

Sterilization of enzyme glucose sensors: problems and concepts

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Abstract

A useful method of enzyme glucose sensor sterilization has not only to ensure the needs of sterility assurance but has also to guarantee the functional stability of the sensors. The action of 2 or 3% alkalized glutaraldehyde solution, as well as gamma irradiation with a dose of 25 kGy caused changes of the in vitro functionality and polymer material irritations, respectively. After a combined treatment by 0.6% hydrogen peroxide solution acting over 4 days with 7 kGy gamma irradiation only a slight loss of sensitivity must be registered. The combination of a specially designed universal homogeneous ultraviolet irradiation over 300s with a 3 days lasting treatment by an inclusion compound of hydrogen peroxide with tensides in urea (0.15% effective hydrogen peroxide concentration) did not cause any influence on the glucose sensor function in vitro. With all methods tested here, a *Bacillus subtilis* spore reduction over 8 log₁₀ cycles from 10⁶ to 10⁻² spores per test object on an average could be proved experimentally. In general, if non-thermal methods must be used it seems to be impossible to guarantee a sterility assurance level of 10⁻⁶ as it is demanded by the pharmacopoeias. Consequently, effective concepts to produce sterile glucose biosensors for medical and biological applications should be based not only on final product treatments but should include germ reducing measures in every manufacturing step. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Sterilization is a main precondition to optimize the in vivo functionality of implantable enzyme biosensors, as well as for its practical clinical application (Koudelka-Hep, 1997). For sterilization procedures, the pharmacopoeias demand a sterility assurance level (SAL) of 10⁻⁶ which 'denotes a probability of not more than one viable micro-organism in 1 × 10⁶ sterilized items of the final product'. According to the pharmacopoeial rules steam and dry-heat sterilization, ethylene oxide gas sterilization, as well as sterilization by ionizing irradiation are recommended methods to prepare sterile products (Ph.Eur., 1997; USP 24/NF 19, 1999). Due to the impossibility to prove practically a SAL of 10⁻⁶ suffi-

cient sterilization process conditions are usually determined by exposure of a limited number of test objects with a known high microbial contamination (bioindicators) to fractions of the sterilization process and extrapolation of the experimental data to the SAL based on the assumption that the microbial inactivation generally follows an exponential first-order kinetics (Baird, 1999; Hoxey and Thomas, 1999). Bacterial spores are in general the most difficult to kill. Therefore, the sporidical activity is widely accepted to characterize a sterilizing process or agent sufficiently.

The main problem of sterilization is that a useful method has not only to ensure the needs of sterility assurance but has also to guarantee the functionality of the sterilized product. Above all to take care of the biosensor functionality, most of the methods, which were recommended up to now to sterilize glucose sensors (Table 1) are 'gentle' methods. Despite most of the authors call it 'sterilization' any proof of the sterility assurance in the sense of the pharmacopoeias is missed.

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From our point of view, any verification of a sterilization procedure, which is useful for implantable biosensors, must include both microbiological as well as functionality tests. Primarily, the biosensor functionality is relatively easy to check first of all by calibration. To verify the antimicrobial efficacy of a biosensor sterilization procedure in a little expensive but meaningful manner some knowledge about the microbiological death kinetics is necessary.

Generally, there is no point of absolute sterility of all goods or test objects during a microbial inactivation process. Rather the microbial inactivation kinetics is characterized by a decrease of the probability of microbial survivors or a continuous decrease of the mean number of surviving germs, respectively. Therefore, in an initial part of a sterilization procedure all products will remain non-sterile. However, because this state may be based on one surviving germ in an extreme example the absolute number of microorganisms per test object could be quite reduced. In this part of the antimicrobial process, the number of surviving germs can be quantified by direct germ counting methods

only. However, shortly after the mean number of surviving microorganisms was very low the first products will become sterile and a further treatment will result in an increasing number of sterile products in relation to the whole number of equally treated products. If this situation is reached, a direct quantification of the surviving micro-organisms on the test objects is no longer necessary. Rather, a so-called end-point method can be used, i.e. the test objects are examined for evidence of microbial growth by visual inspection. Based on the rules of a Poisson distribution, the mean number of surviving germs (m) can be calculated from the number of sterile products (n_0) related to the whole number of products treated (n) (Spicher and Peters, 1975; von Woedtke et al., 1994a):

$$m = -\ln \frac{n_0}{n} \quad (1)$$

Using this method, it will be possible to verify both the antimicrobial efficacy of sterilization procedures and its influence on the biosensor functionality with a relatively low operating expense. Consequently, the in-

