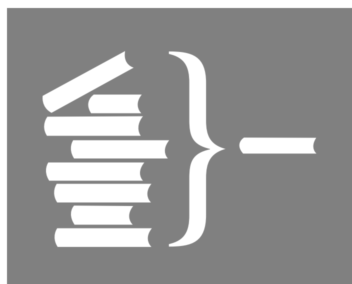

Buckminsterfullerene and Photodynamic Inactivation of Viruses

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SUMMARY

The development of new virus inactivation procedures has become an area of growing interest mainly due to increased demands concerning the safety of biological products. Photochemical processes represent the most promising methods for the future to inactivate viruses. In these methods, dyes are the most widely used photosensitising reagents. The current article covers a new interesting alternative, namely the use of buckminsterfullerene (C_{60}). The unique properties of this molecule make it a valid candidate for future applications in the inactivation of viruses in biological fluids. © 1998 John Wiley & Sons, Ltd.

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INTRODUCTION

The development of new virus inactivation procedures, including studies with new compounds exhibiting inactivation properties, has become a growing field of research. New methods are needed because the safety of biological products (e.g. blood products, recombinant proteins used in medicine) has become a major issue during the past few years. Although to date a high standard of safety has been achieved, it has to be borne in mind that it will never be possible to guarantee absolute safety. Nevertheless, public opinion requires that the safety of biological products fulfil the highest standard according to current scientific knowledge. Therefore, new efforts must be made to further increase margins of safety.

The aim of every procedure for the inactivation of viruses in blood preparations is to achieve maximum virus inactivation with minimal damage to the product or inclusion of material with undesirable chemical or immunogenic activities. It is obvious that the inactivation of viruses in cellular components (e.g. red cells, thrombocytes) presents more complications than the inactivation of viruses in stable products (e.g. immunoglobulins). The inactivation procedure is also defined by the target viruses. Enveloped viruses are inactivated more readily than nonenveloped, as destruction of the lipid membrane is accompanied by loss of virus infectivity. Fortunately, the most important viruses that are transmitted by blood products are enveloped (Table 1). In addition to the enveloped viruses listed in Table 1, two nonenveloped viruses, hepatitis A virus (HAV; positive-RNA virus; *Picornaviridae*) and human parvovirus B19 (single-stranded DNA virus), were also discussed as possible hazardous contaminants in blood products.[1–3] However, these two viruses play a minor role in terms of disease severity and because antibody prevalence and thus immunity of adults in Europe is high, and for hepatitis A a vaccine is available.

There are several means by which the safety of blood products can be improved: selection of blood donors, screening of donated blood for markers of infectivity and clearance of viruses from blood components.

Viruses can be cleared from biological products by either inactivation or removal. Table 2 summarises the methods currently in use or still under development. The established methods (bold in Table 2) are briefly mentioned below (for a review see[3–5]).

In the production of stable blood products one of the most widely used methods is the Cohn fractionation. The ethanol concentration used for precipitation (8%–40%) is not high enough to show virucidal effects, but the viruses are concentrated in certain fractions, whilst other fractions are free from viruses. Chromatographic methods also lead to a decrease of virus load in certain fractions (e.g. ion exchange chromatography).[6] Ultrafiltration through a membrane filter is a method analogous to sterile filtration. To ensure removal of the

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Abbreviations used: $^1O_2$, singlet oxygen; amu, atomic mass units; C_{60}, buckminsterfullerene; IR, infra-red; MVM, minute virus of mice; NMR, nuclear magnetic resonance; O_2; SFV, Semliki Forest virus; $TCID_{50}$, 50% tissue culture infectious dose; UV, ultraviolet; VSV, vesicular stomatitis virus.

