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Antiviral Research 38 (1998) 55–62

Antiviral Research

Inactivation of enveloped viruses by singlet oxygen thermally generated from a polymeric naphthalene derivative

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Received 13 October 1997; accepted 22 December 1997

Abstract

Inactivation of viruses can be induced by singlet oxygen generating agents. The water-insoluble polymeric compound PVNE (poly (1,4-dimethyl-6-vinylnaphthalene-1,4-endoperoxide)) is used as a storage for reactive oxygen and is able to produce thermally generated $^1O_2$ in a dark-reaction. Enveloped viruses from two different families, Semliki Forest virus (SFV, Togaviridae) and vesicular stomatitis virus (VSV, Rhabdoviridae) showed a loss of infectivity of up to 8 $\log_{10}$/ml ($TCID_{50}$) when incubated at 37°C with PVNE in buffered solutions. PVNE produces singlet oxygen by thermal decomposition without irradiation. Such chemically generated oxygen excludes reactions involving radicals (type I photoreactions), a problem often encountered in photodynamic processes utilizing dyes as sensitizers. In addition, the water insolubility of the oxygen-carrier allows an easy removal and recycling from aqueous solutions. Therefore, it may prove useful in the inactivation of viruses in biological systems and may be a helpful tool in studies concerning the inactivation mechanism by $^1O_2$.

Keywords: Virus inactivation; Singlet oxygen; Semliki Forest virus; Vesicular stomatitis 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 large 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).

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0166-3542/98/$19.00
PII S0166-3542(98)00007-2

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 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). Apart from the singlet oxygen mechanism the excited sensitizer can also react in so-called type I or free radical reactions, directly interacting with the substrate to yield free radicals. The generation of free radicals that occurs with most dyes used to produce $^1O_2$ can lead to covalent modifications of proteins by the dye.

An inherent property of most of the above mentioned dyes, is their water solubility, which makes their removal from solutions extremely difficult. In addition, the 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. For this reason, total removal of these $^1O_2$ generators from biological fluids will be required 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).

It is obvious that new methods must be developed in order to overcome these disadvantages. These studies have focused on the use of singlet oxygen-generating agents which can be completely and rapidly removed from aqueous solutions, such as the all carbon compound buckminsterfullerene (C_{60}) (Käsermann and Kempf, 1997).

In this study a chemical source of $^1O_2$ was chosen to exclude free radical (type I) reactions. It is known that polymethylated naphthalene derivatives may store $^1O_2$ as peroxides in a reversible manner. Upon raising the temperature above 10°C, the endoperoxides release $^1O_2$ with yields as high as 65–80% (Turro et al., 1981). These monomeric water-soluble $^1O_2$-carriers are known to inactivate enveloped viruses (Müller-Breitkreutz et al., 1995; Dewilde et al., 1996).

It was found that poly (1,4-dimethyl-6-vinylnaphthalene-1,4-endoperoxide) (PVNE), the polymeric compound of a naphthalene derivative with its properties of water insolubility and reversible capacity of $^1O_2$ storage, is an ideal candidate for virus inactivation by thermally generated singlet oxygen. The fact that the target sample does not need to be irradiated, reduces not only side effects like type I photoreactions but generally represents a more subtle treatment. Therefore heat production or other damaging effects when irradiating target samples like protein or cell containing solutions are reduced or excluded. In this report, the inactivation of enveloped viruses in the presence of PVNE in the dark was studied.

2. Material and methods

2.1. Synthesis of poly(1,4-dimethyl-6-vinylnaphthalene-1,4-endoperoxide) (PVNE)

Poly(1,4-dimethyl-6-vinylnaphthalene) (PVN) was prepared from 1,4-dimethylnaphthalene as described by Saito et al. (1985). The synthesis is schematically depicted in Fig. 1. Briefly, 2-acetyl-5,8-dimethylnaphthalene was prepared by Friedel-Crafts acetylation (a). Further reaction steps included the reduction to the corresponding alcohol by $LiAlH_4$ (b) and the chlorination with $SOCl_2$ followed by dehydrochlorination with *t*-BuOK in dimethyl sulfoxide (c). The purified product 1,4-dimethyl-6-vinylnaphthalene (DVN) was analyzed by ^1H NMR in $CDCl_3$. Radical polymerization of the monomeric compound DVN was carried out in toluene at 70°C in the presence of azobisisobutyronitrile (AIBN) (d). The photooxidation of PVN, leading to the corresponding endoperoxide PVNE, was performed under constant flushing with oxygen at 4°C. A polymer solution (900 mg) in methylene chloride (400 ml) containing methylene blue (0.1 mM) was irradiated with a 24 W fluorescence tube (PL 24W/83; Philips, Holland) (d). The reaction was