Table 1
Sterilization procedures recommended for enzyme glucose sensors

Procedure	Process parameters	Reference
Steam	No specifications	Suzuki et al., 1991
Dry heat	120 °C, 1 h	Moussy et al., 1994
Gamma irradiation	Without dose specification	Rea et al., 1985; Matthews et al., 1988; Ege, 1989
	15 kGy	Churchhouse et al., 1986
	20 kGy	Vadgama et al., 1989
	25 kGy	Koudelka-Hep et al., 1993; Bobbioni-Harsch et al., 1993
Electron beam irradiation	Without dose specification	Johnson et al., 1992; Rebrin et al., 1999
X-ray irradiation	200 Gy	Mayer et al., 1995
UV irradiation	Without dose specification	Moussy et al., 1993
	At least 3 h, no specification of irradiation energy	Moussy et al., 1994
Ethylene oxide gas sterilization	Use of validated sterilizers	Zhang et al., 1991; Bindra et al., 1991; Poitout et al., 1993; Moatti-Sirat et al., 1994
	1 atm, 5 h, 50 °C, degassing in the air 12 h at room temperature	Ohashi and Karube, 1995
<i>Chemicals</i>		
Glutaraldehyde	6%, 6–12 h	McKean and Gough, 1988; Armour et al., 1990
	2.5%, 5 h	Ertefai and Gough, 1989
	2%, 1 h	Pickup et al., 1993
	2.5%	Schindler et al., 1994 (flow-through system)
	2.5%, 5 h	Kerner et al., 1993
Thiomersal	0.024%	Ege, 1989
	2.5%	Moatti-Sirat et al., 1992
	0.1%	Updike et al., 1994
	0.05%, 24 h	Gilligan et al., 1994
Isopropanol	100%, 70%	Vadgama et al., 1989
<i>Antibiotics</i>		
Cefaloridine		Kondo et al., 1982
Sterile sensor fabrication		Linke et al., 1994

tention of our work was to prove systematically traditional methods as gamma irradiation and the treatment by glutaraldehyde, as well as innovative combination procedures using components as hydrogen peroxide or a specially designed universal homogeneous ultraviolet (UHUV) irradiation (von Woedtke et al., 1994a, 1998) with regard to its real usefulness for a safe as well as practicable sterilization of glucose biosensors.

2. Experimental

2.1. Enzyme glucose biosensors

Pencil-shaped, as well as miniaturized catheter-shaped enzyme glucose sensors were used. Functional principle, preparation technique and functional characteristics were similar in both types. Glucose oxidase (GOD, EC 1.1.3.4.; Boehringer Mannheim GmbH, Mannheim, Germany) immobilized onto sepharose 6B microspheres (Pharmacia, Uppsala, Sweden) was placed on the platinum anode surface of pencil-shaped Clark-type electrodes consisting of a platinum anode and a silver/silver chloride cathode (SME/S3, outer diameter (o.d.) 4 mm, ELBAU GmbH, Berlin, Germany). The enzyme layer was covered by a 30 μm thick hydrophobic polyethylene membrane containing a perforation in the anode area providing an 'analyte door' for glucose diffusion control, as well as by a plane hydrophilic outer membrane of regenerated cellulose (PT 150 Cuprophan[®]) (von Woedtke et al., 1994b, 1997). Using the miniaturized Clark-type catheter electrodes (MSME/01, o.d. 0.6 mm, ELBAU GmbH, Berlin, Germany) GOD was co-immobilized directly on the electrode surface by glutaraldehyde (E. Merck AG, Darmstadt, Germany) and human serum albumine (HSA, albumin 20% human, Octapharma GmbH, Langenfeld, Germany). The sensor was covered by polyurethane (Tecoflex[®] EG 80 A, Thermedics, Medextru, Hamburg, Germany) dissolved in dimethyl formamide and tetrahydrofuran (Aldrich, Steinheim, Germany) using a special dip-coat technique including an on-site membrane formation under well controlled environmental conditions (Abel et al., 1999). The glucose sensors were maintained at room temperature on a +700 mV polarization source in a glucose-free imidazol buffer pH 7.0 (Imidazol puriss p.a., Fluka AG, Buchs, Switzerland) for at least 24 h before any functionality test. For calibration steady state sensor current versus glucose concentration was measured. Response times T_{95} were determined based on the results of a step-like glucose concentration change from 5 to 10 mM by non-linear regression analysis (von Woedtke et al., 1991, 1994b). Due to the individual laboratory-scale preparation of the sensors, the functional parameters varied mutually. Consequently, to