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Table 1. Some viruses transmitted by blood and plasma products (reviewed in [3])

Virus	Type
Hepatitis C virus (HCV)	Enveloped positive strand RNA virus
Human immunodeficiency virus (HIV I & II)	Enveloped negative strand RNA virus
Hepatitis B virus (HBV)	Enveloped DNA virus
Human cytomegalo virus (HCMV)	Enveloped DNA virus
Human parvovirus B19	Nonenveloped DNA virus
Hepatitis A	Nonenveloped positive strand RNA virus

Table 2. Virus inactivation methods

Physical	Chemical, biochemical	Combined
Heat	Immunological neutralisation	β-propiolactone/UV
Irradiation (ionising, UV)	Ethanol	Photochemical methods
Partitioning	**pH**	singlet oxygen ($^1O_2$)
Chromatography	Enzymes	
Filtration	**Solvent/detergent**	
	Nucleic acid breaking reagents	

smallest viruses, the membranes needed for filtration must have a pore size of less than 20 nm. However, such filters can create problems with efficient recovery of high molecular weight proteins. For practical use, filters with a pore size of 40–50 nm are also under investigation. The development of multilayered filters has clearly improved the efficiency of removing viruses. Complete elimination of HIV from culture medium by filtration[7] indicates the potential for the future. However, the filtration methods are restricted to solutions and are not applicable to cellular components.

Treatment with heat (pasteurisation) is used for either dry products or for solutions. The principal thermal inactivation method for solutions consists of heating the liquids to 60°C for 10 h (e.g. for albumin). To reduce the extent of protein denaturation, stabilisers such as sugars or amino acids may be added prior to the pasteurisation. To inactivate freeze-dried samples, temperatures of up to 100°C are needed. As most of the viruses likely to be transmitted by blood products are enveloped viruses, organic solvents and detergents may be used to disrupt their membrane leading to inactivation. The solvent usually used is tri-(*n*-butyl)-phosphate and the detergents are Triton-X-100, Tween 80 or sodium cholate.[8] A combination of low pH and treatment with pepsin at 37°C, a method which is commonly used to eliminate the anticomplementary activity in the production of immunoglobulins, leads to the inactivation of enveloped viruses.[9,10] All these procedures help to increase the safety of blood products, but they may only be applied to stable products.

PHOTOINACTIVATION

In recent years interesting attempts have been made to develop alternative methods for virus inactivation. Several photochemical methods look to be the most promising for future improvement of the safety of blood products, including products consisting of cellular components.

The use of photosensitisers for the inactivation of biological material was first reported at the turn of the century.[11] The sensitivity of viruses to such photodynamic procedures was then shown in the 1930s,[12] but only within the past 15 years (and with the occurrence of AIDS) have photodynamic techniques for the inactivation of viruses received growing attention.

There are basically two different photoinactivation methods: (a) irradiation of psoralens, or (b) dyes which produce highly reactive singlet oxygen ($^1O_2$) when illuminated with visible light in the presence of oxygen. Psoralens mainly react (when irradiated with light) in an oxygen independent way with nucleic acids. A disadvantage of psoralens is that they are potential

