. Haemolysis for zirconia (ZrO2)-hydroxyapatite (HA) composite were found to be 1.66 %. It was concluded that graded ZrO2-HA composite bioceramics has good biocompatibility and is suitable for orthopedic biomaterials. Zhongguo Xiu Fu et al, 2006.

Protein shows protected activity at high temperature when immobilized in proper substratum. Horseredish peroxidase (HRP) and met hæmoglobin, when intercalated in the galleries of α-zirconium(IV) phosphate Shows peroxidase activities at elevated temperature (86-90 ^{0}C) and the rates increased to 2-3 times the rates observed at room temperature. Intercalation increased the d-spacings of α-Zr.P from 7.6 A° to 65 A° for Hb-/ α-Zr.P and the layer spacing is consistent with the binding of Hb. The activities of HRP and Hb at elevated temperature illustrate improved thermal stabilities of the intercalated proteins, stabilization of the native state, and destabilization of the denatured state, by the inorganic matrix can account for enhanced thermal stability on a thermodynamic basis. Kumar and Chaudhari (2002).

Hemoglobin can be immobilized on zirconium dioxide nanoparticles. Hb. retains its bioactivity and native structure. Song Liu et. al. (2003).

Erythrocytes membrane has strong ability to tolerate the deformation under pathologic conditions eg. Malaria, blood infection, contamination. At shear stress below the haemolitic threshold the deformed RBCs showed enhanced K^{+} efflux (counter balanced by Na^{+} influx). Oxidation and deformation individually promote passive leak of monovalent cation through RBC membrane and that a synergistic effect is exerted when the two stresses are combined. The findings help to explain the abnormal monovalent cation leak under Oxygen stress condition. Paul A. Ney et al.

Blood compatibility and complement-activating properties of metals used in various medical devices was studied. The metal-coated chips were incubated in direct contact with whole blood in an *in-vitro* chamber model, and the blood was then analyzed for platelet counts, thrombin-

antithrombin (AT), fXIIa-AT, fXIa-AT and fXIIa-C1INH complexes and the complement parameters C3a and sC5b-9. Polyester chips were coated with 50- to 100-nm thick layers of or zirconium using magnetron sputtering. The result shows that zirconium is intermediate activator of thrombogenicity. This study has implication for the selection of metals intended for medical applications. Hong J et al 2005.

Bio-distribution studies were performed with 88Zr-citrate, 88Zr-Df, and 88Zr-labeled mouse serum albumin (88Zr-Df-MSA), modified with different amounts of chelating groups Zr-citrate was found to accumulate in bone and a higher accumulation of Zr was found in liver, kidney, and spleen. The absence of large amounts of 88Zr in bone indicated that in vivo the conjugates (proteins labeled with zirconium (Zr)-isotopes. The bifunctional chelating agent desferal (Df) was coupled to albumins via a thioether bond) are reasonably stable. Meijs WE et al (1996).

Tetravalent zirconium (Zr^{4+}) an oxophilic ion , and a potential metallo-antibiotic, can block Fe^{3+} binding by Fbp and even 1:1 mixtures of apo-Fbp and Zr^{4+} lead to the assembly of trizirconium clusters in periplasmic space which may control iron trafficking. It provides a new insight into bacterial–mineral interface, virulence and the design of novel antibiotics. Haizhong ZHU et al. (2003).

Upon contact with blood plasma, zirconium activates the intrinsic pathway of coagulation and is opsonized with complement factor 3 (C3). The failure to detect properdin and transient presence of factor H at the surface suggest that complement binds to zirconium although the activation becomes quickly down-regulated. Tengvall P et al. (2001).

The plasma membrane of erythrocyte and macrophages is damaged by quartz and induces an inflammatory reaction in lung. Quartz samples which are contaminated with Al_2O_3 are cytotoxic to a less extent (Adamis et al. 1991b;Brown et al 1990) has documented the effects of alumina coating in

decreasing the deleterious action of quartz our investigation has revealed that in-vitro methods can provide valuable data concerning the toxicity of minerals and zirconium salts. The physico-chemical characteristics are of crucial importance in the explanation of the hæmolytic mechanism of different samples. Kriegsciset.al.1987; Valyathan et.al.1995; Fubini, 1998.

Chapter 3.

3 Experimental Design
3.1 Materials and Methods.
3.2 Observations and Analysis.
3.3 Results and Discussion.
3.4 Conclusion

3 Experimental Design

The bioactivity of Zirconium Oxychloride so far has not beer investigated and data on the complexity of interactions taking place with tha of biomaterial with appreciation of the role of surface texture are lacking Therefore the purpose of this study is to investigate bioactivity witl establishing simple *in-vitro* general cellular toxicity to human blood cells like the rate of haemolysis of erythrocytes and the general reactivity with cellula fluids, its minerals etc. The rate of haemolysis as a cytotoxic parameter ha: been established for quartz and other trace metals which we can compare witl that of the physico chemical- properties of Zirconium Oxychloride. Th reactions of Zirconium Oxychloride with commonly occurring cellula minerals are studied with the help of X-ray diffractometer. We want t establish a comparison of the biological activity of ZOO at differen temperatures (4 °C, 40 °C).

3.1 Materials and methods

Materials:

Blood Sample: In these experiments reproducibility is better with freshl drawn blood than with stored Material.

Anticoagulant: Heparin is the preferred anticoagulant and is used in a concentration equivalent to about 20 IU/ml blood. Approximately 0.5 m heparin per 5 ml. of blood is placed in the collecting vessel and the bloo sample is drawn into this, with gentle mixing. The high molecular weigh of heparin ensures that even in concentrations greatly exceeding tha necessary to prevent clotting, there is no detectable osmotic effect on cel volume. This is not true of alternatives like EDTA, Sodium/potassiun Oxalate or Citrate as anticoagulant.

Methods:

X-Ray diffraction patterns at different temperatures are compared t evaluate the bioactivity of Zirconium Oxychloride at different temperatures

Control samples are of pure RBC extract; samples incubated with quartz were also examined and investigated for general ionic reactions.

Five milliliter of blood was voluntary contributed, by the author and withdrawn with heparin at Student Health centre JNU under standard conditions. The Red Blood Cells (RBC) was separated from serum and washed three times with Isotonic Phosphate Buffered Saline. In order to release the hemoglobin, from the RBCs, 0.5 ml of Triton X was added to 2 ml of RBC and shaken gently, it was centrifuged at 2000 rpm for ten minutes to sediment the RBC membrane proteins. These lysed RBCs were then incubated with zirconium Oxychloride (0.002 gm) at temperatures 4 ^{0}C, 37^{0}C, and 80 ^{0}C for 24 hours.

