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Vibrational spectroscopic detection of radiation-induced structural changes in *Chironomus* hemoglobin

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ABSTRACT

Purpose: Chironomus hemoglobin is known to exhibit higher gamma radiation resistance compared to human hemoglobin. In the present study, we have introduced a sensitive method to analyze radiation-induced alterations in *Chironomus* hemoglobin using Vibrational spectroscopy and further highlighting its potential for monitoring radiotoxicity in aquatic environments.

Materials and methods: Vibrational spectroscopic methods such as Raman and FT-IR spectroscopy were used to capture the distinctive chemical signature of *Chironomus* hemoglobin (ChHb) under both *in vitro* and *in vivo* conditions. Any radiation dose-dependent shifts could be analyzed Human hemoglobin (HuHb) as standard reference.

Results: Distinctive Raman peak detected at 930 cm-1 in (ChHb) was attributed to C–N stretching in the heterocyclic ring surrounding the iron atom, preventing heme degradation even after exposure to 2400 Gy dose. In contrast, for (HuHb), the transition from deoxy-hemoglobin to met-hemoglobin at 1210 cm-1 indicated a disruption in oxygen binding after exposure to 1200 Gy dose. Furthermore, while ChHb exhibited a consistent peak at 1652 cm-1 in FT-IR analysis, HuHb on the other hand, suffered damage after gamma irradiation.

Conclusion: The findings suggest that vibrational spectroscopic methods hold significant potential as a sensitive tool for detecting radiation-induced molecular alterations and damages. *Chironomus* hemoglobin, with its robust interaction of the pyrrole ring with Fe, serves as a reliable bioindicator molecule to detect radiation damage using vibrational spectroscopic method.

1. Introduction

Vibrational spectroscopic methodologies have become indispensable assets in biological research, presenting versatile applications for unraveling the intricate molecular behaviors of atoms and molecules. Exploiting the vibrational dynamics of molecular bonds, these techniques offer a potent avenue for elucidating molecular identities and structures. In recent times, vibrational spectroscopy has emerged as a sensitive technique to investigate molecular structure and dynamics as well as molecular fingerprinting based spectral analysis of biomedical samples [1].

These methodologies afford unique spectral profiles spanning a broad spectrum of biological constituents, encompassing both normal and aberrant tissues, cells, Deoxyribose Nucleic Acid (DNA), Ribose Nucleic acid (RNA), and proteins [2]. They serve as cornerstones for structural elucidation, furnishing fundamental insights into the constitution of biological entities. Any deviation observed in these spectral patterns serves as an alarm, indicating potential anomalies or deviations within biological specimens, thus accentuating their significance in the realms of biomedical exploration and diagnostics. Among these
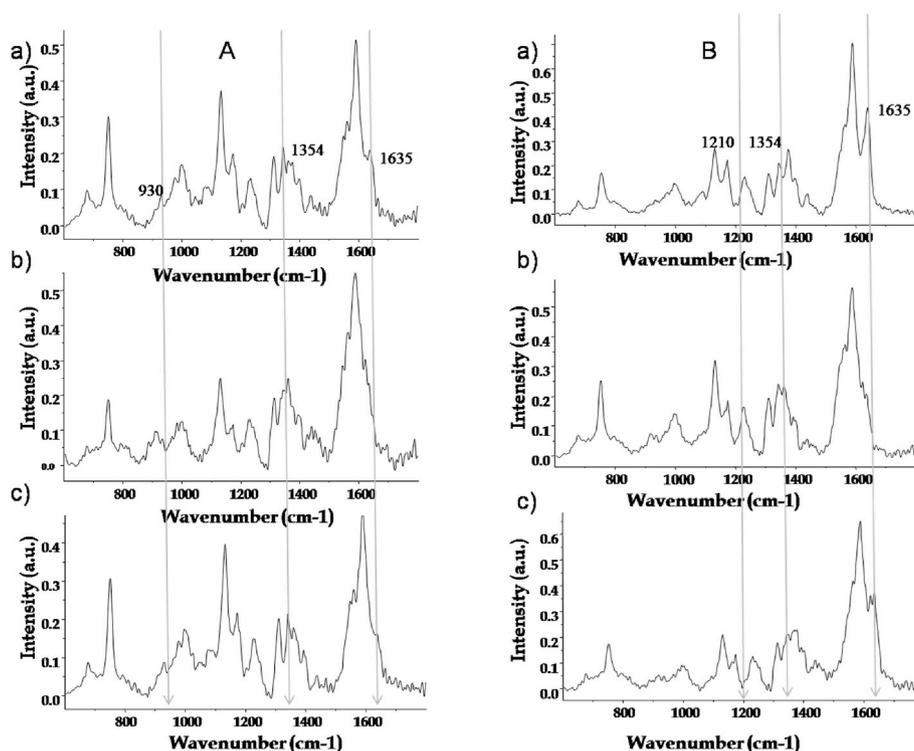
Fig. 1. Micro-Raman spectra recorded from 500 to 1800 cm^{-1} for A: *Chironomus* hemoglobin (ChHb), B: Human hemoglobin (HuHb); a) control, b) 1200 Gy gamma exposure c) 2400 Gy of gamma radiation exposure.

