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RAPID COMMUNICATION

Protective effect of nicotinic acid on human albumin during UV-C irradiation

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Abstract−The potential of nicotinic acid (NiAc), a vitamin B3 compound with antioxidant activity, to protect human albumin against the damage caused by exposure to 254-nm UV-C radiation was investigated. Experiments performed at irradiance levels of 3.5-15 W m^{-2} and application times between 5 and 30 min showed that albumin undergoes rapid dose-dependent aggregation when subjected to irradiation. Addition of 0.2 mM NiAc to the albumin solution resulted in a remarkable reduction of protein damage. Protection provided by NiAc was effective up to radiation doses of about 30 kJ m^{-2}, which are significantly higher than those required for complete microbial and viral inactivation.

Key words: Albumin, Nicotinic Acid, Sterilization, UV-C, Viral Inactivation

INTRODUCTION

Human albumin (HA) is the most abundant protein in blood plasma, with a concentration of about 40 g L^{-1} in healthy individuals. HA has many important physiological functions, including maintenance of colloid osmotic pressure, buffering of pH, antioxidant activity and transport of a variety of endogenous and exogenous substances [1].

The world production of HA obtained from the blood of donors is of the order of hundreds of tons per year [2]. Its primary use is as a plasma expander in situations involving severe blood loss, traumatic shock and the treatment of burns. Moreover, because of the lack of toxicity and immunogenicity, HA is also used as a manufacturing excipient in vaccines and therapeutic protein formulations, for coating of medical devices and as a component in drug delivery systems.

Although the viral safety of HA and other plasma derivatives has been enhanced significantly over the past decade, the risk of transmitting certain pathogens is still of great concern [3]. This is particularly the case for small non-enveloped viruses, such as parvovirus and hepatitis A virus, and prions, which are relatively resistant to inactivation by physicochemical procedures [4].

Recently, there has been a renewed interest in the use of UV-C light as a possible means of virus elimination from blood components and plasma derivatives [5]. In particular, continuous-flow UV-C devices operating at 254 nm have been designed and their ability to eliminate viruses accurately tested [6-8]. The virucidal effect of UV-C radiations (100-280 nm) is due to their inherent capacity to induce the dimerization of adjacent pyrimidines in DNA and RNA, preventing subsequent replication and transcription. In contrast to UV-A and UV-B, UV-C treatments do not require the addition of photosensitizers and can be easily implemented. However, free radicals generated by UV irradiation may have detrimental effects on the proteins, causing loss of activity and the formation of potentially immunogenic aggregates [9].

NiAc is a water-soluble, B-complex vitamin (vitamin B3) which has been used for over 50 years for treating a variety of lipid disorders because of its ability to reduce low-density lipoprotein cholesterol and triglyceride levels, while increasing high-density lipoprotein levels [10]. Furthermore, NiAc has also been demonstrated to exhibit primary antioxidant activity [11]. In particular, Hu et al. reported that NiAc was capable of scaveging ˙OH radicals with a second-order rate constant of 6.8 10^8 M^{-1} s^{-1}, which was about one-third of those of mannitol and thiamin [12]. For these reasons and for the fact that NiAc is considered harmless from a toxicological point of view, we decided to explore its potential as a protective agent in a virucidal UV-C treatment of human albumin. More specifically, we were interested in assessing whether small additions of NiAc to the protein solution could mitigate the detrimental effects of UV-C irradiation. Experiments were performed on recombinant human albumin (rHA), which is structurally identical to the serum-derived protein but entirely fatty acid-free [13]. This allows avoiding the confounding effects resulting from the presence of protein-bound compounds [14].

EXPERIMENTAL

rHA (CAS No. 70024-90-7) and NiAc (CAS No. 59-67-6) were purchased from Sigma-Aldrich (Milano, Italy). rHA, expressed in rice, was obtained as a lyophilized powder with a purity ≥96%. NiAc was supplied in the solid form with a purity ≥99.5%. Both products were dissolved in distilled water just before use.