The XRD pattern was obtained on a Phillips X-Press model using a Cu Kα_1 radiation source and over a range of 10-90 ^{0} at 2^{0} /min. The curved graphite monochromatic is used in diffraction systems with Bragg-Brentano focusing geometry. Measurements were taken with the tube operating at 45 kV and 40 mA. Powders of the dried blood sample as incubated with Zirconium Oxychloride which formed a granular residue were loaded onto a flat, glass substrate. The sample was run, to the incident beam for 45 minutes.

For haemolysis experiment: Freshly prepared heparinised human blood (AB^{+}) was obtained and washed three times in phosphate buffered saline (isotonic, pH 7.2) by centrifugation (2500 rpm for 10 min. in table top Remi centrifuge). A 10 % aliquot of RBC was added to the ZOO in Phosphate buffered saline. As a control, for comparison the same experiment was repeated with Quartz. Quartz 5 mg/ml. was added to RBC suspension 10 % When the 30 minutes time was reached the RBCs were centrifuged at 2500 rpm for ten minutes to sediment the RBC membrane proteins. The absorbance of released hemoglobin was determined in triplicate and their values were averaged. In this experiment it is essential to use well washed cells because even small traces of plasma markedly inhibit the action of ZOO and quartz.

Haemolysis was performed at two different temperatures 4 ^{0}C, 40^{0}C, since all biological activities are stopped at high temperature the same experiment was not performed at 80°C and the results are compared with normal haemolytic potential.

Observations and Analysis with XRD

A brilliant red translucent solution of hemolysed RBCs with Triton X was seen. When the hemolysed RBCs were incubated at temperature (40 °C) and at the higher temperature in a water bath maintained at 80°C, a definite thick possibly denatured coagulum was observed at the higher temperature. On drying the coagulum was easy to lift out and fragmented into a coarse powder. Slides were prepared with the residues, of samples at normal temperature and higher temperature, by drying at room temperature.

Figure 3. XRD pattern of basic material, $ZrOCl_2.8H_2O$. It has Tetragonal structure (a (A°): 17.0800, b (A°):17.0800, c (A°):7.6900, $\alpha=\beta=\gamma= 90^0$) and a measured density of 1.97 and cell volume 2243.38. Reference code: 21-1499.

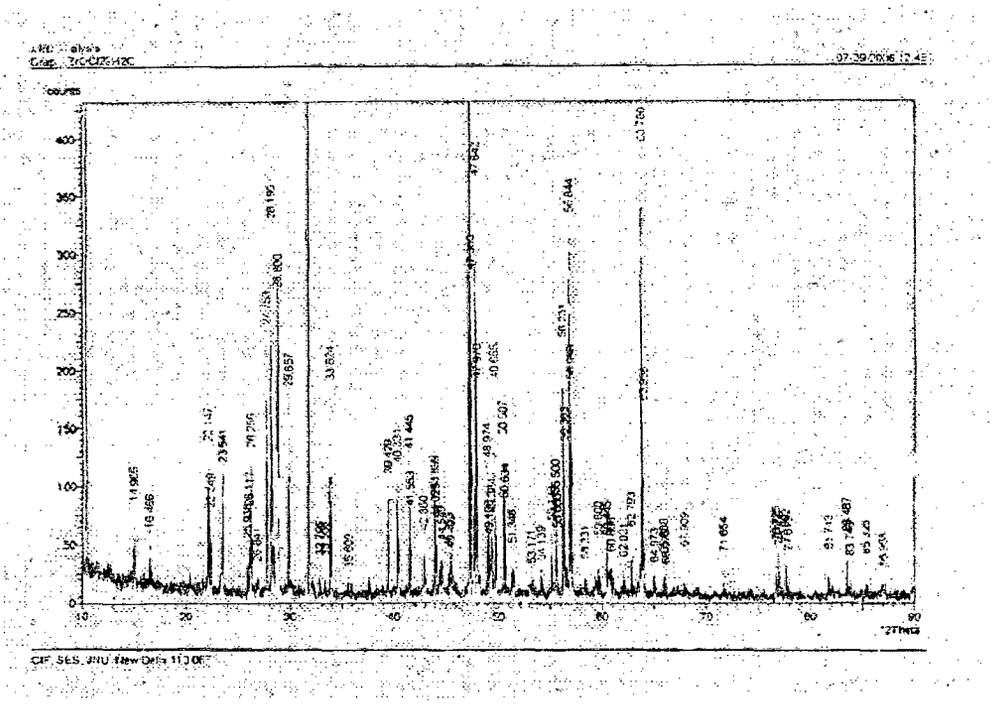


Figure 3.

d- Spacing and peak position shows the presence of two compounds Hafnium along with Zirconium Oxychloride. Hafnium (as hafnium tellurate) is present as impurity (Reference code: 40-0928, Crystal system cubic a=b=c=10.072 A^0, $\alpha=\beta=\gamma= 90^0$, Vol. of cell: 1227.52). Chemical properties of both of these elements are very similar that it is not possible to purify it normally. For advance uses in nuclear science Hafnium free Zirconium is used.

