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Photodynamic inactivation of enveloped viruses by buckminsterfullerene[1]

Fabian Käsermann[a,*], Christoph Kempf[a,b]

[a]*Department of Chemistry and Biochemistry, University of Bern, Freiestraße 3, 3012 Bern, Switzerland*
[b]*ZLB Central Laboratory, Blood Transfusion Service, Swiss Red Cross, Bern, Switzerland*

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Abstract

Photodynamic reactions induced by singlet oxygen-generating agents are known to inactivate enveloped viruses. In this report we demonstrate that the water-insoluble photosensitizer buckminsterfullerene (C_{60}) can be used to mediate the inactivation of enveloped viruses. Viruses from two different families, Semliki Forest virus (SFV, *Togaviridae*) and vesicular stomatitis virus (VSV, *Rhabdoviridae*) were used as model systems. Buffered solutions containing C_{60} plus either of these viruses were illuminated with visible light for up to 5 h, resulting in a loss of infectivity of more than 7 $\log_{10}$/ml ($TCID_{50}$). Furthermore, it was demonstrated that this viral inactivation was oxygen-dependent and equally efficient in solutions containing protein. C_{60} yields singlet oxygen in very high amounts and is completely inert to photo-oxidative destruction. In addition, it can be easily removed and recycled from aqueous solutions. For these reasons, it may prove useful in the inactivation of viruses in biological systems. © 1997 Elsevier Science B.V.

Keywords: Virus inactivation; Singlet oxygen; Buckminsterfullerene; Semliki Forest virus

1. Introduction

It is generally accepted that enveloped viruses can be inactivated efficiently by singlet oxygen-generating agents, amongst which dyes are the most prominent and widely used. Viral inactivation properties have been described for a wide variety of dyes such as phthalocyanines (Horowitz et al., 1991; Rywkin et al., 1994, 1995), merocyanines (Sieber et al., 1992), porphyrin derivatives (Matthews et al., 1992), hypericin and rose bengal (Lenard et al., 1993; Lenard and Vanderoef, 1993) and methylene blue (Mohr et al., 1995).

The photosensitizer is first excited into the short-lived singlet state following the absorption of light. Normally, the singlet state is converted into the triplet state via an intersystem crossing

* Corresponding author. Tel.: +41 31 6314349; fax: +41 31 6314887; e-mail: kempf@ibc.unibe.ch

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mechanism. This mechanism is assumed to be important for the formation of more stable and longer living species. A further step in the pathway is the transfer of energy from the triplet state to the ground state of oxygen. As a result, highly reactive singlet oxygen is formed ($^1O_2$; type II photodynamic reaction).

An inherent disadvantage of most of these dyes is their water solubility, which makes their removal from solution extremely difficult. An additional problem is that many of these dyes, or their newly formed photoproducts, might be toxic or are known mutagens. For most dyes that might be used in the future, no long term studies on their toxicity to humans or animals are available. Hence, total removal of these dyes from biological fluids will be necessary in most cases. To date only one method which uses photosensitizers as inactivators of viruses has become established in the production of blood plasma components. In this procedure, fresh frozen plasma is treated with methylene blue and visible light, which reduces any viral activity without damaging plasma proteins (Mohr et al., 1995). However, this method has proven to be far from applicable when used on labile products, such as erythrocytes or thrombocytes. In addition, the methylene blue remains in the plasma following treatment.

It is obvious that new methods must be developed in order to overcome these disadvantages. Our studies have focused on the use of the singlet oxygen-generating agent C_{60}, which has the additional benefit that it can be completely and rapidly removed from aqueous solutions.

The photochemical properties of C_{60} have been well studied (Krätschmer et al., 1990; Kroto et al., 1991; Stoddart, 1991) and have recently received intensive attention. C_{60} is a potent generator of singlet oxygen (Arbogast et al., 1991; Nagano et al., 1994) and has been used in preparative photooxygenations (Orfanopoulos and Kambourakis, 1994). There is evidence that C_{60} can also act as a sensitizer in aqueous systems (Orfanopoulos and Kambourakis, 1995). In this report, we studied the inactivation of enveloped viruses in the presence of C_{60} when illuminated with visible light.