techniques, Fourier Transform Infrared (FTIR) imaging has been increasingly employed for point-of-care diagnosis, serving as a biochemical fingerprint for cellular analysis [3,4]. Raman spectroscopy, coupled with FTIR analysis, stands out as a highly sensitive and nondestructive method, capable of detecting subtle structural changes in molecules. Previous research has explored Raman and FTIR spectroscopic data to study signatures in human keratinocytes and lymphocytes following radiation exposure, showcasing the potential of these methods in radiation research [5].

Furthermore, spectroscopic techniques have found applications in ecology and evolution, offering reliable indicators of intra- and interspecies variations, environmental influences, selective pressures, and fitness. In the context of environmental stressors, insect pest management, and age-grading studies, various spectroscopic methods have been utilized, highlighting their versatility [6,7]. Recently, near infrared spectroscopy on whole organism was used for age-grading of wild populations of *Anopheles gambiae* [8].

Amid growing concerns about radiation exposure from human-made devices, nuclear accidents, and environmental radioactivity, there is a need for innovative technologies and bioindicator organisms to assess the impact on ecosystems [9]. Aquatic environments, acting as the ultimate sink for a myriad of toxicants, including radioactive wastes, are particularly vulnerable (EC-2008). Chironomid midges, specifically *Chironomus ramosus*, have emerged as model organisms due to their resilience against environmental stressors, including gamma radiation exposure [10–15].

Chironomid midges possess unique extracellular hemoglobin (Hb), earning them the name 'blood worms.' The monomeric/dimeric *Chironomus* hemoglobin (ChHb) exhibits an alkaline Bohr effect and noncooperative binding for oxygen [16,17]. Remarkably, the hemoglobin of these insect larvae can withstand heavy metal stress [18]. In our prior research, we discovered that the aromatic amino acid composition within the heme pocket of *Chironomus* hemoglobin contributes to its resilience to gamma radiation. This stands in contrast to human hemoglobin, which features aliphatic amino acid composition in its heme

pockets, promoting water interaction and the generation of free radicals upon radiation exposure, consequently harming the cell [19].

Building on previous observations studied through UV spectroscopy and CD spectroscopy, our research aims to obtain vibrational spectroscopic signatures of the effects of radiation on hemoglobin protein in *Chironomus in vivo*. This study not only reaffirms our earlier findings but also proposes the development of a high-precision and sensitive vibrational spectral analytical technique to assess the radio-toxicity of aquatic biota, using *Chironomus* hemoglobin as a molecular tool.