Irradiation experiments were performed in a cabinet at room temperature (20±2 °C). A 6-W low-pressure mercury lamp (Spectroline ENF-260C/FE, Spectronics Corporation, Westbury, NY, USA), emitting predominantly at 254 nm, was used for UV-C exposure. To ensure a stable light emission, the lamp was warmed up for at least 5 min before starting the experiment. Then, a given amount of 0.2 μm-filtered protein solution, with or without NiAc, was poured into a stoppered quartz cuvette (path length: 1 cm). The cuvette was positioned perpendicular to the direction of light propagation, at the selected distance from the external surface of the lamp, and irradiated for various time intervals (5 to 30 min). No appreciable change in the temperature of the irradiated solution was observed over this

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period, the maximum increase being <1 ºC. At the end of exposure, the cuvette was removed, gently hand-shaken and the absorbance spectrum was recorded. Measurements were made in a double-beam spectrophotometer (Perkin-Elmer, Lambda 25) against a blank consisting of pure water or NiAc in water subjected to the same conditions as the rHA solution. In all experiments the protein concentration was 1 g L^{-1} (1.5 10^{-5} M) while NiAc, when present, was at a concentration of 0.025 g L^{-1} (0.2 mM). The irradiance level at 254 nm (I_{254}) for the protein solution was varied by changing the distance of the quartz cuvette from the lamp. The distances considered were 2, 3, 5 and 10 cm. A radiometer equipped with a UV-C sensor (AccuMax XR-1000, Spectronics Corporation, Westbury, NY, USA) was used to determine the corresponding irradiances, which were measured to be 15, 12, 7.4 and 3.5 W m^{-2}, respectively.

RESULTS AND DISCUSSION

Fig. 1 shows typical absorption spectra of rHA exposed to increasing doses of UV-C radiation in the absence (Fig. 1(a)) or presence (Fig. 1(b)) of NiAc. As can be seen, protein irradiation results in a progressive increase in absorbance in the 300-340 nm range and at the two peaks located at 255 nm (local minimum) and 279 nm (local maximum). The ratio between the absorbances at these two wavelengths (A_{max}/A_{min}) was chosen by Artyukhov et al. [15] to monitor the structural changes in serum albumin molecules induced by exposure to vacuum UV radiation (λ<200 nm). According to the authors, a decrease in A_{max}/A_{min} can be attributed to chemical alterations of the aromatic amino acid residues of the irradiated protein, whereas the increase in absorbance at 300-340 nm range is a reflection of the formation of rHA aggregates following protein denaturation [16].

Under the conditions of the present study, we found that A_{max}/A_{min} was essentially independent of the radiation dose applied, suggesting that photochemical alterations of the aromatic amino acid residues of rHA are not likely to occur. Conversely, at all irradiance levels tested we observed a progressive increase in absorbance with exposure time in the 300-340 nm region, indicating that UV-C irradiation induces aggregation of rHA molecules. Therefore, to quantify the extent of protein damage, we used the following parameter [14]: $\alpha = A^*_{320} - A_{320}$, where A^*_{320} and A_{320} are the absorbances at λ= 320 nm (the intermediate wavelength between 300 and 340 nm) of irradiated and unirradiated rHA, respectively.

The effects of exposure time and irradiance level on α are illustrated in Fig. 2. Inspection of these plots reveals three important points. First, an increase in irradiance (from 3.5 to 15 W m^{-2}) causes an increase of α, both in the absence and presence of NiAc. Second, at each irradiance level, NiAc has a marked protective effect on rHA. Finally, while in the presence of NiAc α varies linearly with exposure time, in the absence of the additive the time dependence of α is better described by a second-order polynomial. These results may be indicative of some changes in the degradation pathway of rHA occurring in the presence of NiAc. The linear dependence of α with time is characteristic of a zero-order kinetic process, that is, the aggregation rate is independent of aggregate concentration and numerically equal to the rate constant (k) of the process. We also note, however, that at lower radiation doses (up to about 10-15 kJ/m^2) the kinetic data obtained in the absence of NiAc do not deviate significantly from linearity. Accordingly, we determined the apparent zero-order rate constants (k_0) from the linear portion of the plots. The values of k and k_0 were estimated by least-square analysis of the data, which gave the values listed in Table 1.