2. Material and methods

2.1. Cells and media

Aedes Albopictus cells, clone C6/36 (Igarashi, 1978), were grown at 28°C in Mitsuhashi–Maramorosch medium (MM-medium; Amimed, Switzerland), supplemented with 16% foetal calf serum (FCS), 100 mg streptomycin and 100 U penicillin/ml. Cells were passaged weekly by 1:20 dilution.

Vero cells were grown at 37°C in RPMI 1640 medium (Amimed, Switzerland), containing 10% FCS, 100 mg streptomycin and 100 U penicillin/ml. The cells were passaged weekly by a 1:15 dilution.

2.2. Virus propagation

Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV) were propagated in Aedes cells. Briefly, cells were infected with approximately five plaque forming units per cell. At 24 h post infection the medium was harvested and cellular debris removed by centrifugation (600 g, 10 min). The virus-containing supernatant was aliquoted and stored at −80°C. Virus titres were determined on Vero cells by endpoint titration according to established methods.

2.3. Inactivation assay

A suspension of C_{60} (Aldrich, USA) was prepared by sonification with a sonifier converter tip (sonifier model 110; Branson) for approximately 5 min (position 3, 3 A, D.C.). The C_{60}-suspension (1 mg/ml) in MES-buffered saline (MBS; 400 mOsm, pH 7.4) was spiked with stock virus to obtain an initial virus titre of approximately 10^9 $TCID_{50}$ per ml (50% tissue culture infectious dose). The virus-C_{60} suspension (in MBS containing 10% MM-medium) was stirred constantly throughout the duration of the inactivation assay. O_2 saturation of this suspension was achieved by a constant flow of O_2 (bubbling) which started 5 min prior to, and continued during, the illumination.

Photoillumination of the virus suspensions (2.3 ml, on ice) in a 1 cm glass cuvette was performed

using a 350 W short arc mercury lamp (HBO 350 W; Osram, Germany) positioned at a distance of 28 cm from the sample and equipped with a 495 nm long pass filter (GG 495; Schott, Germany). The light beam was focused on the cuvette, producing a light intensity of approximately 2×10^5 lux (measured with a MX4 lux-meter (Metrowatt, Germany)). To determine the kinetics of inactivation, 70 μl samples were taken after different illumination times. C_{60} was removed by centrifugation and residual virus determined by endpoint titration.

2.4. Determination of virus titer

To determine the virus titers, Vero cells grown to 80–100% confluency in 96-well tissue culture plates (TPP, Switzerland) were infected with 50 μl aliquots of 1 in 10 serial dilutions (in RPMI-medium) of virus samples (eight wells per dilution). After incubation for four days at 37°C in 5% CO_2, cytopathic effects (cell destruction) were visualized by staining the remaining viable cells with crystal violet (0.5% in methanol; Fluka, Switzerland). Virus titres were calculated according to the method of Spearman (1908) and Kärber (1931) and are indicated as $\log_{10}$ ($TCID_{50}$).

3. Results

The photosensitizer C_{60} was tested for its ability to reduce virus infectivity. A suspension of C_{60} in buffer (MBS) was prepared by sonification with a sonifier converter tip. This suspension was spiked with stock virus and illuminated with visible light at wavelengths higher than 495 nm under constant stirring and flushing with oxygen.

Fig. 1 shows the resultant time-dependent loss of infectivity of SFV. The points indicated represent mean values and standard errors from three independent experiments (●). From this curve the reduction factors after 3 and 5 h, respectively, of illumination were calculated. Reduction factors are defined as the logarithm of the ratio of the initial and the residual titer. Values of 7 $\log_{10}$ for 5 h and more than 5 $\log_{10}$ for 3 h of illumination were obtained. As a control, SFV was illuminated in the absence of C_{60} (○), or incubated with 1 mg C_{60} per ml at 4°C without illumination (▽). The observed maximal non-specific inactivation was in the range of one $\log_{10}$ after 5 h incubation.