estimate influences of sterilization parameters on the sensor function, the results of the sensitivity, as well as the response time measurements were compared individually using the paired Student's *t*-test.

2.2. Gamma irradiation

For gamma irradiation Cesium-137 (B. Braun Melsungen AG, Melsungen, Germany) or Cobalt-60 radioisotope sources (Hahn Meitner Institute Berlin GmbH, Berlin, Germany), respectively, were used. Based on dosimetric measurements, the absorbed radiation doses were controlled by the exposure time.

2.3. Universal homogeneous ultraviolet (UHUV) irradiation

A low-pressure mercury-vapor discharge tube generating high intensity ultraviolet (UV) resonance radiation at 254 nm was specially designed for biosensor irradiation (Institute of Nonthermal Plasma Physics, Greifswald, Germany). A UV generating plasma is circulating around the object to be irradiated which is localized inside a double-wall quartz glass tube (inner diameter (i.d.) 35 mm, length 100 mm). Due to this spatial arrangement, an universal and nearly homogeneous irradiation of the sensor can be realized (Jülich et al., 1998). In this study, the germicidal lamp operated with a constant irradiation energy of 0.3 mW/cm².

2.4. Reagents

Glutaraldehyde solution (50% in water, Fluka AG, Buchs, Switzerland) was diluted with distilled water to concentrations of 2 and 3%, respectively. Immediately before starting the antimicrobial treatments, the solutions were alkalized (pH 7.5–8.5) by addition of 0.3% sodium hydrogen carbonate.

Hydrogen peroxide (H₂O₂) test solutions were prepared from a 30% stock solution (Ph.Eur., stabilized, Roth GmbH, Karlsruhe, Germany) by dilution with distilled water.

An inclusion compound from H₂O₂ and tensides in urea (Institute of Applied Chemistry Adlershof, Berlin, Germany) containing 15% H₂O₂ and 5% lauryl sorbitan ester (Span 20) was prepared in a water-free system (Jülich et al., 1999). For test purposes, the compound was dissolved in distilled water.

2.5. Microbiological tests

In each single test 10–20 commercially available *Bacillus subtilis* spore test strips (6 × 31 mm; *B. subtilis* var. *niger* ATCC 9372, 0.5–1.5 × 10⁶ spores per strip; BAG BioStrips, Biologische Analysensysteme GmbH, Lich, Germany) were used as test objects. After any

Table 2
Sensitivity of enzyme glucose sensors before and after several antimicrobial treatments (mean \pm S.D.)