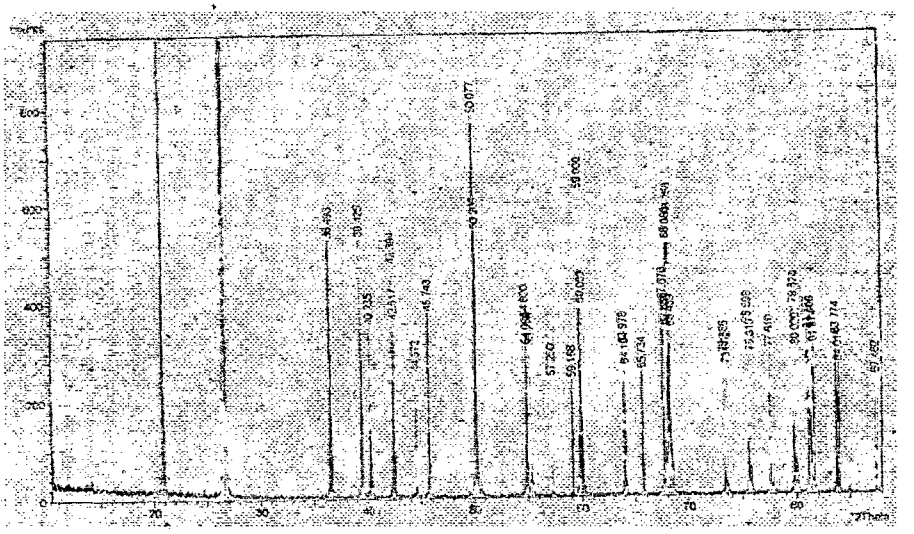


Figure 4

Figure 4: XRD peak pattern of Quartz (control sample, reference material) analytical grade. Reference code: 46-1045, PDF index name: Silicon Oxide, Crystallographic parameters: Crystal system: Hexagonal (a (A°): 4.9134, b (A°):4.9134, c (A°):5.4052, $\alpha=\beta=90^0$ $\gamma=120^0$).Measured density: 2.66 g/cm^3, Volume of unit cell: 113.01

Figure 5:- Control run of incubated blood (10%) with $ZrOCl_2$ at 40^0C for 24hrs. On comparison with PDF (powder diffraction files) and according to peak lines the following compounds were identified. Halite (NaCl), Calcium Zirconium Oxide ($CaZrO_3$), Calcium Silicate ($CaSiO_3$), Potassium Iron Oxide (KFeSi), and Iron Silicon (Fe_3Si).

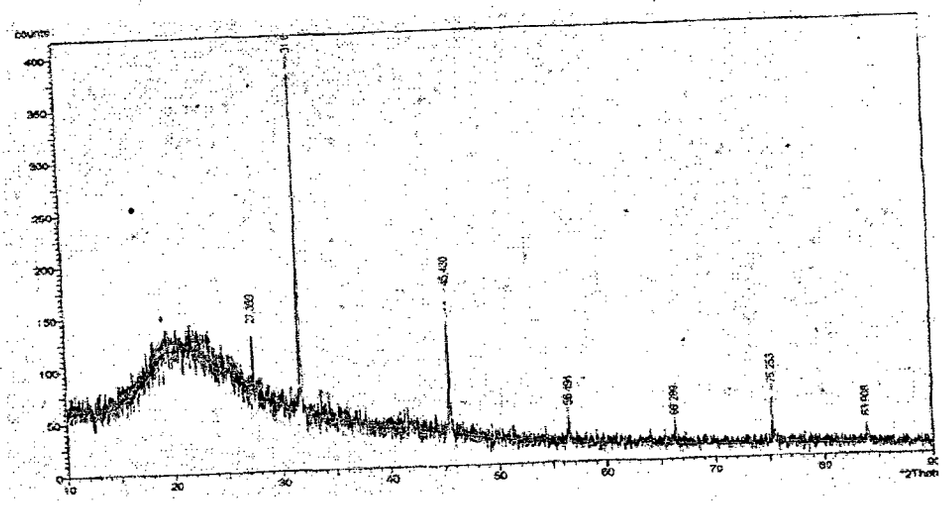


Figure 5.

Figure 6: Shows the results of incubation of RBCs with Zirconium Oxychloride at 80^0C after 24 hours. While Calcium Zirconium Oxide ($CaZrO_3$), Calcium Silicate ($CaSiO_3$), Potassium Iron Oxide (KFeSi), and Iron Silicon (Fe_3Si) were found. The halite was conspicuously missing (due to evaporation at high temperature). High temperature treatment of sample only adds to

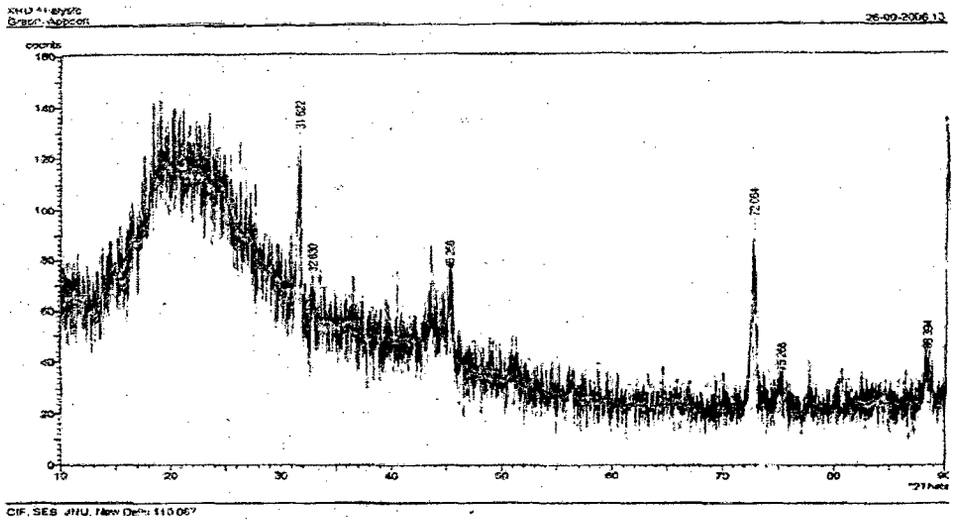


Figure 6.

the background as shown in figure 3. Peak intensity got lowered. Several small peaks are possible, not visible due to amorphous background. However this temperature is not common to biological systems.

(In harsh environment some species are exposed to such high temperature but some proteins are well adapted to tolerate it for example some bacteria live in hot water springs and several species of giant worms are adapted to live near mid oceanic ridges and sea smokers).

Figure 7: shows the results of incubation of RBCs with Zirconium Oxychloride at 4^0C after 24 hours. Calcium Zirconium Oxide ($CaZrO_3$), Calcium Silicate ($CaSiO_3$), Potassium Iron Oxide (KFeSi), and Iron Silicon (Fe_3Si) was present. Iron is present as hemoglobin protein which is released by disruption of erythrocytes.