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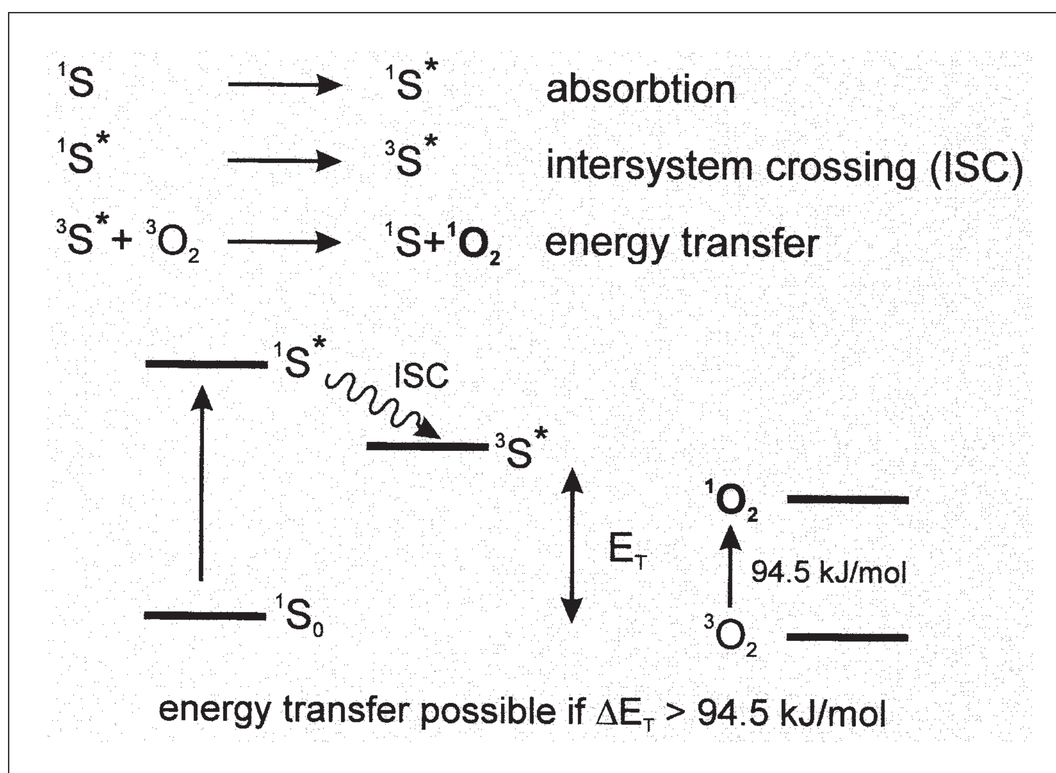

Figure 1. Diagram of the energy levels in the photosensitised generation of singlet oxygen ($^1O_2$). Where ^1S, $^1S^*$, $^3S^*$ are the singlet ground state, the singlet excited state, and the triplet excited state respectively of the sensitiser S (e.g. C_{60}), and $^3O_2$ and $^1O_2$ are the triplet ground and singlet excited state, respectively of oxygen.

mutagens due to their ability to bind to double-stranded nucleic acids via intercalation.[13,14]

It is well known that enveloped viruses can be inactivated efficiently by agents which generate singlet oxygen. Among these agents, dyes are the most prominent and widely used. Viral inactivation properties have been described for a wide variety of dyes such as phthalocyanines,[15–17] merocyanines,[18] porphyrin derivatives,[19] hypericin and rose bengal,[20,21] and methylene blue.[22]

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. For this reason, total removal of these dyes from biological fluids will be necessary in most cases. To date only one method which uses photosensitisers 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.[22] 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 though efficient elimination of methylene blue is under study.

The reaction mechanism which generates singlet oxygen may be briefly described as follows (Figure 1). The photosensitiser is first excited into the short-lived singlet state ($^1S^*$) following the absorption of light. Normally, the singlet state is converted into the triplet state ($^3S^*$) 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 of the sensitiser to the ground state of oxygen $^3O_2$ ($^3\Sigma_g$). As a result, highly reactive singlet oxygen is formed, $^1O_2$ ($^1\Delta_g$) (Table 3a) (type II photodynamic reaction).

Although there has been intensive research, only limited data are available concerning the specific action of $^1O_2$ on virus structures. It has to be kept in mind that photodynamic actions of most dyes include singlet oxygen (type II photoreaction) as well as mechanisms involving radicals (type I reaction) (Table 3b). Therefore discrimination between the two mechanisms is not always possible. In studies using rose bengal as sensitising dye, inhibition of fusion in vesicular stomatitis virus infections was found to be due to crosslinking of membrane proteins (G protein).[20] Singlet oxygen reacts with aromatic and sulphur-containing amino acids but reacts mainly with histidine residues of peptides in solution.[23] In addition it was also reported that $^1O_2$ acts on the nucleic acids of certain enveloped viruses. Thus, the detailed mechanism of photodynamic virus inactivation remains to be elucidated.

