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Mendeleev Commun., 2023, **33**, 858–860

Mendeleev Communications

The effect of tritiated water on the alkaline phosphatase inactivation after ultrasound and gamma-rays irradiation

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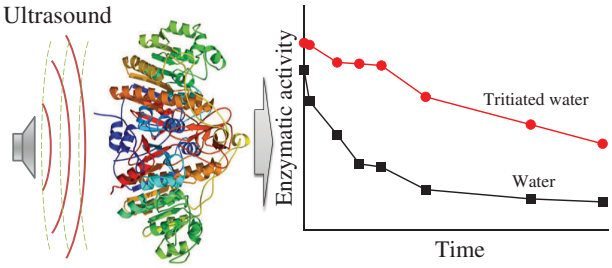
DOI: 10.1016/j.mencom.2023.10.040

Comparative analysis of the alkaline phosphatase post-inactivation dynamics in natural and tritiated water (H_2O and HTO, respectively) after ultrasonic or γ-irradiations was performed. It has been established that in HTO, unlike in H_2O, the enzyme retains its activity up to 15 days. It is hypothesised that different hydrogen bond structure of tritiated water results in a lower effect of ultrasound or gamma-irradiation reactive species onto enzyme globules.



Keywords: alkaline phosphatase, inactivation, ultrasound, ionising radiation, tritiated water, combined treatment.

One of the most prevalent areas of modern medicine is the design of an individualised therapy regimen. Developments in the combination cancer therapy field are, potentially, an extensive way of dealing with the personal therapy requirements and restrictions of a patient's condition. As such, tumour treatment based on combined ionising radiation and ultrasound action is a promising method, the reason being that the curative potentials of each component are complementary.

The influence of ultrasound (US) is most commonly witnessed upon extracellular matrices. Under the effect of US the cancerous cell membranes and vessels are degraded and drug diffusivity (including radiopharmaceuticals) is increased. US-mediated hyperthermia enhances the therapeutic action of ionising radiation.

Ionising radiation (IoR) is destructive to a wide range of vital molecules within cancerous cells and also to permeability of cell nuclei membranes, and mitochondria membranes.

Therefore, combining both these methods for one therapy regimen may result in an increase in initial cellular damage probability[1] and the radiopharmaceuticals bioavailability, thus decreasing cancer cell regeneration ability.

The scope of US and IoR combination therapy potential has been considered since the middle of the last century.[2–4] Such studies were reviewed by Kremkau[5] in the 170-article assay, however, there was no comprehensive verdict if this combination would yield an effective therapy method. The results reported vary from synergistically effective to polar opposite, causing severe damage to healthy tissues. The assumed reason behind such diversity is the lack of standardised approach in the study, in particular, the chosen tumour type and US treatment conditions. In the last decade, interest in this combination for therapy has again increased. Nowadays, the US treatment is reduced to sonodynamic therapy (low-intensity US exposure) which largely improves upon its medicinal capabilities.[6,7] A range of successful experiments both *in vivo* and *in vitro* were reported.[8] At the same time, there is a lack of concern for consequences of bodily exposure to US physical factors. These consequences should be prioritised for consideration in therapy regimen design.

In this work, we analysed the alkaline phosphatase (the model object chosen for the study) inactivation dynamics after the combined sequential US treatment and β-radiation of tritium, present as tritiated water (HTO) with specific radioactivity 9.1 mCi ml^{-1}. Estimated absorbed dose in the used HTO constitutes 1.1 Gy in 1 hour (800 Gy in 30 days). Considering the long-lived biological effects of both IoR and US, the main objective was to study the enzyme inactivation dynamics after the treatment is over (for details, see Online Supplementary Materials). The results of these experiments were compared to those obtained with γ-irradiation replacing the US.

Alkaline phosphatase (ALP) (EC 3.1.3.1) is a two-subunit cell membrane bound enzyme. The fluctuations in its activity during and after treatment signal cell membrane damage as well as repair mechanism activation.[9]

Tritium is generally considered one of the least hazardous radionuclides. Therefore, it is widely used as a radioactive tracer in chemical and biochemical studies.[10] It emits β-particles with a maximum energy of 18.59 keV, the average energy being 5.7 keV. Up to 90% of β-particles are stopped by 1 μm of water resulting in high ionisation density within the latter. The primary chemical processes that occur during the decay of tritium in the composition of tritiated water can be represented as follows:

$$H–O–T \rightarrow H–O–{}^3He^+ + e^-, \quad (1)$$

$$H–O–{}^3He^+ \rightarrow H–O^{\cdot +} + {}^3He, \quad (2)$$

$$H–O^{\cdot +} + H_2O \rightarrow [O] + {}^{\cdot}H_3O^+. \quad (3)$$

In addition, the β-particles transfer energy to the media with formation of a large number of ions and excited molecules. High stopping power of tritium radiation may affect charge dependent processes in the surrounding molecule matrix.