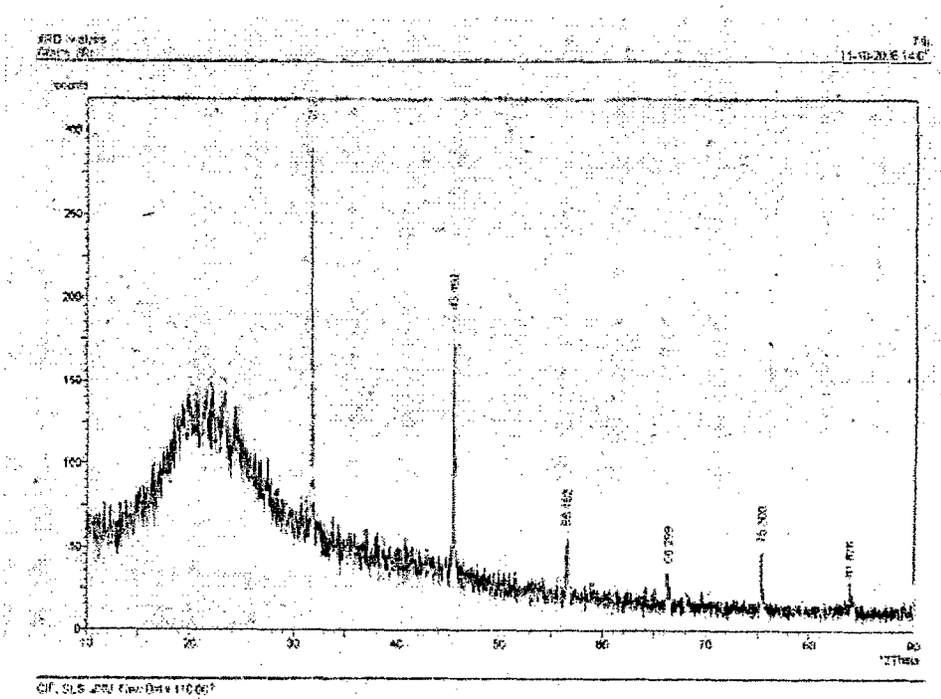


Figure 7.

TABLES and CHARTS:

Table3. A compilation of the XRD (PHILIPS-X Press) with a Cu kœ source for 10-90°with a 2°/min at 45KV/40mA has been done. The samples are of a control series of human red blood corpuscles (RBC) extract (1 RBCs+$ZrOCl_2$at 80 °C (2) RBCs+$ZrOCl_2$ at 40 °C (3) RBC alone at 40°C (4) RBCs+$ZrOCl_2$+DMSO at 40 °C (5) RBCs+ Quartz at 40°C (6) RBCs+$ZrOCl_2$ at 4 °C. Exposure of the RBCs with $ZrOCl_2.8H_2O$ record scores of the compounds detected and matched by the XRD pattern lists with exposures for 24 hours at 4°C, 40°C, 80 °C.

Table		Temperatures of Exposure of RBCs with Zirconium Oxychloride					
	Compounds detected by XRD.	RBCs+ $ZrOCl_2$	RBCs+ $ZrOCl_2$	RBCs Alone	RBCs+ DMSO+ $ZrOCl_2$	RBCs+ Quartz	RBCs+ $ZrOCl_2$
		80 °C	40°C	40°C	40°C	40°C	4°C
1	Calcium Zirconium Oxide						
	Score	21	21	-	15	-	13
	Peak Area	9.55,6.67	42.07, 15.06, 4.47	-	35.81, 16.77, 4.79, 2.38	-	92.13, 19.59
2	Potassium Iron Oxide						
	Score	15	19	24	24	9	24
	Peak Area	3.06	42.07, 15.06, 4.47,	92.13, 19.59	35.81, 4.79	13.13, 2.25, 3.14, 1.58, 4.41	92.13
3	Calcium Silicate						
	Score	10	0	4	0	11	4

	Peak Area	6.67, 6.51	4.97, 42.07, 15.06, 2.43	92.13	4.42, 16.77	13.13, 2.25, 2.24, 4.80, 3.53, 1.90	9.97
4	Iron Silicon						
	Score	35	48	27	39	36	27
	Peak Area	9.55, 6.67, 10.45	4.97, 15.06, 4.47, 2.43, 6.38	6.71, 92.13, 10.52, 4.63, 19.59, 9.97	4.42, 35.81, 4.79, 2.38, 5.03	13.13, 7.11, 1.58, 2.79	6.71, 92.13, 10.52, 19.59, 9.97, 4.63
5	Halite/NaCl (added as an ingredient of PBS buffer)						
	Score	-	81	51	76	56	51
	Peak Area	-					
6	Cesium Hafnium Chloride						
	Score	-	-	-	-	-	-
	Peak Area						
7	Cadmium Lead Oxide						
	Score	16	-	-	-	-	-
	Peak Area	3.06,	-	-	-	-	-
		10.44, 14.16.					
8	Potassium Chromium Oxide Fluoride						
	Score	-	-	-	-	14	-
	Peak Area	-	-	-	-	1, 5.70, 3.50	-

Scores:

X'Pert High Score can automatically accept the best matching candidates from the list of materials, using a sophisticated filter function that combines several selection criteria such as score, abundance and number of new matching lines. The basis for this "automatic phase identification" is a feature known as auto residue scoring.

This is extremely powerful way of dealing with multiphase problems. The decision is primarily on the unexplained profile features and peaks in the diagram. After identifying a phase, the auto residue scoring function automatically re-evaluates all remaining candidates. Candidates similar to phases already identified now score lower and are shifted down the list.

Observations and Analysis with the Rate of Hæmolysis

Haemolysis: Control experiment Quartz: the results of haemolysis are given in terms of % haemolysis. Here we are comparing the haemolytic activity of both materials at same concentrations ie. 6mg / ml. the logic behind taking this concentration is that 6mg is the daily intake of zirconium from different sources as reported in previous chapter.

In a study by Harley and Margolis (1961). The haemolysis is shown to increase with increasing surface area i.e. with decreasing the particle size of silica.

Percentage of haemolysis induced by different concentrations of quartz dust at different time interval: -

Table 4:

% Haemolysis at different concentration of Quartz at room temperature.