From the irradiance level at 254 nm (I_{254}) and the exposure time (t), the radiation dose (D_{254}) received by rHA can be easily determined as $D_{254}=I_{254}\cdot t$. The resulting values were in the range of 1.05-27.0 kJ m^{-2}. Interestingly, when the experimental data were represented as α against D_{254}, the kinetic plots shown in Fig. 2 coalesced into two well-defined curves, the upper one corresponding to systems not containing NiAc (Fig. 3). This suggests that, under the experimental conditions employed, the effects of irradiance level and exposure time on rHA degradation are separable. As can be seen from the data in Fig. 3, the presence of NiAc reduced the extent of UV-induced protein damage by approximately 30 to 60%, the protection increasing with radiation dose.

While the precise nature of the damage caused by UV radiation to proteins has not been unequivocally demonstrated, it is known that both direct and indirect mechanisms are involved [17]. Direct, or primary, effects arise from the absorption of UV radiation by chro-

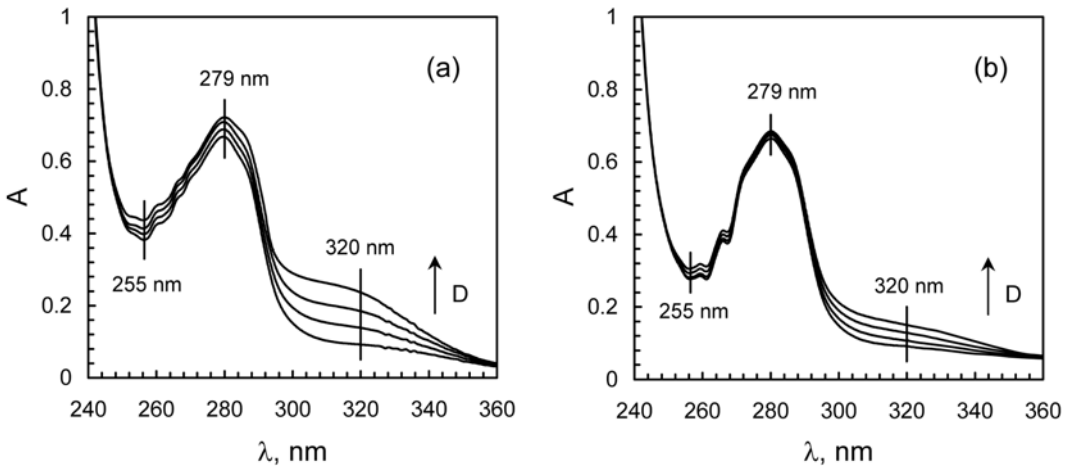

Fig. 1. Effect of increasing doses (D) of UV-C radiation (from 0 to 7 kJ m^{-2}) on the absorption spectrum of rHA at 1 g L^{-1} in the absence (a) and presence (b) of 0.2 mM NiAc.