2. Materials and Methods

2.1. Rearing of Chironomus larvae and gamma radiation exposure

Chironomus ramosus were cultured at a controlled temperature of 25 ± 2 °C, with a relative humidity of 50 ± 10 % and a photoperiod of 14 h of light followed by 10 h of darkness in the insectory of Bhabha Atomic Research Centre (BARC), Mumbai, India. Larvae were fed on every alternate day with dry moss extract along with supplements as described by Nath & Godbole [20]. As fourth instar larvae is fully developed stage of larvae and possess higher percentage of hemoglobin in their circulation this stage has been chosen for all the experiments. A fourth instar larva can be identified when it reaches the 'read-head stage' [21]. Fourth instar larvae were exposed to gamma radiation doses of 1200 Gy or 2400 Gy from a ^{60}Co source (dose rate 55.6 Gy/min, Gamma Cell 5000, BRIT, Mumbai, India) while being submerged in tap water. Control groups were maintained under identical conditions outside the gamma chamber. Human hemoglobin (H7379, Sigma Aldrich) solution, referred to as HuHb, was prepared in a buffer solution consisting of 50 mM Na-phosphate and 100 mM NaCl at pH 7.0. As described previously [19], *Chironomus* hemolymph was collected from the larval body using glass capillary, centrifuged and pellet consisting hemocytes were discarded. Supernatant containing *Chironomus* hemoglobin (ChHb) was used for further experiments.
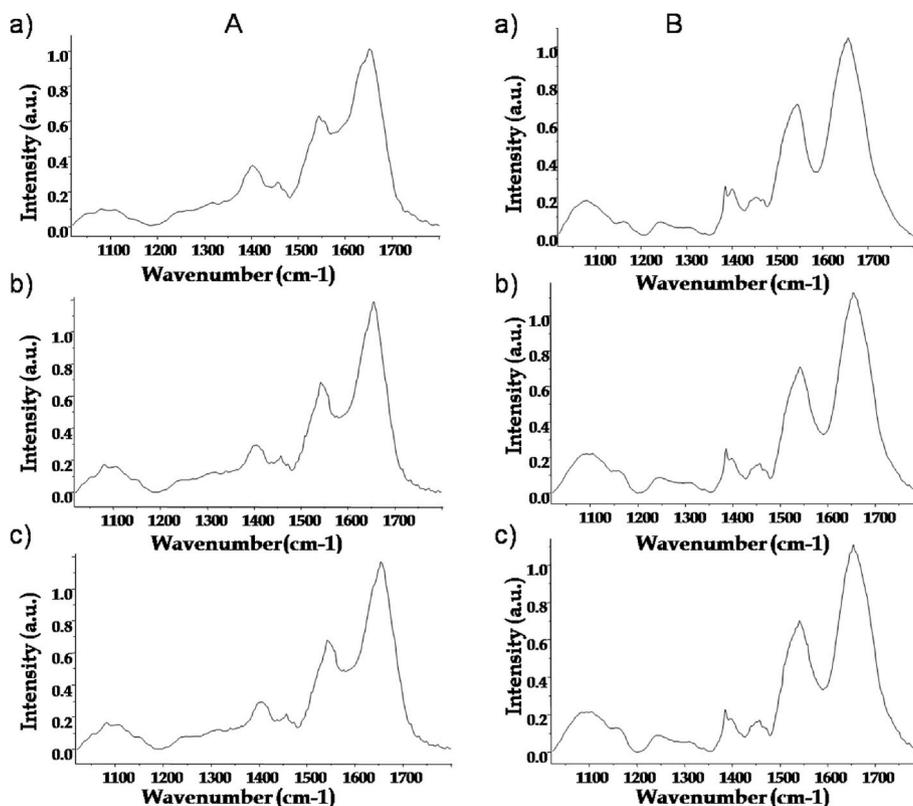
Fig. 2. FT-IR spectra obtained after 1200 Gy and 2400 Gy of gamma radiation exposure from 900 cm^{-1} to 1800 cm^{-1} for A. ChHb and B. HuHb; where a) Control, b) 1200 Gy and c) 2400 Gy gamma radiation exposure.

2.2. In-vitro Raman spectroscopy

ChHb and HuHb samples were exposed to 1200 Gy and 2400 Gy of gamma radiation. Following radiation exposure, 10 µl of sample were dried on silicon wafers to form a single layer. Raman spectra were recorded using a Witec 300 R alpha confocal Raman spectrometer equipped with a He–Ne laser operating at 532 nm and a 10× objective. Spectra were collected in back-scattered geometry within a detection range of 500–1800 cm-1.

2.3. In-vivo Raman spectroscopy

Whole larvae (both control and irradiated) were subjected to spectral recordings using a Witec 300 R alpha confocal Raman instrument with a 532 nm laser (10 mW power) focused on the larval samples through a 10× objective (Zeiss, NA 0.9). Spectra were acquired with an integration time of 25 s and 10 accumulations per larvae. To minimize movement artifacts, larvae were gently blotted on tissue paper after being removed from water. Spectra were recorded from the larval tail region, devoid of other tissues, ensuring target-specific vibrational data.

2.4. Data Pre-processing and analysis for Raman spectroscopy

Acquired spectra (600-1800 cm-1) specific to proteins were interpolated and processed through five-point smoothing and fifth-order polynomial fitting to correct baseline distortions. Raman spectra were then vector normalized. Multivariate analysis techniques, including Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA), were employed to reduce the data dimensionality and maximize variance between groups. The analysis was validated using the cross-validation method. Data analysis was conducted using Unscrambler® X software version 10.4.1 (CAMO Software AS, Oslo, Norway).