Con. mg/ml	TIME min.											
	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.	35 min.	40 min.	45 min.	50 min.	55 min.	60 min.
5	3.0	7.2	19.6	34.0	58.5	82.0	91.6	97.0	98.0	99.0	99.0	99.6
4	3.0	5.0	14.7	22.0	31.4	46.0	57.5	68.0	76.6	83.1	86.0	87.0
3	2.5	4.0	19.5	17.1	26.0	34.0	43.2	51.0	58.0	64.0	69.3	71.0
2	2.0	3.0	7.3	11.0	16.4	21.5	29.3	39.0	48.0	56.0	61	62.0

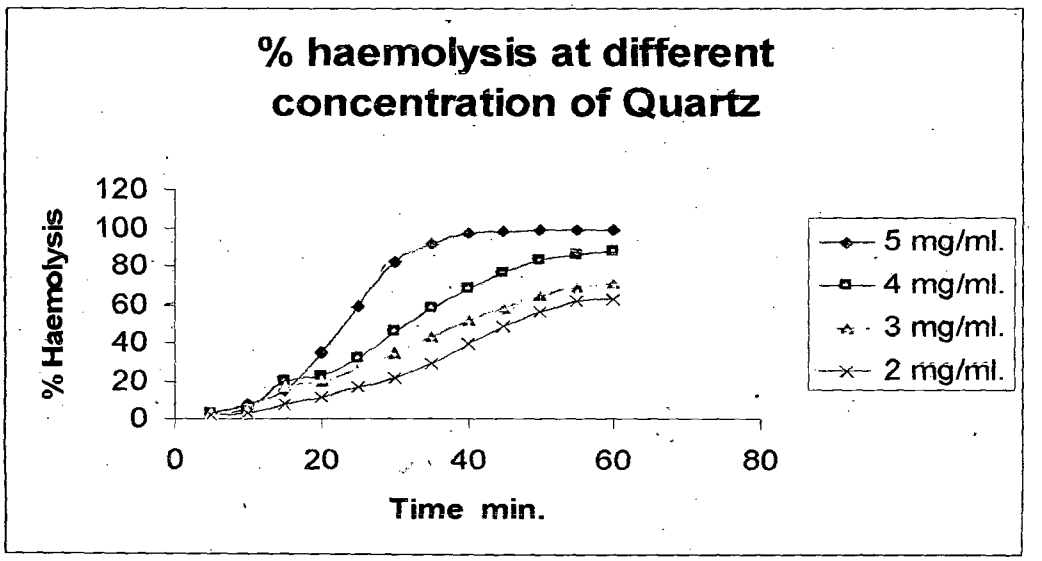


Figure 7: % Hemolysis at different concentration of Quartz at room temperature

Haemolysis induced by different concentrations of Zirconium Oxychloride:

Table 5:

Table 5: % Hemolysis at different concentration of Zirconium Oxychloride Octahydrate ($ZrOCl_2.8H_2O$)

Con. mg/ml	TIME Min.										
	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.	35 min.	40 min.	45 min.	50 min.	60 min.
2	2	2	3	5	8	12	15	18	20	21	21
3	2	2	4	8	12	18	24	31	37	43	47
4	2	3	6	13	21	30	39	48	61	69	72
5	2	4	7	24	43	63	79	89	94	94	95
6	2	10	33	70	96	96	97	97.5			

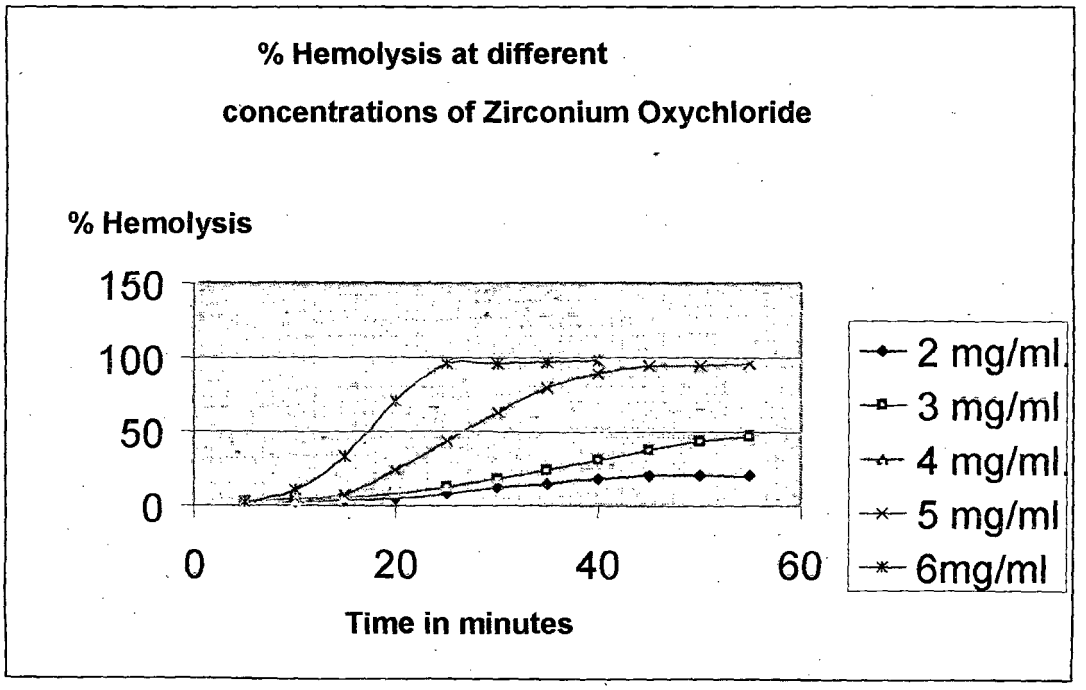


Fig.8: % Hemolysis at different concentration of Zirconium Oxychloride Octahydrate ($ZrOCl_2.8H_2O$)

Interquartile range obtained from the straight line of two curve 75-25/time period.

k_1 =5.5.

k_2 =5 .0

T_1=313 ^{0}K

T_2=227 ^{0}K

For calculation equation (3) and (4) are substituted as following

$$E = \frac{2.303 \times 1.987 \times T_1 \times T_2 (\log k_1 - \log k_2)}{T_1 - T_2}$$

= 39.273 (log k_1-log k_2)

= 4940.4066 Kilocalories per mol.

For log A, the substitution is

$$\log A = \frac{298}{298-288} (\log k_1 - \frac{288}{298} \log k_2)$$

= 29.8(log 5.5-0.9664 log 5)

= 2.53613 % per min.