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Table 3. Spectroscopic states of oxygen and photoreactions

(a) Spectroscopic states of oxygen

State	Spin assignment	Energy [kJ/mol]
$3\Sigma_g$ (triplet ground state)	⇅ — antiparallel	94·5
$^1\Delta_g$ (singlet excitated state)	↑ ↑ parallel	0

(b) Two types of photoreactions

Type I quenching of the excited sensitiser by substrate production of radicals involved	$^3S_1 + RH \rightarrow \cdot SH + R\cdot$ $^3S_1 + RH \rightarrow S^{\cdot -} + RH^{\cdot +}$
Type II quenching of the excited sensitiser by O_2 ($^3\Sigma_g$) singlet oxygen generation	$^3S_1 + O_2(^3\Sigma_g) \rightarrow {}^1S + O_2(^1\Delta_g)$

The generation of radicals, that occurs with most dyes used to produce singlet oxygen, represents an additional pathway for virus inactivation (type I reaction). However, radicals may also produce undesired covalent modifications of proteins, which create an increased risk of neo-antigen formation. Thus, a further prerequisite of new $^1O_2$-generators is the exclusion of free radical reactions. One such method was reported using endoperoxides of naphthalene compounds that generate $^1O_2$ in a thermic reaction.[24–27] The water-soluble compounds show a virucidal activity when tested on enveloped viruses.[28,29] Such methods using the generated $^1O_2$ as the exclusive reaction species may also aid in the investigation of the mechanism underlying singlet oxygen-mediated virus inactivations.

To overcome the disadvantages of water-soluble compounds and to have only singlet oxygen as the reactive species, a polymeric naphthalene derivative was synthesised. This polymer is water-insoluble and has the benefit that it can be removed easily from aqueous solutions. The polymeric compound was loaded in a photodynamic reaction to its endoperoxide. Exposure of the endoperoxide to 37°C led to the inactivation of enveloped viruses due exclusively to the action of the $^1O_2$ generated (Käsermann and Kempf unpublished data).

However, this method is rather complicated because it includes procedures such as photodynamic loading with oxygen, subsequent purification of the $^1O_2$-generator and preparation of a suspension. Therefore new studies have focused on the use of singlet oxygen-generating agents which have the same advantages but are easier to handle. Buckminsterfullerene (C_{60}) was chosen because it is water-insoluble, excludes the generation of radicals, and is commercially available.[30]

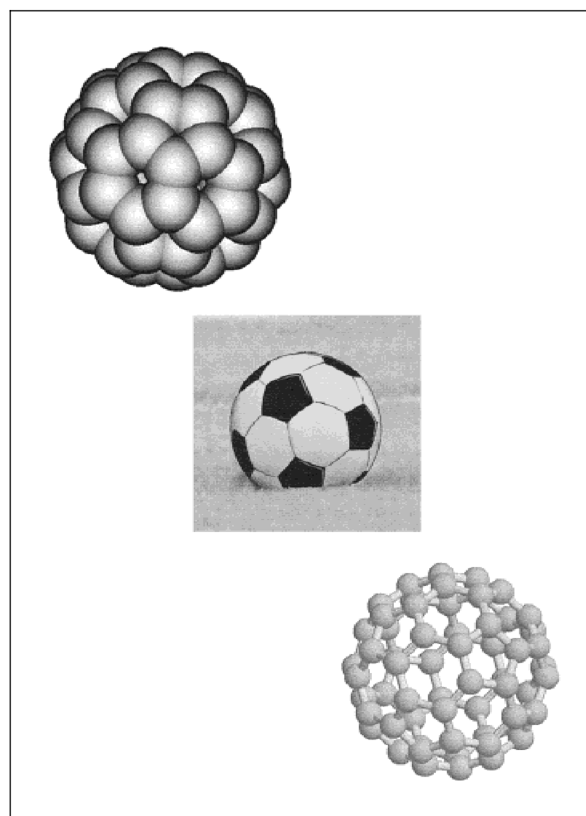

Figure 2. Schematic representation of buckminsterfullerene (C_{60}). Top: space filling model; centre: European football (soccer ball) model; bottom: ball and stick model.