2.5. FT-IR spectroscopy

Fourier transform spectra for ChHb and HuHb were recorded within the range of 900 cm-1 to 1800 cm-1 using Bomen, MB Series equipment, with dual SiC/NIR as an internal source, KCl optics, and an MIR-NIR DTG detector.

2.6. Statistical analysis

Micro-Raman spectra and FT-IR spectra were normalized and smoothed using Origin Pro 8.5 software. Data comparison was performed through linear regression analysis using SPSS software, with a significance level set at $p < 0.05$.

3. Results

3.1. Raman spectroscopy

The Raman spectra showed alterations in vibrational spin for both *Chironomus* hemoglobin (ChHb) and Human hemoglobin (HuHb) following exposure to gamma radiation. Fig. 1 illustrates that ChHb, subjected to a 1200 Gy dose of gamma radiation, exhibited changes in peak intensity at 930 cm-1, specifically related to oxygenated hemoglobin (ox-Hb) and pyrrole deformation. This shift indicated C–N stretching in the heterocyclic ring surrounding the iron atom, which protected the heme from degradation and stabilized the *Chironomus* hemoglobin molecule. Notably, this distinctive peak was absent in HuHb samples. Instead, consistent peaks at 1354 cm-1 were observed in control samples, with intensification noted in irradiated samples.

Following exposure to 1200 Gy of gamma radiation, significant damage was observed in HuHb, with a transformation from the deoxygenated hemoglobin (deoxyHb) state to met-Hb at 1210 cm-1, hindering oxygen binding to heme. Additionally, the peak position of ferrous

Table 1

Confusion matrix after PC-LDA analysis for *Chironomus* control and test larval samples (T1: 1200 Gy and T2: 2400 Gy).

Predicted Actual	C	T1	T2
C	37	12	17
T1	08	18	03
T2	14	19	32

hemoglobin at 1370 cm-1 in HuHb shifted to ferric Hb at 1377 cm-1 after doses of 1200 Gy and 2400 Gy. In contrast, the peak position of ChHb samples remained unchanged at 1635 cm-1 after gamma radiation exposure, while a peak shift was observed in irradiated HuHb samples, indicating the formation of deoxy-Hb. These findings suggest a significant impairment in the efficiency of oxygen binding to heme after high doses of gamma radiation, rendering human hemoglobin more susceptible to radiation-induced damage [position for Fig.1].

3.2. FT-IR spectroscopy

FT-IR spectroscopy was utilized in this investigation to elucidate the compositional alterations in ChHb and HuHB, subsequent to exposure to high doses of gamma radiation. As depicted in Fig. 2, the FT-IR spectra displayed discernible variations in the characteristics of ChHb and HuHb. Noteworthy changes in band intensity were observed for both ChHb and HuHb within the 1400 cm-1 to 1700 cm-1 region, attributable to amide I and amide II bands arising from C═O stretching vibrations of the peptide linkage.

A broadened amide I band was discernible for ChHb following gamma radiation exposure (1200 Gy and 2400 Gy), suggesting a prevalence of alpha-helical structure in comparison to HuHb. Given that the α-helical structure plays a crucial role in maintaining the integrity of the protein's secondary structure, the radiation-induced feature at 1652 cm-1 in the case of ChHb indicates a protective mechanism against hemoglobin degradation. Furthermore, a notable increase in the intensity of the porphyrin peak at 990 cm-1 was observed in ChHb samples following radiation exposure compared to HuHb samples. This observation suggests the likelihood of a robust interaction between the pyrrole ring and Fe exclusively in *Chironomus* hemoglobin, essential for mitigating radiation-induced protein damage [*position for Figure 2]

3.3. In-vivo Raman spectroscopy for Chironomus whole larvae

In vivo Raman spectroscopy was conducted to validate the radiation tolerance characteristics of *Chironomus* hemoglobin (Hb) at the organism level, given our data suggesting that gamma radiation exposure did not induce structural changes in *Chironomus* hemoglobin (as indicated in Table 1). As depicted in Fig. 3A, Principal Component Analysis (PCA) revealed distinct clustering among all three groups - Control, 1200 Gy, and 2400 Gy - indicating minimal variation between Raman spectra of

Chironomus larval hemoglobin in the control and treated groups. Similarly, the confusion matrix (Table 1) and Fig. 3B demonstrated significant overlap between factor one (C, i.e., control) and factor two (T, i.e., treated), further supporting the lack of substantial differences between the two groups.