Statistical Analysis

Table 6: Regression Analysis for data output at 40°C

Regression Statistics	
Multiple R	0.700365
R Square	0.490511
Adjusted R Square	0.426825
Standard Error	31.96718
Observations	10

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	7870.694	7870.694	7.702014	0.024101
Residual	8	8175.206	1021.901		
Total	9	16045.9			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	-64.965	46.46562	-1.39813	0.199619	-172.115	42.185
X Variable 1	92.89297	33.47192	2.77525	0.024101	15.70652	170.0794

Table 7: Regression Analysis for data output at 4°C

Regression Statistics	
Multiple R	0.982559
R Square	0.965423
Adjusted R Square	0.958507
Standard Error	7.937695
Observations	7

ANOVA

	df	SS	MS	F	Significance F
Regression	1	8796.014	8796.014	139.6038	7.64E-05
Residual	5	315.035	63.007		
Total	6	9111.049			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	-354.207	34.84018	-10.1666	0.000158	-443.767	-264.648
X Variable 1	268.9474	22.76244	11.8154	7.64E-05	210.4347	327.46

Table 8: Variable 3 is time in minutes and variable 4 is % Hemolysis at 40°C

Correlations

		VAR00003	VAR00004
VAR00003	Pearson Correlation	1	.874(**)
	Sig. (2-tailed)	.	.001
	N	10	10
VAR00004	Pearson Correlation	.874(**)	1
	Sig. (2-tailed)	.001	.
	N	10	10

** Correlation is significant at the 0.01 level (2-tailed).

Table 9: Variable12 is time in minutes and variable 11 is % Hemolysis at 4°C

Correlations

		VAR00011	VAR00012
VAR00011	Pearson Correlation	1	.978(**)
	Sig. (2-tailed)	.	.000
	N	7	7
VAR00012	Pearson Correlation	.978(**)	1
	Sig. (2-tailed)	.000	.
	N	7	7

** Correlation is significant at the 0.01 level (2-tailed).

High positive correlation is found between % Hemolysis (X Axis) and time (Y axis) in min. (as independent variable) with a correlation coefficient of 0.874 (for data set at 40°C) and .978 (for data set at 4°C).

Kinetics of haemolysis: Quartz at very low concentration is more hemolytic as compared to zirconium Oxychloride and the rate of haemolysis increases significantly with concentration. The rate of hemolysis increases linearly with time at initial stage up to 40 min. thereafter a plateau is formed.

Table 4. Shows the percent haemolysis with time, for different concentrations of control sample. It is generally accepted that the crystalline structure and surface characteristics influence the pathogenic interactions of quartz and various silica modifications (Kriegsciset.al.1987; Valyathan et.al.1995; Fubini, 1998). Quartz damages the plasma membrane of erythrocyte and macrophages and induces an inflammatory reaction in lung. Quartz samples which are contaminated with Al_2O_3 are cytotoxic to a less extent (Adamis et al. 1991b;Brown et al 1990) have also documented the effects of alumina coating in decreasing the deleterious action of quartz our investigation has revealed that in-vitro methods can provide valuable data concerning the toxicity of minerals and zirconium salts. The physico-chemical characteristics are of crucial importance in the explanation of the hemolytic mechanism of different samples.

Slow release of hæaemoglobin is is observed after 5 min. respectively for different concentrations. The released hæaemoglobin gets adsorbed on quartz particles which slow down further haemolysis activity.

MHT is taken as the index of hæmolytic potential. Lower value of MHT represent greater toxicity i.e. MHT is inversely proportional to toxicity of a compound.

- Minimal hæmolitic dose (MHD) is the concentration of an agent producing 50 % hæmolysis (Scnitzerand Pundsak, 1970).
- Minimum hæmolitic time (MHT) is the incubation time of an agent that produces at least 50 % hæmolysis.
- Maximum hæmolytic inhibition time (MHIT); incubation time at which maximum inhibition hæmolysis Started.

Table 10: Comparison of MHT between Quartz and Zirconium Oxychloride.

Hæmolytic material Concentration.	MHT (min.) Quartz	MHT (min.) Zirconium Oxychloride
2 mg/ml.	46.5	*
3 mg/ml.	39.0	+63.5
4 mg/ml.	31.5	39.0
5 mg/ml.	24.0	24.0

* found less than 50.0 %

+ Above 60.min.

Quartz exhibits very high hæmolytic activity even at very low concentration. As concentration of quartz increases the Minimum hæmolytic time (MHT) decreases.

Quartz is a better standard to compare with hæmolitic activities of other materials as Zirconium Oxychloride Octahydrate.

Results and Discussion

Results:

- Hafnium (as Hafnium Tellurate) was present as impurity with Zirconium Oxychloride.(Fig.3)
- Heating of sample at elevated temperature (80^0C) degrades it as shown in background. .(Fig.6)
- Zirconium Oxychloride on oxidation binds with calcium (present in cellular fluid and as membrane component) to form Calcium Zirconium Oxide ($CaZrO_3$).
- Minimum hæmolytic time (MHT) for Quartz is less for Zirconium Oxychloride than for Quartz. It shows higher haemolytic potential of Quartz as compared to $ZrOCl_2.8H_2O$.
- Hæmolysis is pseudo first order reaction.
- Rate of haemolysis was found to be 2.53613 percent per Minute.
- Hæmolysis at 40^0C was higher than at 4^0C. (Erythrocytes are more fragile at higher temperature).
- Hæmolysis at 4^0C was not significant (up to 15 min.) at a dose of 5 mg/ml.
- High positive correlation is found between percent hæmolysis (X Axis) and time (Y axis) in min. (as independent variable) with a correlation coefficient of 0.874 (for data set at 40°C) and .978 (for data set at 4°C).

Discussion

The Erythrocytic membrane and Hæmolysis:

Hemolysis is blood-sample-specific. For example, the mean cell volume (MCV) for RBCs vary from species to species (MCV human blood > MCV bovine blood), blood flow conditions, e.g., the hemo-incompatibility of the blood contacting surfaces, blood-air contact, thermal hemolysis, etc., can affect the hemolysis measurements.

The red blood cell is a bag-full of antioxidants such as catalase, glutathione and superoxide dismutase, within a living envelope permeable to both H_2O_2 and O_2. The flexible and elastic meshwork of proteins in the stromal cytoskeleton lying within this lipid bilayer, plasma membrane and is responsible for maintaining the bi-concave shape, reversible deformability and the structural integrity of the red blood cell. *In-vitro* hæmolysis as a function of the disruption of the membrane and graded spilling from the cytoskeleton is amenable to a fairly precise qualitative and quantitative change, as a result of insults from toxic materials. The kinetics of hæmolysis is better studied with varying the concentration of the toxic material and determining the hæmolysis over time as was first used for free silica and mineral dusts and fibers.

