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Elucidation of *In-vitro* toxicity screening of carboxylated Multi-Walled Carbon Nanotubes using Red Blood Cells

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ABSTRACT

Carbon nanotubes (CNTs) possess variable hemolysis activity against Red Blood Cells (RBCs). Hemolysis Activity of CNT varies according to functional group present on CNT surface, concentration of CNT and length of exposure period. There is a need to have more studies reporting hemolysis by carbon nanotubes. This study attempts to provide further incite to understand Hemolysis of RBC by Carbon nanotubes. In this study, we have studied hemolysis activity on RBCs by carboxylated Multi-Walled Carbon Nanotubes (MWCNT-COOH) within 1 hour using an universal method of determining nanoparticle hemolytic properties in vitro. This study provides an useful incite to understand ability of carbon nanotubes to cause hemolysis of RBCs. This study clearly indicating that hemotoxicological profile is a mandatory step toward the development of clinically relevant medications or contrast agents based on carbon nanotubes.

Key words: Carboxylated Multi Walled Carbon Nanotube; Hemolysis of RBCs; Nanotoxicity.

INTRODUCTION

Due to their unique physical (mechanic, electronic, thermal) and chemical properties, CNTs are intensively studied since 15 years by the scientific community [1]. CNTs are widely utilised in numerous applications (computers, aircraft airframe, and sporting goods, bicycles, golf irons) and have also emerged as efficient drug delivery carriers in the biomedical field [2]. Today, the annual global market in CNTs is estimated to be of the order of hundreds of tons. They are most broadly synthesised by Chemical Vapor Deposition (CVD). Being among the most guaranteeing materials in nanotechnology, they are expected to revolutionize medicine [3]. The exposure of workers and consumers in both occupational settings and environmentally is becoming more likely with the introduction of CNT-based nanotechnological products and, therefore, validated methodologies for the assessment of risk associated with these materials are urgently required. Unfortunately, some necessary information (e.g. the exposure scenario) is still lacking [4].

So far, various determinants affecting nanotoxicity of Carbon nanotubes on living organisms have been identified [5]. Mechanisms of nanotoxicity on Humans and mammals through Oxidative and Non-oxidative stresses are known [6]. Study on the long-term accumulation and naotoxicity of intravenously injected SWCNTs in the main organs (such as liver, lung and spleen) in mice is reported [7]. Also, Influences of ambient carbon nanotubes on toxic metals accumulation in Daphnia magna is been studied [8].

A comparison between the different pharmacological studies using nanotubes published today, offers a broad and not conclusive, albeit very informative overall picture. The indication is that chemical functionalization of CNT can

lead to significant and rapid urinary excretion. On the other hand, pristine or non-covalently coated CNT show preferential and predominant accumulation in liver and spleen [9]. The modification of CNT surfaces has rendered nanotubes dispersible in physiologically-relevant, aqueous environments, revealed their interaction with the biological milieu and allowed for their investigation in biomedical applications [5,10].The biodistribution profiles determined for different CNT types have already shown a strong dependence on the nature of surface modification [11].

It seems that the common critical parameters that determine CNT hemocompatibility remain the chemical nature of surface modifications, surface charge, nanotube structure, and nanotube surface area available for interactions. Surface characteristics are the major parameter influencing the interactions with blood components and therefore the overall biological impact [12]. So far there are very few studies revealed hemolysis activity of Multi walled carbon nanotubes.

According to Yu.I. Prylutskyy et. al., the influence of MWCNT on cells depends on MWCNT concentration and duration of incubation. At low concentrations, ≤25µg/ml MWCNT do not affect the stability of erythrocytes to hemolysis, a number of viable thymocytes in suspension and the rate of MTT reduction in thymocytes. At concentration 50 µg/ml the negative effect of MWCNT was observed: the acceleration of the hemolysis process [13]. Donkor et al. reported Carboxylated SWCNT possess neither hemolysis nor internalization in red blood cells [14]. Whereas Sachar and Saxena studied hemolysis of RBC by pristine and carboxylated SWCNT. They found Carboxylated SWCNT induces dose and time dependent hemolysis [15]. On the other hand, Meng et. al. studied hemolysis of RBC by aminated and carboxylated MWCNT. They reported no internalization in RBC but morphological changes and hemolysis. Meng et al. investigated also the impact of four different MWCNTs (either long ones (50 µm): long-aminated and long-carboxylated; or short ones (0.5–2 µm): short-aminated and short-carboxylated) on human erythrocytes. They reported nanotoxicity level as: short-aminated > long aminated > short carboxylated > long carboxylated [16].

Nanotoxicity of carbon nanotubes is a rising problem with increasing use of carbon nanotubes in various fields. Understanding the ecological transformation of MWCNTs is vital for their life cycle evaluation and potential environmental footprints.In this project, we have demonstrated that the Acinetobacter sp. (MTCC 10497) grows many folds in presence of carboxylated MWCNT. This finding will help engineers to prepare a protocol to reduce nontoxicity and accumulation of carbon nanotubes.[17]

From these studies it is very difficult to come to a concluding remark. There for there is a need have more studies reporting hemolysis by carbon nanotubes. This study attempts to provide further incite to understand Hemolysis of RBC by Carbon nanotubes.