BUCKMINSTERFULLERENE

The new form of carbon, the fullerenes, enriched the family of carbon allotropes, because it represents a new variety with interesting properties. In contrast to graphite or diamond, the fullerenes are spherical molecules. They are insoluble in water but soluble in a variety of organic solvents. The latter property is an important requirement for chemical manipulations. Thus, synthetic chemists in particular became interested in elemental carbon. The fullerenes are built up of fused pentagons and hexagons. The pentagons, which are absent in graphite, provide the curvature. The smallest stable, and most abundant,

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fullerene is the buckminsterfullerene C_{60}. Buckminsterfullerene has a shape which will be familiar to all virologists (Figure 2). Compared with small two-dimensional molecules, e.g. the planar benzene, the structure of the three-dimensional systems appeals also from an aesthetic point of view. The beauty and the unique architecture of the fullerene cages immediately attracted the attention of many scientists and buckminsterfullerene became one of the most intensively investigated molecules.

Discovery of C_{60}

In 1985 Kroto and Smalley were interested in the conditions prevailing in the atmosphere of red giant stars. It was known that carbon forms clusters under such conditions. Among other species, they detected C_{60} for the first time.[31] The main feature of C_{60} is that it possesses unique physicochemical properties. The extra stability is due to the spherical structure of icosahedral symmetry. The proposed model of the fullerene cage with a truncated icosahedral structure was a very elegant explanation of the unique behaviour. The model was in agreement with many earlier observations on bulk carbon. In addition, some previously unexplained phenomena in carbon chemistry became interpretable by the model.[32]

In their experiments Kroto and Smalley applied a laser beam to solid graphite. The nature of the species produced during laser vaporisationwas analysed by mass spectroscopy. Conditions were found for which the mass spectra were completely dominated by the C_{60} signal.[31]

Due to the geodesic structural concepts which were proposed, the molecule was named after Buckminster Fuller, the inventor of geodesic domes. Buckminsterfullerene is the chosen name for C_{60}, whereas the name fullerene is conveniently used for the whole family of closed carbon cages. The interest in buckminsterfullerene might be partly due to the high degree of symmetry, as mankind has always been fascinated by symmetric objects. Indeed, scientists performed theoretical studies on symmetric compounds. In the course of searching for new three-dimensional π-systems, the C_{60} molecule was first predicted in 1970. Still prior to its discovery, several molecular orbital calculations for buckminsterfullerene were carried out (reviewed in)[32,33].

In the first few years after the proposal of the C_{60}-structure, the properties of the species were tested in order to confirm the proposed cage structure, which apparently was produced only on observation of a single strong mass spectra peak at 720 amu. It was thus necessary to establish methods for the synthesis of C_{60} in macroscopic amounts.

The second breakthrough, after the discovery of buckminsterfullerene in 1985, was achieved in 1990 by Krätschmer *et al*. when they vaporised graphite rods in a helium atmosphere.[34]. The IR spectra of the soot showed similarities to the predicted spectra for buckminsterfullerene. Fullerenes were isolated from the soot by benzene extraction. This allowed verification of the proposed structure by crystallographic and spectroscopic methods (x-ray, IR, UV/visible, ^{13}C NMR and mass spectrometry). Fullerenes can be separated by chromatographic methods (e.g. on alumina with hexane or hexane/toluene as eluent or by flash chromatography of a concentrated toluene extract on silicagel/charcoal).[33]

The predicted structure of buckminsterfullerene was confirmed and from then onwards fullerene was available in bulk. In the following years, research in the fullerene field grew in an explosive way. Today C_{60} is commercially readily available. Therefore not only synthetic research and physico-chemical studies, but also biochemical investigations and studies of biological applications have become possible.