The three major mechanisms of responses of erythrocytes are:

I). Colloid osmotic lysis with increased permeability of the cell membrane due to disturbances in cation gradients or mechanical pore formation, resulting in cell shape changes and leakage of the Hemoglobin.

II).Catalytic peroxidation of membrane lipids mainly with phosphate ester groups, or particle interactions with secondary groups on stromal protiens, and

III).Physical perforation of the cell fragmentation, and erythrophagocytosis leading to the release of free hemoglobin.

Hypothesis regarding the membranolytic effect of quartz:

- Catalytic lipid peroxidation (Chavapil et al.1976).
- Lipid peroxidation and in-vitro hypothesis are two independent processes (Singh and Rahman1987).
- Damage or rupture of membrane is through particle interaction with phosphate ester groups a phospholipid component, (Stadler and Staber 1965, Nash et al 1966, Allison 1971, 1978).
- Particle interaction with secondary amine groups of proteins is the main cause. (Harly and Margolis 1961).

The Red Cell membrane is a multilayered material and the processes of Zirconium salts as a family of layered inorganic compounds has been found to involve several dynamic steps. These can be the bond formation between surfaces and proteins, the lateral diffusion, conformational and/or orientational rearrangement of adsorbed proteins. These conformational changes can be in secondary and tertiary structures based on the electrostatic interactions between oppositely charged γ-ZrP. (Geng). A thick gel like formation which appears at 80^0C is a good indicator of such re formations at higher temperatures. Intercalation of Red Cell derived Hæmoglobin at various higher than body temperatures can compensate for the loss of enzymatic activity by adopting a process of intercalation into galleries with Zirconium compounds particularly α-ZrP (Kumar)

Plasma membrane Ca $^{2+}$ATPase in erythrocytes is vital for the maintenance of intracellular Ca^{2+} levels. The formation of $CaZrO_3$ in this study, as found by powder X –ray Diffraction is suitable for the study of the changing properties of cell plasma membrane i.e. deformation from its basic structure, which may ultimately leads to hæmolysis. $CaZrO_3$ is a bio-inorganic complex and change conformation which resulting in orientation and re-

arrangements of red cell proteins. Analytical Studies of the mineral and pure forms show important mechanisms involving oxygen interstitial sites and defect calculations on $CaZrO_3$ to predict that small trivalent cations show Zr-site selectivity with oxygen vacancy compensation, while larger cations show Ca-site selectivity with Ca vacancy compensation (Islam). In biological systems, like blood and plasma, the advantageous effect of toughening outweighs the adverse effect of increased porosity associated with the incorporation of ZrO_2 (Chang) $KFeO_2$.

Fig 9. Shows structure of $CaZrO_3$.

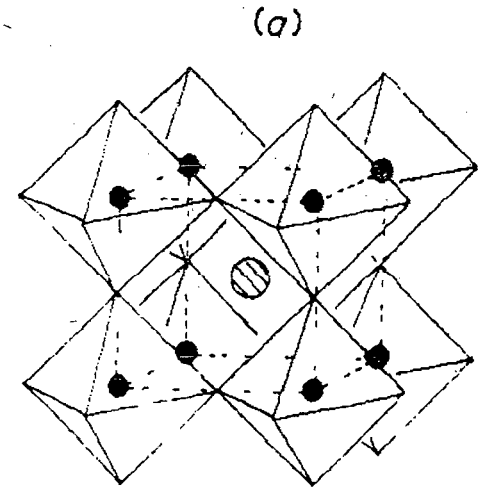


Figure 9.

Figure 9: Crystal structure of cubic perovskite (Common structure of $CaZrO_3$).

This shows the twelve fold co-ordination of O^{2-} ions around the CaZ * ions.

The O^{2-} ions at the corners of the octahedra are omitted for clarity.

Black spheres: Zr^{4+}

Central sphere: Ca^{2+}

Scanning Electron Micrograph of CaZrO3 (high density).

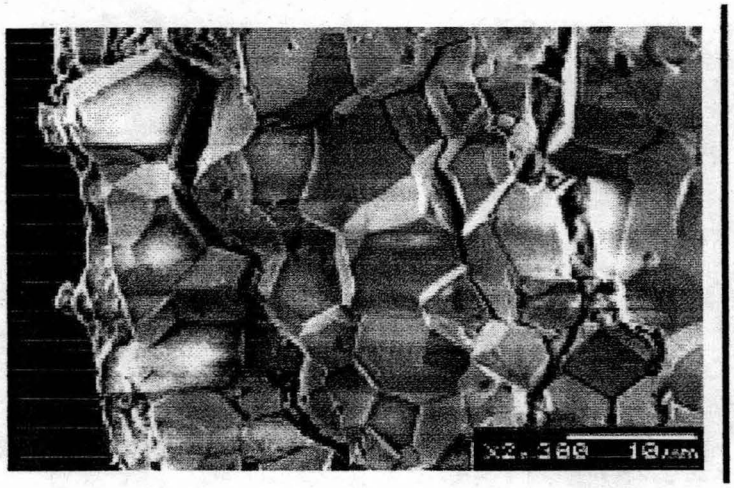


Figure 10

Calcium Zirconium Oxide ($CaZrO_3$) poses a serious problem in stabilized ZrO_2 ceramics, in the sense that it results in the attenuation of the typical strengthening and toughening mechanism i.e. the phase transformation of tetragonal ZrO_2 (Hae-Won Kim, Biomaterials).

$CaSiO_3$ (Wollastonite ceramics) having strong bone like apatite formation ability with good bioactivity within 24 hours of soaking in the body fluids. One hypothesis is that Quartz/silica is causing the hæmolysis of RBCs through the interaction of negatively charged surface of the siliceous materials at physiological pH with the Quaternary amines of the Phospholipids at the cell membrane surface. (Aleska V et al, 2006)

The mechanism of lyses of erythrocytes by the quartz involves the adsorption of lipids from the erythrocyte membrane. The reaction between silica and the cell membrane may be visualized in general terms as a two-stage process: first, a rapid adsorption of the particles by the cell surface, due mainly to the ionic attraction between silica and the amino-groups at the cell surface and further reinforced by hydrogen bonds and Vander walls forces; secondly protein denaturation, the extent of which may depend purely on the geometrical relation between the colloidal particles and the protein molecules, as was previously suggested in connection with blood clotting. All membranes including those of mitochondria are composed of substantial amounts of lipids

as are microsomes. In isolated biological systems silica has been shown to interfere with some enzymes, activate blood clotting and pharmacologically active substances in plasma and interfere with the red cell envelope. Most of these effects are probably due to the adsorption and partial denaturation of proteins at the surface of silica particles. Harley J.D., Margolis J. (1961).