Antimicrobial treatment	<i>n</i>	Sensitivity before treatment <i>b</i> ₁ (nA/mM)	Sensitivity after treatment <i>b</i> ₂ (nA/mM)
Gamma irradiation dose: 25 kGy	4	1.1 \pm 0.4	1.1 \pm 0.1
<i>2% alkalized glutaraldehyde solution</i>			
Treatment time: 0.5 h	6	1.7 \pm 0.6	1.8 \pm 0.6
Treatment time: 4 h	8	1.6 \pm 0.4	1.8 \pm 0.6
Treatment time: 8 h	6	1.2 \pm 0.4	1.5 \pm 0.5 ^a
<i>0.6% Hydrogen peroxide solution</i>			
Treatment time: 4 days	12	1.4 \pm 0.3	1.4 \pm 0.5
<i>Combination of gamma irradiation and 0.6% H₂O₂ solution</i>			
Irradiation dose: 7 kGy, H ₂ O ₂ treatment time: 4 days	6	0.9 \pm 0.5	0.5 \pm 0.4 ^b
<i>Universal homogeneous ultraviolet (UHVU) irradiation</i>			
Irradiation time: 300 s, Irradiation energy: 0.3 mW/cm ²	7	0.9 \pm 0.8	0.9 \pm 0.8
<i>H₂O₂ inclusion compound solution</i>			
Concentration: 3.1% (corresponding to 0.46% H ₂ O ₂), treatment time: 4 days	7	2.0 \pm 0.5	1.9 \pm 0.9
Concentration: 6.2% (corresponding to 0.93% H ₂ O ₂), treatment time: 4 days	6	1.4 \pm 0.2	0.1 \pm 0.1 ^c

^a $P < 0.05$.

^b $P < 0.01$.

^c $P < 0.001$ (paired Student's *t*-test).

antimicrobial treatment the spore strips were incubated in trypticase soy broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) at 37 °C for 7 days. Following this incubation, the culture media were examined for evidence of microbial growth by visual inspection. The mean number of surviving spores per test object (*m*) was calculated according to Eq. (1).

3. Results

3.1. Gamma irradiation

Immediately after gamma irradiation by 25 kGy, the individual sensor calibration characteristics were not changed (Table 2). But, during a following 6-week storage time, a total uselessness of the sensors was ascertained because of spontaneous breaks and fissures of the polymer materials.

3.2. Glutaraldehyde treatment

Using 2 and 3% alkalized glutaraldehyde solutions a germ reduction by about 6 log₁₀ cycles starting from a mean contamination of 10⁶ spores per test object was reached within the first hour of action time. A prolongation of the glutaraldehyde exposure time up to 5 h led to an additional spore reduction by 1 log₁₀ cycle only. Consequently, using 2 or 3% glutaraldehyde, a total spore reduction by 7 log₁₀ cycles within 5 h could be proved experimentally (Fig. 1). The sensitivity of

enzyme glucose sensors increased significantly after a treatment with 2% glutaraldehyde over 8 h (Table 2). But, an action of 2% glutaraldehyde over 0.5 h already induced a significant increase of the sensor's response time T₉₅ continuing even 144 h after the end of the glutaraldehyde exposure (Table 3).

3.3. Combination of gamma irradiation with hydrogen peroxide (H₂O₂)

Testing the effect of 0.6% H₂O₂ on the sensor function, no influence on sensitivity was measured even after a 4 days treatment (Table 2). The sensor's response time was not affected, too (data not shown).

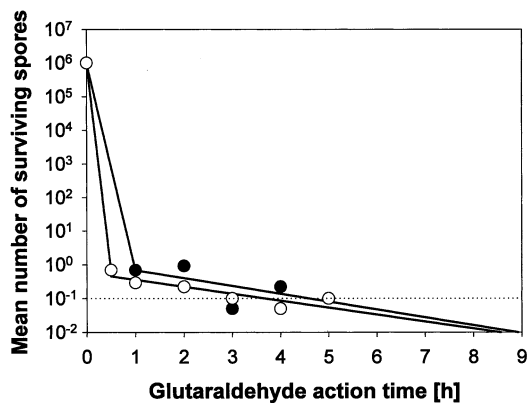


Fig. 1. Time dependent inactivation of *B. subtilis* spores by alkalized glutaraldehyde solutions 2% (filled circles), as well as 3% (open circles).

Table 3

Response times T95 of enzyme glucose sensors before and after treatment with 2% alkalized glutaraldehyde solution (mean \pm S.D.)