A further control was performed by chasing the oxygen from the solution. To reduce the oxygen available during the inactivation, the suspension was flushed with argon 5 min prior to and during the illumination. This treatment resulted in a significant loss of the inactivation capacity of C_{60} (☆), thus confirming that the photodynamic inactivation process is oxygen-dependent. The fact that C_{60} retains some residual inactivation capacity even after argon treatment may be due to traces of oxygen still present in the inactivation suspension. Inclusion of 2 mM glutathione or hydroquinone (scavengers of free radicals) in the assay had no effect on virus inactivation by C_{60} (data not shown), suggesting that no radical mechanism is involved in the inactivation process.

Since a potential application of this process is the inactivation of viruses in biological fluids, the photoinactivation was also examined in a proteinaceous solution. C_{60}-suspensions containing 2% bovine serum albumin (BSA) were spiked with virus and illuminated under the same conditions as described above. As shown in Fig. 2, the

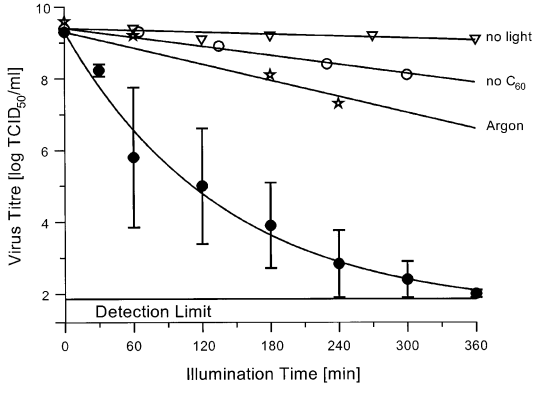

Fig. 1. Kinetics of the photodynamic inactivation of SFV by C_{60}. SFV was illuminated with visible light in the presence of C_{60} under constant stirring and O_2 bubbling (●). Mean values and standard errors from three independent experiments are shown. Controls include the incubation of SFV with C_{60} without illumination (▽), the illumination of SFV without C_{60} (○) and the illumination with C_{60} under constant stirring and flushing with argon (☆).

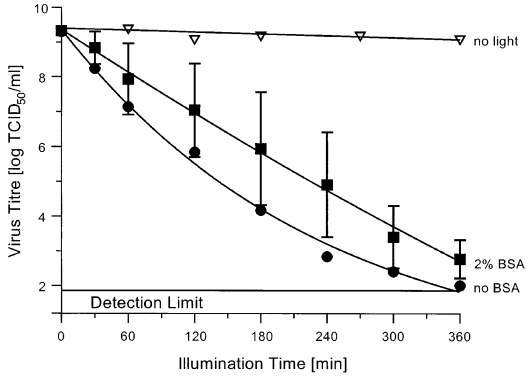

Fig. 2. The effect of proteins on the inactivation kinetics. SFV was illuminated with C_{60} in the presence of 2% BSA (■), and in the absence of BSA (●).

inactivation kinetics were slightly slower in the presence of additional protein. The reducing factor after 5 h illumination was calculated as 6 $\log_{10}$.

In an additional set of experiments, the C_{60}-mediated photoinactivation of vesicular stomatitis virus (VSV) was tested. Mean values and standard errors from three independent experiments are shown in Fig. 3 (●). The non-specific elimination or inactivation of VSV, after incubation with C_{60} for 5 h without illumination, was less than one $\log_{10}$ (▽). Illumination of VSV for 5 h in the absence of C_{60} reduced virus infectivity by less than one $\log_{10}$ (○). It should be noted that the stock-virus titer for VSV was lower than that for SFV; thus the final concentration of MM-medium was approximately 45%. This corresponds to a protein concentration of 0.5–1% in the inactivation suspension and can therefore be considered to mimic a biological fluid. The reduction factor at 5 h of illumination was more than 7 $\log_{10}$. Thus, VSV was efficiently inactivated even in the presence of proteins (0.5–1%).