monitored by the disappearance of the UV absorption band of the naphthalene ring at 290 nm. Purification of the polymeric endoperoxide PVNE was achieved by injection of the concentrated irradiation-mixture into cold methanol, to give a white precipitate. This product was dried, redissolved and precipitated several times. The resulting PVNE was stored for several months in the freezer without significant decomposition to PVN.

2.2. 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% fetal calf serum (FCS), 100 μg 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 μg streptomycin and 100 U penicillin/ml. The cells were passaged weekly by 1:15 dilution.

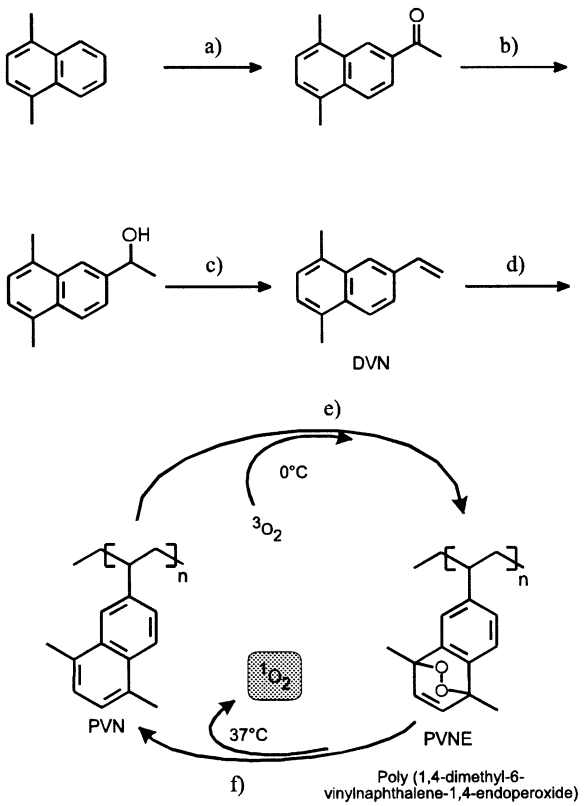

Fig. 1. The reaction scheme for the synthesis of poly(1,4-dimethyl-6-vinylnaphthalene-1,4-endoperoxide) (PVNE). (a) Friedel-Crafts acetylation. (b) Reduction with $LiAlH_4$. (c) The treatment with $SOCl_2$ followed by dehydrochlorination with *t*-BuOK. (d) Radical polymerization. (e) The photooxygenation with methylene blue as sensitizer. (f) The thermal decomposition of polymeric endoperoxide and generation of singlet oxygen.

2.3. Virus propagation

Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV) were propagated in Aedes cells. Briefly, cells were infected with virus at a multiplicity of infection (moi) of approximately 5 plaque forming units (p.f.u.)/cell. At 24-h post infection (h.p.i.) the medium was harvested and cellular debris removed by centrifugation ($600 \times g$, 10 min). The virus-containing supernatant was aliquoted and stored at -80°C.

2.4. Virus purification

Aedes cells were infected with virus at a moi of 10 p.f.u./cell. At 6 h.p.i. the medium was replaced with MM-medium diluted 1:10 with phosphate-buffered saline (PBS; adjusted to 400 mosm) containing 0.2% (w/v) bovine serum albumin (BSA). At 24 h.p.i. the medium was harvested and cellular debris removed by centrifugation ($3000 \times g$, 10 min). The virus was purified and concentrated by centrifugation through a cushion of 12% sucrose in PBS at $130\,000 \times g$ for 2 h 40 min. The virus pellet was resuspended in PBS or MES-buffered saline (MBS; 20 mM Morpholinoethanesulfonic acid, 190 mM NaCl) and analyzed by SDS-PAGE. Protein concentrations were determined by the method of Lowry et al. (1951).