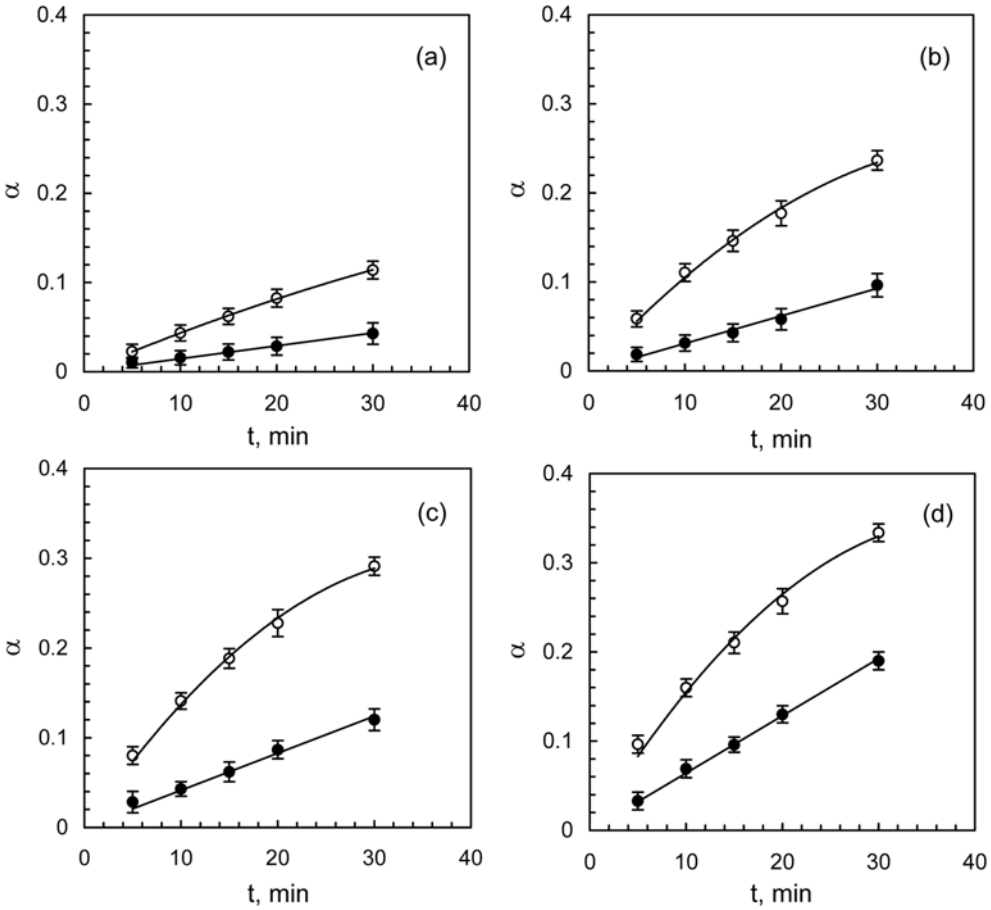

Fig. 2. Effect of exposure time (t) and irradiance level (I_{254}) on rHA in the absence (open symbols) or presence (solid symbols) of NiAc. α is the difference between the absorbances at 320 nm of irradiated and unirradiated rHA. Irradiance levels were: (a) 3.5 W m^{-2}, (b) 7.4 W m^{-2}, (c) 12 W m^{-2}, (d) 15 W m^{-2}. The curves represent best fits to the corresponding data points.

Table 1. Estimated rate constants for rHA aggregation in the absence (k_0) or presence (k) of 0.2 mM NiAc at different irradiance (I_{254}) levels. R_0^2 and R^2 are the coefficients of determination of the corresponding regression equations

I_{254} (W m^{-2})	k_0 (h^{-1})	R_0^2	k (h^{-1})	R^2
3.5	0.272±0.003	0.999	0.087±0.003	0.982
7.4	0.690±0.020	0.995	0.186±0.005	0.986
12.0	0.893±0.032	0.996	0.248±0.007	0.983
15.0	1.003±0.044	0.991	0.385±0.004	0.998

mophores in the protein molecule. Phe, Tyr, Trp and Cys residues are the main targets of UV-C. Light absorption and excitation by these residues is followed by a series of events that can ultimately lead to impairment or complete loss of protein activity. In addition, Phe and Tyr residues are capable of transferring their excited state energy to Trp residues, further enhancing the deleterious effects of UV radiation. Indirect, or secondary, effects are determined by the production of reactive oxygen species (ROS) such as hydroxyl radicals ($^{\cdot}OH$), superoxide anion radicals ($^{\cdot}O_2^-$) and singlet oxygen ($^{\cdot}O_2$). ROS cause oxidative damage to proteins and changes in their secondary and tertiary structure [18].