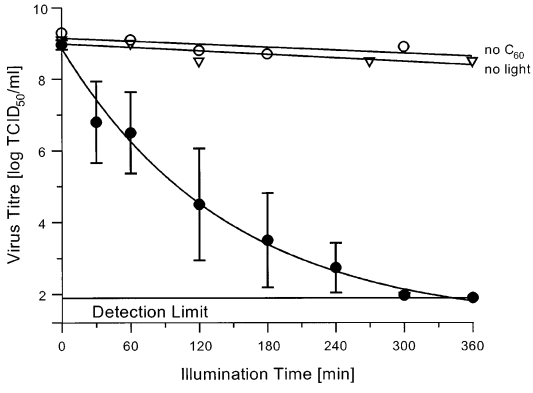

Fig. 3. Kinetics of the photodynamic inactivation of vesicular stomatitis virus by C_{60}. VSV was illuminated with visible light in the presence of C_{60} under constant stirring and O_2 bubbling (●). Mean values and standard errors from three independent experiments are shown. Controls include the incubation of VSV with C_{60} without illumination (▽) and the illumination of VSV without C_{60} (○).

4. Discussion

It is well known that enveloped viruses can be inactivated by the action of singlet oxygen ($^1O_2$) (Blood Cells, 1992). Singlet oxygen can react with aromatic and sulphur-containing amino acids but reacts mainly with histidine residues in proteins (Michaeli and Feitelson, 1994, 1995). The photodynamic inhibition of the viral fusion process in vesicular stomatitis and influenza virus by rose bengal was found to be due to a crosslinking of membrane proteins (Lenard et al., 1993; Lenard and Vanderoef, 1993). However, it was also reported that singlet oxygen acts on the nucleic acids of certain enveloped viruses (Lambrecht et al., 1991). Thus, the detailed mechanism of virus inactivation by singlet oxygen remains to be elucidated.

Since C_{60} is a very potent generator of singlet oxygen (the triplet state of C_{60} sensitizes the formation of $^1O_2$ with a quantum yield of 0.96 when irradiated at 535 nm (Arbogast et al., 1991; Nagano et al., 1994)), we evaluated its ability to inactivate viruses upon illumination with visible light in the presence of oxygen. An irradiation wavelength greater than 500 nm was used, in order to optimize $^1O_2$ production whilst simultaneously minimizing nonspecific light-mediated damage to components of the inactivation mixture. Model viruses belonging to two different families could be efficiently inactivated. Reduction factors of greater than 7 $\log_{10}$ $TCID_{50}$ were obtained. This inactivation was dependent on the presence of oxygen. Chasing the oxygen with argon resulted in a dramatic reduction of the inacti-

vation capacity, thus strongly suggesting that singlet oxygen was the reactive species.

The generation of radicals, that occurs with most dyes used to produce singlet oxygen, although it represents an additional pathway for virus inactivation (type I photodynamic reaction), can lead to covalent modifications of the proteins by these dyes. Radical generation by illuminated C_{60} has never been reported and, in agreement with this, we did not observe any reduction in the inactivation capacity when known scavengers of free radicals (glutathione and hydroquinone) were present during the illumination of C_{60}. Thus, the use of C_{60} excludes the possibility of protein modification by compounds other than singlet oxygen. In consequence the likelihood of producing neoantigens, when inactivating viruses in biological fluids, is drastically reduced with this compound.

Two further advantages of buckminsterfullerene are that it is totally insoluble in aqueous solutions and extremely stable. Therefore, C_{60} can be removed from the incubation mixture by procedures such as centrifugation, filtration, or by introducing special properties (e.g. magnetism) into the carbon cages (Edelmann, 1995). Removal of C_{60} from solutions should help to reduce any toxic effects or undesirable complications arising from the use of this photosensitizer in biological fluids; such problems are often encountered with conventional photosensitizers, e.g. hypericin. Its stability makes it also possible to recycle C_{60}; clearly an economical advantage.

C_{60} can also be used in proteinaceous solutions, e.g. biological fluids, as demonstrated by the fact that the presence of bovine serum albumin, or increased amount of culture medium, barely decreases the inactivation kinetics.

It should be mentioned that many parameters that might affect the inactivation kinetics (e.g. temperature, supply of oxygen, the formation of highly dispersed C_{60} suspensions, stirring process) have yet to be examined in detail. By optimising further parameters it is very likely that faster kinetics of virus inactivation could be achieved.

In conclusion, the data presented and the unique properties of buckminsterfullerene, make this system a valid candidate for future application in the inactivation of viruses in biological fluids.

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