It's important to note that this methodology could not be applied to *in vivo* studies involving human hemoglobin. Our findings substantiate the hypothesis that this unique feature of *Chironomus* larvae could serve as a valuable tool for biomonitoring background radiation in potentially contaminated water bodies. [Please refer to the indicated positions for Fig. 3A and B, and Table I].

4. Discussion

Present study highlights vibrational spectroscopic signature of invertebrate hemoglobin, an abundant extracellular protein in *Chironomus* larvae. This study marked the pioneering use of a radiation-tolerant invertebrate model organism for Raman probing and its potential application for radiation toxicity assessment in aquatic environments using hemoglobin of Chironomid midges as molecular bio-indicator.

Previous studies suggested that FT-IR peaks could arise at the region of 1589–1730 cm-1 which is specific for beta sheets, turns and coils while 1474- 1589 cm-1 explained alpha helical structure of a protein; whereas porphyrin stretching arises at 900 -1200 cm-1 [22,23]. Polakovs et al. [3] employed FT-IR spectroscopy to investigate the radiation-induced alterations in human blood. Their findings revealed that ion Fe^{2+} within the heme of hemoglobin undergoes oxidation to Fe^{3+} upon radiation exposure. This transformation into methemoglobin poses a significant concern for the vital functions of the human body. As documented in our prior research, the presence of aromatic amino acids surrounding the heme pocket in ChHb inhibits the formation of methemoglobin, thereby demonstrating its resilience to gamma radiation exposure [19].

Presently, ChHb and HuHb samples, when subjected to vibrational spectroscopic assays, represent two extreme dose-threshold endpoints for gamma radiation stress. The small size of *Chironomus* larvae (~1 cm) and their abundant hemoglobin content make them ideal candidates for *in-vivo* probing. Additionally, *Chironomus* larvae have proven to be resilient survivors in radioactively contaminated water bodies, as evidenced after the Chernobyl accident (cross references in Ref. [15]).

Furthermore, the differentially identified FT-IR peak at 1652 cm-1 between *Chironomus* and Human hemoglobin holds promise as a potential marker for high doses of gamma radiation bio-monitoring. Although *in vivo* measurement with FT-IR spectroscopy is limited due to water absorption, whole larval *Chironomus* Raman spectroscopy offers a distinct advantage in measuring subtle changes in hemoglobin. The presence of radioresistant hemoglobin molecules in these midges can thus be harnessed as a potential whole organismal biosensor, enabling
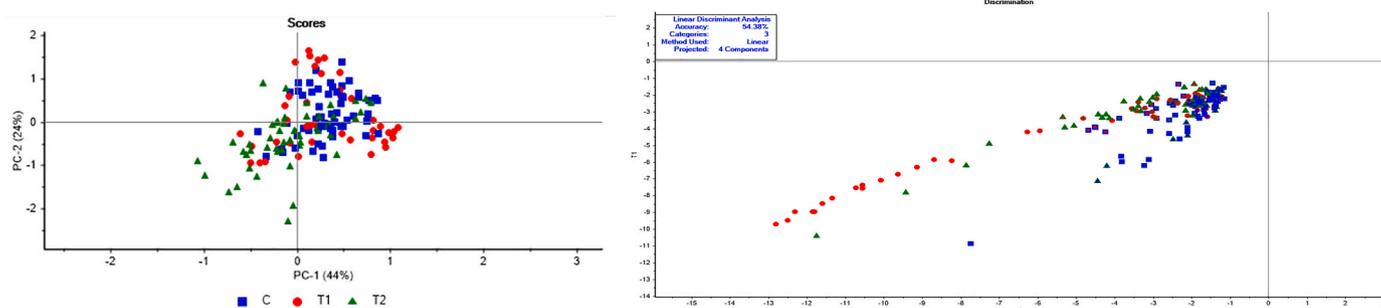

Fig. 3. A. PCA plot showing group clustering between *Chironomus* whole larvae samples, control (Blue), 1200 Gy (Green) and 2400 Gy (Red) after *in-vivo* Micro-Raman spectroscopic analysis; B. LDA analysis showing score between experimental conditions for *Chironomus* larvae between factor one (C for Control) and (T1 for Test 1200 Gy) in the form of scatter plot.

the evaluation of very high radiation levels in contaminated water bodies.

CRediT authorship contribution statement

Declaration of competing interest

The authors report no conflict of interest.

Data availability

No data was used for the research described in the article.

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