MATERIALS AND METHODS

Multi-Walled Carbon Nanotubes (MWCNT)

MWCNTs (TNMC3, -COOH, 10-20nm, >95%) were purchased from Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences. No.16, South section 2, the first Circle road, Chengdu, P.R.China, 610041.

Other Reagents

- Tri-sodium citrate from HiMedia TC249-500G, 6132-04-3.
- NaCl from Himedia TC046-1KG, 7647-14-5.
- Citric acid from HiMedia GRM1023-500G, 77-92-9

Hemolysis Activity of MWCNT against RBCs

A universal method for testing in vitro nanoparticle hemolysis proposed by McNeil [18] was employed to investigate the hemolytic activity of Carboxylated Multi Walled Carbon Nanotubes (MWCNT-COOH) [17].

1. 3.8 gm Tri-sodium citrate in 100ml Distilled water was taken.
2. pH of Tri-Sodium Citrate (TSC) was adjusted to 5.6 with citric acid.
3. Blood sample was collected using sterile syringe.
4. 4.5ml of blood was added to 0.5ml TSC in a centrifuge tube.
5. This sample was centrifuged at 2000 rpm for 10 minutes.

6. Supernatant was discarded.
7. RBCs were re-suspended in 50ml 0.9% NaCl.
8. 0.2 ml of bulk sample was transferred to 2ml centrifuge vials.
9. Further additions of Bulk sample, MWCNT-COOH, 0.9% NaCl and Distilled water are listed in table 1. These centrifuge vials were kept for 90 minute incubation at room temperature.
10. After incubation vials were centrifuged at 5000 rpm for 5 minutes.
11. O.D. was measured at 540nm.

Table 1: Samples addition procedure for measurement nano toxicity on RBC

Sample	RBC (in ml)	SAMPLE	0.9% NaCl (in ml)	Distilled water (in ml)
Negative Control	0.2	-	0.8	-
Positive Control	0.2	-	-	0.8
MWCNT				
25µg	0.2	5µl	795 µl	-
100µg	0.2	20µl	780 µl	-
250µg	0.2	50µl	750 µl	-
500µg	0.2	100µl	700 µl	-

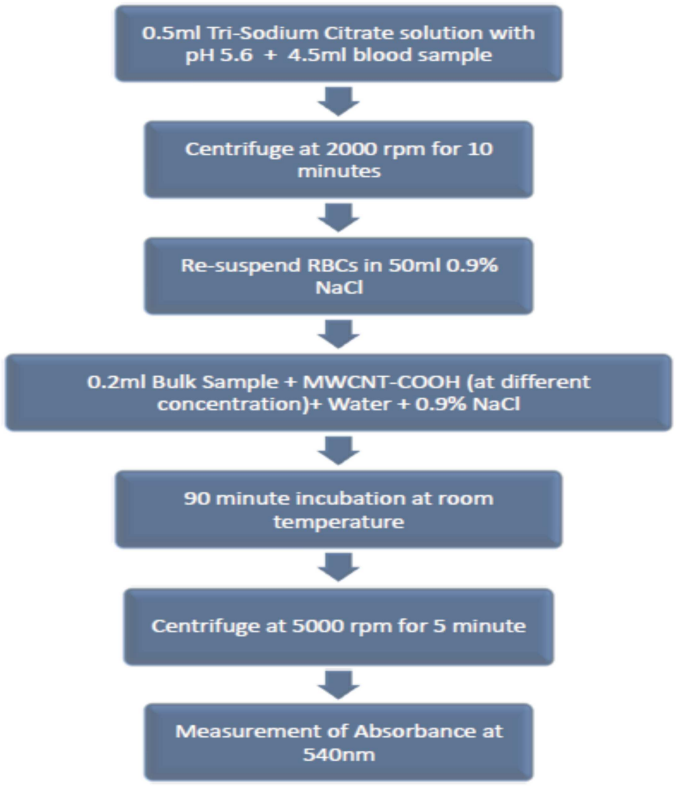

Figure 1: Flow chart of procedure for measurement of Hemolysis Activity against RBCs

RESULTS AND DISCUSSION

No red colour was observed in test vials, which led to no absorbance at 540nm. This indicated Carboxylated multi walled carbon nanotubes are unable to break red blood cells even at 500 µg/ml within 1 hour. Following table 2 illustrates absorbance of different samples at 540nm.

Table 2: Absorbance of samples measured at 540nm

Sample	O.D.$_{540}$
Negative Control	0.0
Positive Control	0.323
MWCNT	
25µg/ml	0.0
100µg/ml	0.0
250µg/ml	0.0
500µg/ml	0.0

Figure 2: Centrifuged MWCNT-COOH & RBCs samples

CONCLUSION

MWCNT-COOH don't have ability to cause hemolysis of RBCs within 1 hour incubation period at 500 µg/ml. Which is a confirmatory incite to prove, MWCNT-COOH are unable to cause Hemolysis in short time. They may cause hemolysis in case of lengthier incubation periods. Therefore, this study demands to check hemolysis activity of MWCNT-COOH at different concentration in lengthier incubation periods.

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