2.5. Inactivation assay

PVNE (4 mg) in 50 μl CH_2Cl_2 was suspended in 2 ml MBS by injection and subsequent evaporation of the solvent under reduced pressure and constant stirring on ice. This suspension (containing approximately 10 mM of the monomeric

naphthalene subunit) was spiked with purified virus to obtain an initial virus titer of approximately 10^{11} (SFV) or 10^{7} (VSV) $TCID_{50}$/ml (50% tissue culture infectious dose) and stirred constantly. To start the decomposition process the temperature was raised to 37°C. To determine the kinetics of inactivation, 70 μl samples were taken after different incubation times. PVNE was removed by centrifugation and residual virus infectivity determined by endpoint titration.

2.6. Determination of virus titer

Virus titers were determined by the endpoint dilution method. Briefly, Vero cells grown to 80–100% confluency on 96-well tissue culture plates (TPP, Switzerland) were infected with 50 μl aliquots of one in ten serial dilutions (in RPMI-medium) of virus samples (8 wells/dilution). After incubation for 4 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). The virus titers and corresponding errors were calculated according to the method of Spearman (1908) and Kärber (1931) and are indicated as $\log_{10}$ ($TCID_{50}$).

3. Results

3.1. Synthesis of PVN

PVN was prepared from 1,4-dimethylnaphthalene as shown in Fig. 1. The overall yield of the monomeric compound 1,4-dimethyl-6-vinylnaphthalene (DVN) was 20% and the structure was confirmed by ^{1}H NMR. δ: 2.64 (s, 3H),2.66 (s, 3H), 5.34 (dd, 1H, $J = 11$, 1 Hz), 5.89 (dd, 1H, $J = 18$, 1 Hz), 6.93 (dd, 1H, $J = 11$, 18 Hz), 7.18 (m, 2H), 7.68 (dd, 1H, $J = 9$, 2 Hz), 7.90 (d, 1H, $J = 2$ Hz), 7.95 (d, 1H, $J = 9$ Hz).

PVN was loaded with oxygen to the corresponding endoperoxide PVNE by illumination of the photosensitizer methylene blue. The time for the loading was dependent on the procedure used. In a small scale preparation (in a 2-ml cuvette), when using a 350-W short-arc mercury lamp (HBO 350 W; Osram, Germany), the photooxygenation was terminated after 2 h, whereas the oxygenation of a large batch took approximately 18 h (400 ml; 24-W fluorescence tube; Philips, Holland).

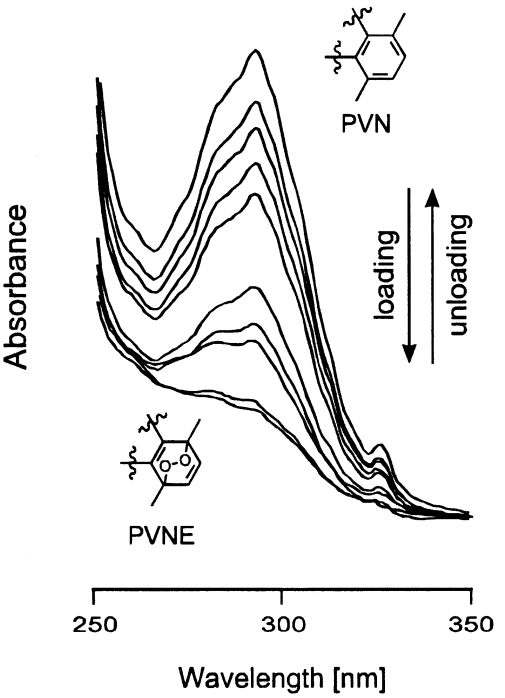

Fig. 2. The UV spectral change in the loading and unloading process. The loading and unloading was monitored by the UV spectra after different times of illumination or incubation at elevated temperatures, respectively.

3.2. Kinetics of thermal decomposition of PVNE

A solution of PVNE in ethylene dichloride was incubated at different temperatures. The subsequent decomposition to PVN by the release of $^1O_2$ was confirmed by monitoring the appearance of the 290 nm UV-absorption band of the naphthalene ring (Fig. 2). The kinetics of the reaction at 30 and 37°C are shown in Fig. 3. The UV absorption values at 292 nm are normalized according to the following equation:

$$y = \frac{A_{292}(t) - A_{292}(\infty)}{A_{292}(0) - A_{292}(\infty)} \quad (1)$$