Therefore, the tritium decay effect on organisms may be both activating and detrimental. Even for low activity HTO, 'trigger' function of tritium decay products was shown.[11–13] Thus, HTO as the IoR source for treatment was selected.

The dynamics of alkaline phosphatase inactivation was assessed by the change in activity after the cessation of exposure. The combined treatment was executed by administering the ALP HTO solution with a concrete US or γ-irradiation dose. Such doses that the ALP activity change from direct exposure does not exceed 10% were selected.

Considering the use of the same standardised vessel for treatments as well as the constant ALP concentration, solution volume and method parameters throughout all experiments, the results are presented in arbitrary relative units. The initial value of the activity of the native enzyme under experimental conditions (20 μmol min^{-1} mg^{-1}) was taken as a conventional unit of activity.

It was revealed that enzymatic activity in H_2O drops by 10% after US treatment and it continues to decrease to 20% after the treatment stops across 20-day containment (at 10 ± 2 °C and 20 ± 2 °C) (Figure 1). The character of dependence in this case is retained in the temperature range of 10–20 °C.

The rate of the initial activity drop $r_{0,US} = 0.068 \pm 0.011$ day^{-1} shows no correlation with temperature changes. After that the enzyme was shown to have unchanging activity over a long period of time. Substituting US for γ-irradiation yields a similar ALP inactivation pattern (Figure 2) with an initial inactivation rate of $r_{0,\gamma} = 0.060 \pm 0.008$ day^{-1}. In this case, the doses for US and γ-irradiation were chosen such that at the end of exposure the activities of enzymes treated with US and γ-radiation were close.

On the contrary, the same US treatment in HTO results in different inactivation dynamics. The pattern is similar to the one obtained from a control sample (not treated with US or γ-irradiation, H_2O) activity analysis. γ-Irradiation of the ALP solution in HTO (Figure 2) yields a less prominent yet alike pattern. It was shown, however, that an untreated enzyme solution in HTO has a dissimilar pattern of inactivation.

It should be noted that the thermal induced ALP inactivation in HTO solution showed a greater activity decrease than the one in H_2O. These inactivation dynamics are no longer discrepant from the ALP in HTO without the US treatment. The inactivation pattern does not persist (Figure 3).

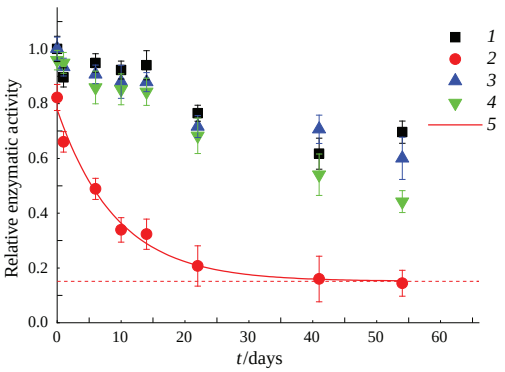


Figure 1 US-induced ALP inactivation: *1* – blank, *2* – ultrasound, *3* – tritiated water, *4* – tritiated water and ultrasound, *5* – approximation of data points (curve *2*).†

† See details in Online Supplementary Materials (Table S1).

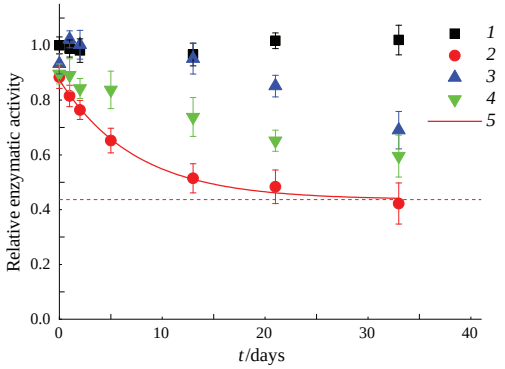


Figure 2 IoR (γ-rays) induced ALP inactivation: *1* – blank, *2* – γ-rays, *3* – tritiated water, *4* – tritiated water and γ-rays, *5* – approximation of data points (curve *2*).†

US treatment of aqueous solutions yields the formation of free radicals and their transformations products under the conditions of cavitation, which is the main factor causing the enzyme inactivation. This hypothesis has been proven by IoR and US exposure of the dry ALP. In diluted solutions, mainly H and OH radicals and H_2 and H_2O_2 molecules are present. Radicals exist only while sustained by US treatment, but the peroxide formed persists after the cessation of treatment. It is assumed that H_2O_2 is the main factor contributing to post-inactivation. Its content may correlate with post-inactivation process rate and extent. Dry samples were irradiated under the same conditions as their solutions. In the case of irradiation in air, subsequent dissolution of the samples did not reveal any changes in their activity.