Properties of fullerene

The solubility of C_{60} in polar solvents is quite low (in water it is nearly insoluble).[35] To achieve a good accessibility of the apolar molecule in biological fluids, biological applications of fullerenes were guided by the search for either water soluble derivatives of fullerenes or methods to solubilise them in polar solvents. Besides this, it was also reported that a suspension of C_{60} in water was stable for long periods and could be delivered to cells.[36] C_{60} was reported to be highly stable (high kinetic stability),[37] i.e. it does not decay under biological conditions to the thermodynamically more stable graphite. For photochemical applications it is important that C_{60} does not show any photodegradations under the chosen conditions. Buckminsterfullerene in hexane shows several strong absorption bands in the UV range and some weak bands in the visible part of the spectrum.[34,38] The symmetry-forbidden transitions between 410 and 620 nm are responsible for its purple colour and for the efficient generation of singlet oxygen. C_{60} can be excited by visible light to its singlet state ($^1C_{60}$), (Figure 1).[39] Since little fluorescence is observed (quantum yield 10^{-5} to 10^{-4}), the predominant decay mode for fullerene singlets is an intersystem crossing to the triplet state ($^3C_{60}$) which occurs with almost unity.[39] This is explained by the relatively large spin orbit coupling of C_{60} due to its spherical geometry.[40] The triplet lifetime in solution is 130 μs and is quenched by ground state oxygen ($^3O_2$). If oxygen is present, the $^3C_{60}$ efficiently (almost 100%) sensitises the formation of singlet oxygen ($^1O_2$). $^1O_2$ is a highly reactive form of excited O_2 and is known to damage biomolecules such as DNA and proteins. Therefore, the effective generation of $^1O_2$ by photo-excited buckminsterfullerene and its stability makes C_{60} a candidate for photodynamic processes in biological applications.

Biological applications

New production methods have made buckminsterfullerene also available for biologists and we are only at the starting point of the investigation concerning its biological applications. Several studies have been performed, which indicate a possible role of buckminsterfullerene in biological systems. A few examples are mentioned in the following section.

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Tokuyama *et al*. showed that several water soluble fullerene derivatives were able to inhibit enzymes like cysteine proteases (papain) and serine proteases (trypsin, plasmin and thrombin) when exposed to light.[41] The mechanism of inhibition was proposed to involve $^1O_2$.

Friedman *et al*. showed that C_{60} competitively inhibited recombinant HIV-1 protease.[42] Their work was initiated by molecular modelling that showed a good fitting of C_{60} into the enzyme's active site.

A water soluble C_{60} derivative was tested for antiviral activity. The compound showed a potent and selective activity against HIV-1 in acutely and chronically infected cells, due to direct interaction with the virus and inhibition of virus protease and reverse transcriptase.[43] No cytotoxicity with up to 100 μM of the C_{60} derivative was observed in several cell lines. In addition, none of the 18 tested mice died within 2 months when administered 50 mg kg day of the compound intraperitoneally. Many other water soluble derivatives showed a similar anti-HIV activity.[44]

It was shown that C_{60} may cleave DNA in a photodynamic process.[41] However, cleavage of DNA may also occur via a $^1O_2$ independent mechanism. For example, in one study a C_{60}-oligonucleotide was used to bind DNA. It was shown that the DNA was cut specifically at guanidine residues near the fullerene terminus of the oligonucleotide and an electron transfer mechanism was proposed.[45] Further studies, showing that fullerenes are able to translocate electrons over lipid bilayers when illuminated, or to trap radicals in blood samples, indicate the broad potential applications of fullerenes to biological systems.[44]

Due to its high level of singlet oxygen generation, buckminsterfullerene has a strong potential for photodynamic actions in biological systems. Some initial reports describe the use of fullerenes in photodynamic therapies (reviewed in [44]).

PHOTODYNAMIC INACTIVATION OF VIRUSES BY C_{60}

The photochemical properties of C_{60} have been well studied[32,34,46] and have recently received intensive attention. Buckminsterfullerene is a very potent generator of singlet oxygen.[39,47] Thus, this compound is a good candidate to be used for singlet oxygen mediated virus inactivations. This is further supported by the fact that C_{60} has been successfully used in preparative photooxygenations[48] and the evidence that C_{60} can also act as a sensitiser in aqueous systems.[49] An irradiation wavelength greater than 500 nm was used, in order to optimise $^1O_2$ production whilst simultaneously minimising non-specific light-mediated damage to components of the inactivation mixture.