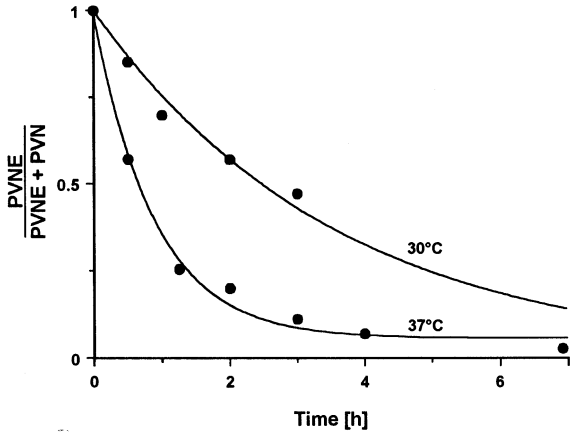

Fig. 3. Kinetics of thermal decomposition of PVNE. The UV absorption values are normalized according to equation (1) and are plotted against the incubation time of PVNE.

The half-life of PVNE at 37°C was in the range of 40 min (2.5 h at 30°C, respectively).

3.3. Virus inactivation

Oxygen loaded PVNE ($\geq 90\%$ of the naphthalene groups peroxidized) was tested for its ability to reduce virus infectivity. A suspension of PVNE in buffer was prepared by injection of a stock solution of PVNE (in methylene chloride) into MBS. The organic solvent was removed by subsequent evaporation under reduced pressure and constant stirring on ice. This suspension was spiked with purified virus and incubation was performed under constant stirring. In Fig. 4 the time-dependent decrease of infectivity of Semliki Forest virus (SFV) when incubated with PVNE at 37°C (filled circles) is shown. From this curve the reduction factors after 1 and 3 h of incubation, respectively were calculated. Reduction factors are defined as the logarithm of the ratio of the initial and the residual titer. Values of 7 $\log_{10}$ $TCID_{50}$ (50% tissue culture infectious dose) per ml for 1 h and more than 8 $\log_{10}$ for 3 h of incubation were obtained. To monitor the non-specific inactivation, SFV was incubated with 2 mg PVNE/ml at 4°C. No reduction in virus infectivity could be observed (open circles). To control whether the inactivation was due to the presence of $^1O_2$, PVNE was unloaded completely by incubation overnight at room temperature. The PVN produced by this procedure was then tested for its virus inactivation ability at 37°C. The maximal $^1O_2$ independent inactivation was found to be less

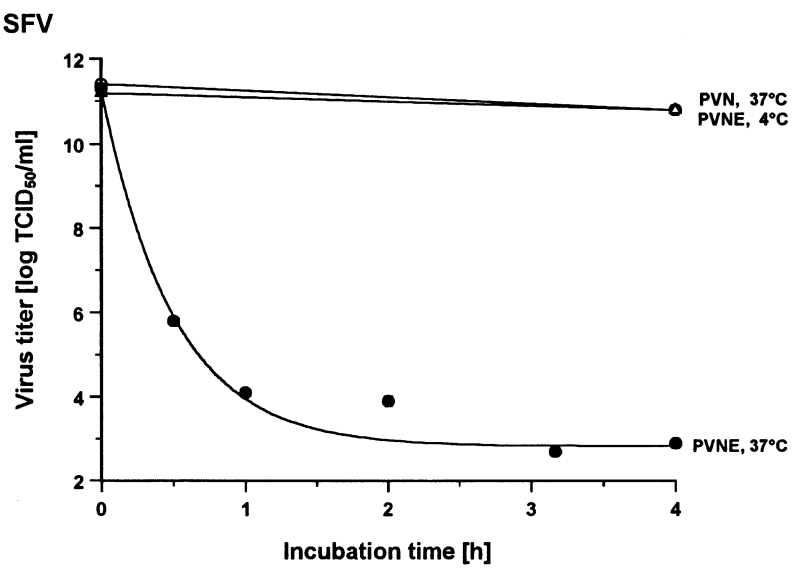

Fig. 4. Kinetics of the inactivation of Semliki Forest virus (SFV) by PVNE. SFV was incubated with PVNE under constant stirring at 37°C (●). Controls include the incubation of SFV with PVN at 37°C (▽) and the incubation of SFV with PVNE at 4°C (○). The inactivation kinetics of a typical experiment are shown. Errors of the single points vary between 0.15 and 0.25 log $TCID_{50}$/ml.