It was shown that the same dose results in higher H_2O_2 content in H_2O than in HTO; H_2O_2 quantity decreases in post-inactivation process; US treatment results in higher content of H_2O_2 in H_2O than γ-irradiation and its content correlates with initial rate of inactivation; H_2O_2 formation in HTO as a result of US treatment is only slightly higher than after γ-irradiation.

Therefore, the H_2O_2 content formed during treatment correlates with post-inactivation process rate and extent. It was revealed that US-induced inactivation and post-inactivation mechanisms are different.

Adding H_2O_2 to ALP solutions in H_2O or HTO does not affect the enzymatic activity and does not show any 'protective' capabilities unlike HTO against γ-irradiation or ultrasonication. Both the close contact with reactive species and globule structure changes during the treatment may be assumed.

Individual cavitation bubbles may be considered as independent (of each other) reactive species source. Therefore, the target theory may be applied in order to evaluate a mean number (N_m) of reactive species hits onto an enzyme molecule

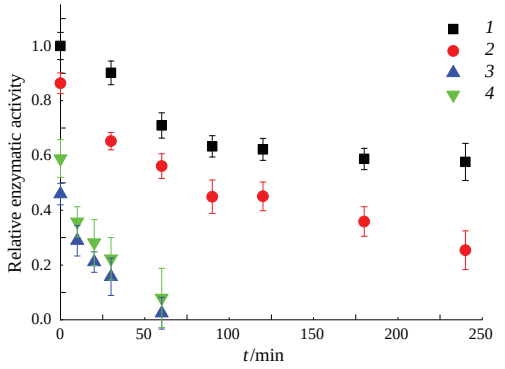


Figure 3 Thermal (60 °C) induced ALP inactivation: *1* – blank, *2* – ultrasound, *3* – tritiated water, *4* – tritiated water and ultrasound.

for both treatment methods. This evaluation is possible, given the number of unaffected molecules N to their initial number N_0 : $N_m = -\ln(N/N_0)$.

If it is assumed that inactivation of the part of the enzyme molecules that have not been exposed to active particles occurs in the same way as in the native enzyme (study supports this hypothesis with the persistent values of Michaelis Constant), then inactivation graph exponential approximation remaining constant activity value equals N/N_0 (see Figures 1 and 2).‡ US treatment is characterised by $N/N_{0,US} = 0.151 \pm 0.026$ and γ-irradiation by $N/N_{0,\gamma} = 0.437 \pm 0.015$. Such an estimate then gives the following N_m values: $N_{m,US} = 1.89 \pm 0.17$, $N_{m,\gamma} = 0.83 \pm 0.03$.

There are at least two possible explanations of such increase in enzyme γ- or US treatment resistance in HTO compared with H_2O. The first, it might be caused by a higher cavitation nucleation threshold in HTO that results in a lower reactive species effect onto the enzyme, yet this hypothesis was not confirmed by experimental data. The second, we can assume that HTO has a different solution matrix.

Tritiated water contains reactive species of tritium decay of tritium decay (such as H and OH radicals, H_3O^+, e_{aq}^-, OH^-, H_2, H_2O_2, *etc*.), all of them are characterised by different mean lifetime and equilibrium concentration, depending on many factors. e_{aq}^-, a strong reducing agent, has the longest lifetime among such species,[14] therefore, its presence can affect the transformations of other reactive species generated by additional high-intensity IoR or US. It may be assumed that the presence of such reductive reactive species results in a decrease of hydrogen peroxide formation rate. It is possible that the addition of tritiated water performs a protective function when treating ALP solutions with IoR (γ-ray and US).

Reactivity of aqueous solutions after IoR treatment is determined by the hydrogen bond matrix. In HTO, due to constant weak radiation processes, disorder of hydrogen bond matrix may cause a weaker impact of physical factors upon globular enzymes.[15] An enzyme subjected to constant HTO irradiation might potentially become more resistant to such effects.

Meanwhile, at 60 °C thermal induced ALP inactivation protective function of HTO is no longer observed (see Figure 3). Furthermore, its effect on enzyme inactivation becomes the most prevalent. It is possible that the impact of free radicals and their transformations products is enhanced at 60 °C. It may be assumed that such synergy is caused by a higher rate of hydrogen bond matrix disarray (instigated by tritium decay) at higher temperatures. At 60 °C the tritiated medium loses its ‘protective’ properties with respect to the action of ultrasound. Moreover, it accelerates the thermal inactivation process.

‡ See details in Online Supplementary Materials.

In conclusion, with implementation of combined US and IoR treatment on biological objects it is crucial to estimate the probability of antagonistic effects occurring. Special attention to the study of post effects mechanisms and their dependence on the temperature and content of the medium should be paid.

The study was partly supported by state assignment of Lomonosov Moscow State University ‘Obtaining and application of radionuclides and labeled compounds for the purposes of nuclear medicine, the study of biologically significant processes and the interaction of living organisms with ionizing radiation’ (project registration no. 122012600116-4).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.10.040.

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Received: 8th June 2023; Com. 23/7189