Therefore, the potential of buckminsterfullerene to inactivate viruses by a singlet oxygen mediated mechanism has been investigated. Enveloped model viruses belonging to two different families, Semliki Forest virus

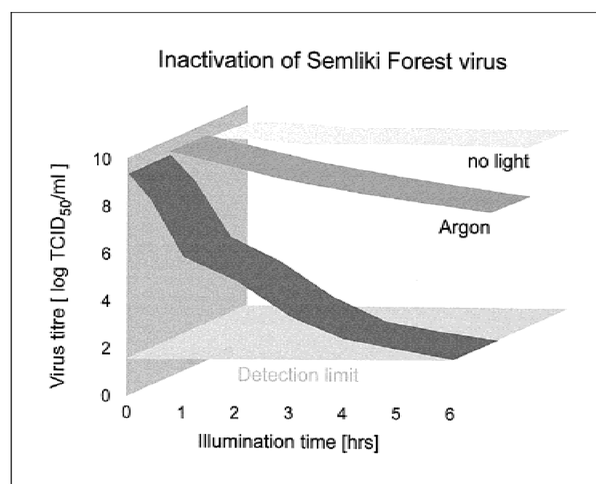

Figure 3. Kinetics of the photodynamic inactivation of Semliki Forest virus by C_{60}. SFV was illuminated with visible light in the presence of C_{60} under constant stirring and O_2 bubbling. Mean values of three independent experiments are shown. Controls include the incubation of SFV with C_{60} without illumination (no light) and the illumination in the presence of C_{60} under constant flushing with argon (argon). Virus titres were determined by the endpoint dilution method on Vero cells. Calculated errors were less than 0·5 log. However, between independent series variable inactivation kinetics could be observed due to differences in C_{60} preparations.

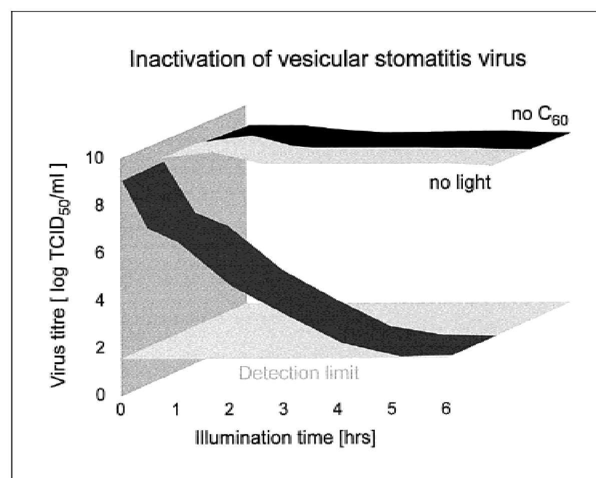

Figure 4. Kinetics of the photodynamic inactivation of vesicular stomatitis virus by C_{60}. VSV was illuminated with visible light in the presence of C_{60} under constant stirring and O_2 bubbling. Mean values of three independent experiments are shown. Controls include the incubation of VSV with C_{60} without illumination (no light) and the illumination of VSV without C_{60} (no C_{60}).

(SFV, family: *Togaviridae*) and vesicular stomatitis virus (VSV, family: *Rhabdoviridae*) were used in the following studies. Both viruses are frequently utilised in virus inactivation studies. Briefly, solutions were spiked with virus and illuminated at 0°C in the presence of C_{60} and oxygen. Residual virus titres were determined at various time intervals. As depicted in Figures 3 and 4, inactivations of $>7\ \log_{10}$/mL $TCID_{50}$ were obtained.[30] This inactivation was clearly dependent on the presence of oxygen. Chasing the oxygen with argon resulted in a dramatic reduction of the inactivation capacity (Figure 3). Inclusion of glutathione or hydroquinone (scavengers of free radicals) in the assay had no effect on virus