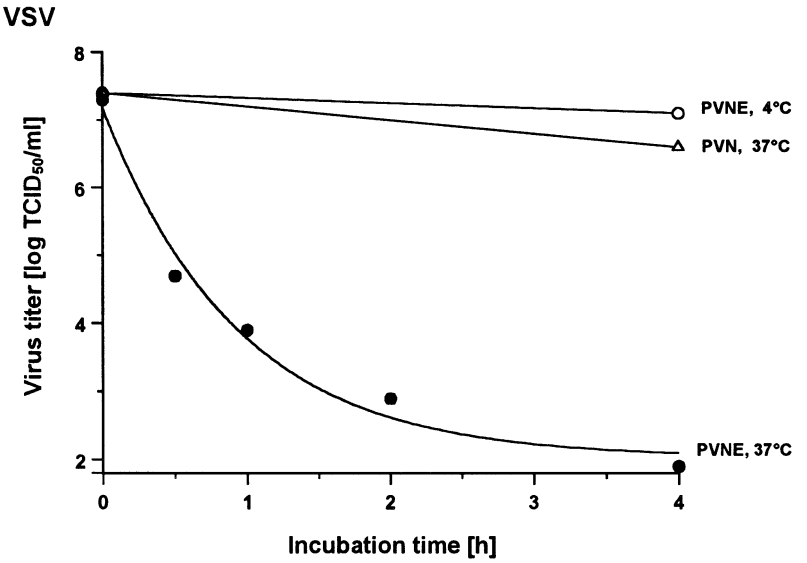

Fig. 5. Kinetics of the inactivation of vesicular stomatitis virus (VSV) by PVNE. VSV was incubated with PVNE under constant stirring at 37°C (●). Controls include the incubation of VSV with PVN at 37°C (▽) and the incubation of VSV with PVNE at 4°C (○).The inactivation kinetics of a typical experiment are shown. Errors of the single points vary between 0.15 and 0.25 log $TCID_{50}$/ml.

than one log_{10}/ml after 4-h incubation (open triangles).

In an additional set of experiments, the $^1O_2$-mediated inactivation of vesicular stomatitis virus (VSV, Rhabdoviridae) was tested. In Fig. 5 the kinetics of a typical inactivation of purified VSV by PVNE (filled circles) is shown. The non-specific elimination or inactivation of VSV, when incubated with PVNE on ice or with the unloaded compound PVN, was less than one log_{10}/ml over a period of 4 h (open circles).

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 sulfur-containing amino acids but acts mainly on 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.

In this study the inactivation capacity of PVNE, a singlet oxygen carrier, was demonstrated. Model viruses belonging to two different families could be efficiently inactivated. Reduction factors up to 8 log_{10} $TCID_{50}$ were obtained. This inactivation was dependent on oxygen loaded on the polymer.

The generation of radicals, that occurs with most dyes used to produce singlet oxygen in photodynamic processes represents an additional pathway for virus inactivation (type I photodynamic reaction), although it can lead to covalent modifications of the proteins by these dyes. The only active species produced by PVNE in a dark reaction is singlet oxygen. Thus, the use of PVNE excludes the possibility of protein modification by compounds other than singlet oxygen. In consequence the likelihood of producing neo-antigens, when inactivating viruses in biological fluids, is drastically reduced with this compound.

Another advantage of PVNE is its complete insolubility in aqueous solutions. Thus, PVNE can be removed from the incubation mixture by procedures such as centrifugation or filtration. Removal of PVNE from solutions should help to reduce any toxic effects or undesirable complications arising from the use of this $^1O_2$-carrier in biological fluids; such problems are often encountered with conventional photosensitizers, e.g. hypericin. The possibility of reloading enables PVNE to be recycled. The temperature used to perform the singlet oxygen generating reaction is in an optimal range for biological systems.

In addition, it was shown that PVNE was able to inactivate SFV in a complex protein mixture (10% MM-medium, 2% FCS) (data not shown). The efficiency of the inactivation system can most likely be improved by optimizing the accessibility to the singlet oxygen carrying group (e.g. by improving the suspension production).

In conclusion, the data presented make this system a valid candidate for future application in the inactivation of viruses in biological fluids. In addition the properties of the $^1O_2$-carrier PVNE suggest that this compound may be useful in studies of the mechanism of singlet oxygen mediated virus inactivation.

Acknowledgements

This work was supported by the Swiss National Science Foundation (Grant No. 31-049217.96 to C.K.).

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