Although more complete studies are needed to elucidate the mechanisms by which NiAc protects rHA, including monitoring structural and conformational changes in rHA during irradiation, it can be speculated that its efficacy is related to its ability to neutralize

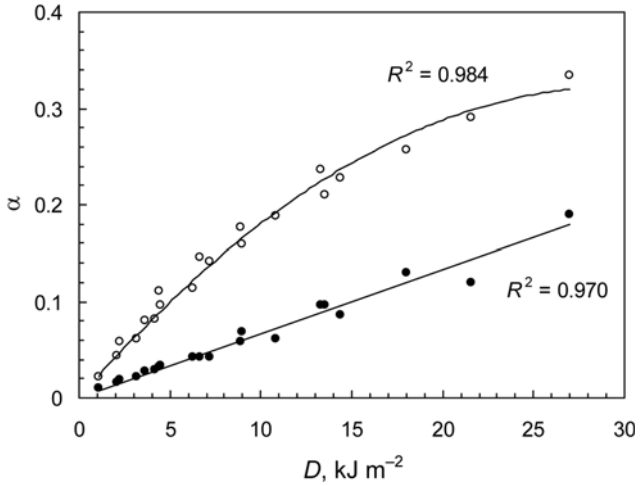

Fig. 3. Effect of UV-C radiation dose (D) on rHA in the absence (open symbols) or presence (solid symbols) of NiAc. α is the difference between the absorbances at 320 nm of irradiated and unirradiated rHA. The curves represent best fits to the corresponding data points.

free radicals. In particular, evidence has been provided that NiAc can quench hydroxyl radicals [12]. These species can be produced during UV-C irradiation of aqueous solutions by the generation of either H_2O_2 or superoxide which, in turn, lead to the formation of $^{\bullet}OH$ in the bulk of the solution. Hydroxyl radicals are known to be powerful reactive agents and cause protein cross-linking and aggregation [19]. According to the Lumry-Eyring model, protein denaturation can be represented by the following two-step process, $N \leftrightarrow D \rightarrow I$, where N is the native protein, while D and I are the partially and irreversibly denatured forms, respectively [20]. ROS generated by UV-C irradiation cause a progressive increase in the concentration of partially unfolded protein molecules which, in order to minimize their solvent-exposed hydrophobic surfaces, form non-native protein aggregates [18,21]. The presence of an antioxidant in the protein medium can thus be expected to protect the protein by displacing partial unfolding toward the native, biologically active, protein form. Such a view is consistent with the few available reports on the subject. So, rutin, a flavonoid glycoside with antioxidant activity against both type I and type II ROS, was found to increase the recovery of coagulation factors from human plasma subjected to virucidal UV-C treatment [22]. The same compound was also found to prevent fibrinogen degradation during UV-C irradiation [23].

Overall, the evidence provided by the present study indicates that NiAc is capable of protecting rHA against the damage caused by exposure to UV-C radiation. We also note that the dose values used here are significantly higher than those considered to be lethal to viral pathogens. In this regard, a recent study using a spiral-flow UV-C reactor showed that doses ranging from 0.74 to 2.16 kJ m^{-2} were sufficient to produce a 4-$\log_{10}$ inactivation of reovirus type 3 and adenovirus type 5, two double-stranded viruses characterized by a large genome size and a comparatively low sensitivity to UV-C radiation [7]. Since the deleterious effects of UV-C on viruses result almost entirely from their direct interaction with DNA (or RNA) molecules and not from free-radical damage [22,24], it can be speculated that the presence of antioxidants in the solution to be treated does not affect the effectiveness of the treatment.

CONCLUSIONS

We have shown for the first time that NiAc added at low concentrations to an aqueous albumin solution is capable of reducing to a considerable extent protein aggregation induced by UV-C. The protection afforded by NiAc was effective even at radiation doses much higher than those required for complete inactivation of viruses and bacterial spores.

Of course, further research is needed to fully assess the real advantages of such an approach including, most obviously, the evaluation of possible interfering effects of NiAc on virus elimination. The possible formation of immunogenic peptides and/or NiAc oxidation products should also be investigated. Nevertheless, the results presented here clearly indicate that the addition of an antioxidant to the protein solution may be an effective means of protecting albumin from the damaging effects of UV-C radiation.

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