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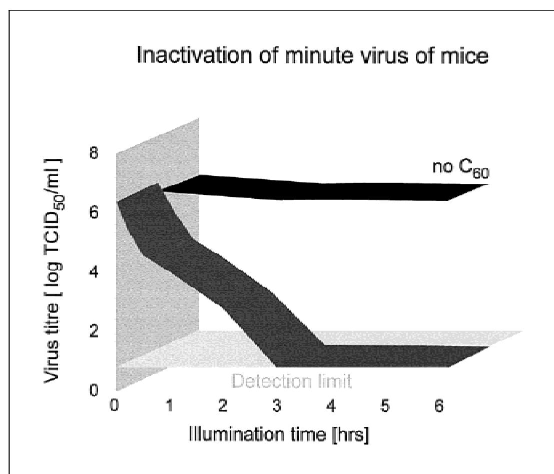

Figure 5. Photodynamic inactivation of minute virus of mice. MVM was illuminated at 45–50°C with visible light in the presence of C_{60} under constant stirring and O_2 bubbling. For controls, MVM was illuminated at 45°C without C_{60} (no C_{60}). The mean values of two independent experiments are shown. Virus titres were determined by endpoint dilution on A9 cells.

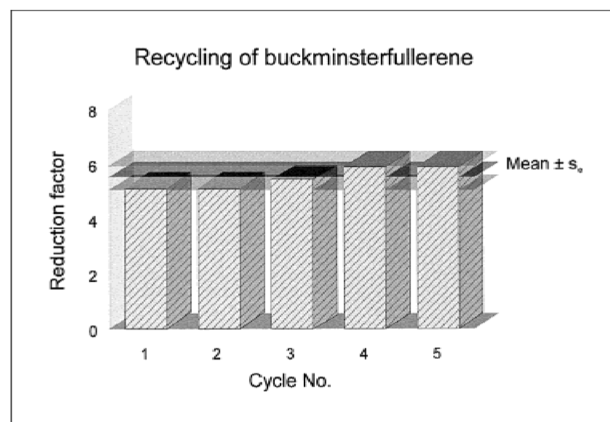

Figure 6. Recycling of buckminsterfullerene. Buckminsterfullerene was utilised in five subsequent inactivation assays. After each cycle C_{60} was washed and resuspended in buffer. Such recycled C_{60} was then tested in the next cycle for its virus inactivation abilities on SFV. The reduction factors (log $TCID_{50}$/mL) obtained upon 2 h illumination are shown.

inactivation by C_{60}, suggesting that no radical mechanism is involved in the inactivation process.

In addition to the inactivation of enveloped viruses, buckminsterfullerene may also be used to inactivate viruses without a lipid membrane. In preliminary experiments it was shown, that when illuminating minute virus of mice (MVM, family: *Parvoviridae*) at elevated temperatures (approximately 45°C), in the presence of C_{60} and oxygen, a reduction of virus infectivity of more than 5 log $TCID_{50}$/mL could be achieved within 3 to 5 h of illumination (Figure 5).

As mentioned in the preceding section, the generation of radicals, that occurs with most dyes used to produce singlet oxygen, may lead to covalent modifications of proteins by the dyes. Radical generation by illuminated C_{60} has never been reported and, as mentioned above, the inactivation capacity was not reduced by radical scavengers.[30] Thus, the use of C_{60} excludes the possibility of protein modifications 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.

Two further advantages of buckminsterfullerene are that it is totally insoluble in aqueous solutions, and extremely stable. Therefore, C_{60} can be removed from solutions by procedures such as centrifugation or filtration, or from suspensions by introducing special properties (e.g. magnetism) into the carbon cages.[50] Removal of C_{60} from the incubation mixture should help to reduce any toxic effects or undesirable complications arising from the use of this photosensitiser in biological fluids; such problems are often encountered with conventional photosensitisers, e.g. hypericin. It is also possible to recycle C_{60}, due to its stability to photo-oxidative degradations, which clearly represents an economical advantage. Indeed our experiments have demonstrated that buckminsterfullerene, utilised up to five times in inactivation assays, showed no loss in its inactivation potential. Following each inactivation experiment, C_{60} was washed, resuspended and tested for its ability to inactivate SFV (Figure 6). Furthermore 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 decreased the inactivation kinetics.[30]

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 these parameters it is very likely that faster kinetics of virus inactivation may be achieved.

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

ACKNOWLEDGEMENTS

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