Intercellular and intracellular ionic concentrations particularly calcium and potassium content interact with zirconium compounds. Formation of Calcium Zirconium Oxide is sensitive probe for the assessment of the cell integrity alterations inducible by exposition of Zirconium Oxychloride Octahydrate. Changes of the cellular ionic concentration are accompanied by oxidative metabolism; we conclude that investigation of cellular homeostasis together with functional activities of cells can improve the evaluation of zirconium cytocompatibility along with other biomaterials.

Effect of Temperature

Thermal stability of protein is maintained at high temperature (86-90 ^{0}C) upon attachment in the galleries of α-zirconium (IV) phosphate. The d-spacing increases and is consistent with the size of hæmoglobin. Intercalation increased the d-spacings of α-Zr.P from 7.6 A° to 65 A° for Hb-/ α-Zr.P. Kumar and Chaudhari (2002) used XRD technique to show this protein (Horseredish peroxidase (HRP) and met hæmoglobin) activity at elevated temperature.

Application of CH4 produces hæmolysis at pressure far below the hydrostatic Pressure known to disrupt membrane or protein structure. CH4 is known to act synergistically with detergents in hæmolysis. At sufficiently high concentration all cells are hæmolysed. Temperature enhanced the hæmolytic effect the results obey the 1st order rate law. (Batlivala H, Somasundram T, Uzgiris E. E., Makowski L).

Conclusion

Minimum hæmolytic dose (MHD) and minimum hæmolytic time (MHT) is large for zirconium Oxychloride as compared to Quartz as standard material. (As shown in Table No. 10).It is in accordance with the earlier studies on hæmolysis.

Zirconium Oxychloride on upon oxidation binds with calcium and results in the formation of Calcium Zirconium Oxychloride as shown from XRD figures, intensity vs. 2θ and matched with powder diffraction files (PDF). Heating the samples above physiological temperature degrades the proteins and other compound. It adds the background to the figure.3.Peak intensity decreases to a large extent.

The RBCs from a young human subject has provided some insight into the interactions of organic layered structures like the RBC membrane and its proteins similar to those that are being used for nanotechnology (Proteins are known to immobilized on nanoparticles without affecting its properties) with the help of trace elements like Zirconium and its intercalating galleries with membranes both ex-vivo and in-vitro. A zirconium compound upon incubation with mature RBC at very high concentration (5 mg/ml.) is hæmolytic and increases its fragility and disrupts its membrane structures. The effect of temperature on hæmolysis is very prominent as shown in table 7. Normally as temperature increases the absolute hæmolysis as well as its rate increases (Table 7.)

Hæmolytic activity reflecting toxicity is allayed by the formation of stable membrane intercalations because of at non uniform/heterogeneous binding with various proteins as large as Hemoglobin sites and membrane other proteins. Further investigations are needed to identify the mechanisms of the specific stimulatory effects of different ions and ion combination. However in-vivo studies are required to explore the bioactivity of Zirconium Oxychloride Octahydrate. Previous studies have shown that zirconium

compounds are biocompatible and therefore can be used for medical application along with Titanium, Aluminum oxide and ceramic materials.

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Appendix I

EMERGENCY FIRST AID PROCEDURES

Eye Exposure

If zirconium compounds get into the eyes, the eyes should be washed immediately with large amounts of water, lifting the lower and upper lids occasionally. If irritation persists after washing, get medical attention. Contact lenses should not be worn when working with this chemical.

Skin Exposure

If zirconium compounds get on the skin, soap or mild detergent must be applied and washed thoroughly with water.

Breathing

If a person breathes in large amounts of zirconium compounds, the exposed person should be moved to fresh air at once. If breathing has stopped, artificial respiration shall be performed. The affected person should be kept warm and at rest.

Swallowing

When zirconium compounds have been swallowed and the person is conscious, large quantities of water shall be given to the affected person immediately. After the water has been swallowed, try to get the person to vomit by having him touch the back of his throat with his finger. An unconscious person must not be forced to vomit. Medical attention is provided as early as possible.

Rescue

Early evacuation from hazardous exposure is must and affected person shall be placed in open air if the exposed person has been overcome, notify someone else and put into effect the established emergency rescue procedures. Unnecessary gathering around affected person should be avoided. Understand the facility's emergency rescue procedures and know the locations of rescue equipment before the need.

Appendix II

HEALTH HAZARD INFORMATION

Routes of exposure:
Zirconium compounds can affect the body if they are inhaled or if they come in contact with the eyes or skin.

Effects of overexposure
Skin rash has been reported from exposure to zirconium-containing deodorants.

Reporting signs and Symptoms:
A physician should be contacted if anyone develops any signs or symptoms and suspects that they are caused by exposure to zirconium compounds.

Recommended medical surveillance:
The following medical procedures should be made available to each employee who is exposed to zirconium compounds at potentially hazardous levels:

1. Initial Medical Screening: Employees should be screened for history of certain medical conditions (listed below) which might place the employee at increased risk from zirconium compounds exposure.

-Chronic respiratory disease: Zirconium compounds (silicate) have been reported to cause radiographic changes in animals due to pulmonary retention. Zirconium hexachloride may be irritating to the mucous membranes of the respiratory tract.

-Skin disease: Zirconium may cause granulomas of the skin. Persons with pre-existing skin disorders may be more susceptible to the effects of this agent.

2. Periodic Medical Examination: Any employee developing the above-listed conditions should be referred for further medical examination.

Appendix III.

WORKPLACE EXPOSURE LIMITS

The following exposure limits are for zirconium compounds (measured as *Zirconium*)

- **OSHA:** The legal airborne permissible limit (PEL) is 5 mg/m^3 averaged over 8-hour work shift.
- **NIOSH:** The recommended air borne exposure limit is 5 mg/m^3 averaged over 10-hour work shift and 10 mg/m^3, not to be exceeded during any 15 min work period.
- **ACGIH:** The recommended airborne exposure limit is 5 mg/m^3 averaged over 8-hour work shift and 10 mg/m^3 as a STEL (short term exposure limit).