Glutaraldehyde treatment time (h)	n	T95 (min) before treatment	T95 (min) 24 h after treatment	T95 (min) 48 h after treatment	T95 (min) 144 h after treatment
0.5	6	3.6 \pm 2.5	6.8 \pm 3.0 ^a	8.4 \pm 2.7 ^b	10.6 \pm 3.7 ^c
4	8	6.8 \pm 1.4	15.6 \pm 4.0 ^c	17.7 \pm 5.4 ^c	24.0 \pm 6.4 ^c
8	6	3.2 \pm 1.0	15.4 \pm 4.0 ^c	17.0 \pm 3.5 ^c	15.1 \pm 3.3 ^c

^a $P < 0.05$ (paired Student's *t*-test related to the data before GA treatment).^b $P < 0.01$ (paired Student's *t*-test related to the data before GA treatment).^c $P < 0.001$ (paired Student's *t*-test related to the data before GA treatment).

However, a prolongation of the H₂O₂ treatment time led to an increasing loss of sensitivity up to a total functional failure of all sensors tested following a 14 days H₂O₂ action (data not shown). Taking into consideration the restrictions concerning the polymer material strain gamma irradiation with reduced doses was combined with the action of H₂O₂. A dose of about 9 kGy was able to reduce the mean spore count by 7 log₁₀ cycles from 10⁶ to 10⁻¹ spores per test object on an average. Paralleled by a 4 day action of 0.6% H₂O₂, a corresponding spore reduction by 7 log₁₀ cycles was reached with 2–4 kGy gamma irradiation only (Fig. 2). A significant decrease of glucose biosensor's sensitivity was measured if a 4 day treatment with 0.6% was combined with a 7 kGy gamma irradiation (Table 2). Influences of the response time T95, as well as long-term material irritations could not be detected after any treatment with this combination.

3.4. Combination of universal homogeneous ultraviolet (UHUV) irradiation with H₂O₂ inclusion compounds

A UHUV irradiation over 300 s resulted in a total spore reduction by about 7 log₁₀ cycles (Fig. 3). Testing the H₂O₂ inclusion compound, a 3 day action of an effective H₂O₂ concentration of 0.15% only resulted in a spore reduction by more than 6 log₁₀ cycles (Fig. 4). After a paralleled action of both components, 100 spore test strips were found to be sterile. This corresponded to a total germ reduction by at least 8 log₁₀ cycles, which could be proved experimentally.

An UHUV irradiation over 300 s influenced the sensor calibration characteristics just as little as a 4 day action of the inclusion compound with an effective H₂O₂ concentration of 0.46%. However, a duplication of the inclusion compound concentration resulted in a significant loss of sensitivity (Table 2).

4. Discussion

Well established highly efficient sterilization procedures are based on intensive physical or chemical treatments. However, the same active principles, which

damage vital biochemical structures of microorganisms are able to influence the materials, as well as the functionality of the products, which are sterilized. Therefore, any heat sterilization of glucose biosensors has to be excluded from the first with respect to the functional stability of the enzyme, as well as the polymer structures. Toxic gases as ethylene oxide should not be used to sterilize products for medical and biological applications because of the risk of toxic and carcinogenic residues. For ionizing irradiation, a minimum dose of 25 kGy is recommended to meet the demands of sterility assurance (Ph.Eur., 1997; USP 24/NF 19, 1999; Russell, 1999). Using this dose, no acute influence on the functional parameters of glucose sensors could be measured in our study. Other authors who sterilized enzyme glucose, as well as lactate sensors assigned for in vivo application by this method had to accept sensitivity reductions by about 20–30%, but experimental in vivo applications of these sterilized biosensors were possible anyhow (Koudelka-Hep et al., 1993; Bobbioni-Harsch et al., 1993; Pfeiffer et al., 1998). However, the capability to penetrate into matter which is the main reason for the high antimicrobial effectiveness of ionizing irradiation on the one hand may induce several changes of materials, as well as biological active substances as enzymes on the other. The spontaneous breaks and fissures of the polymer materials arised

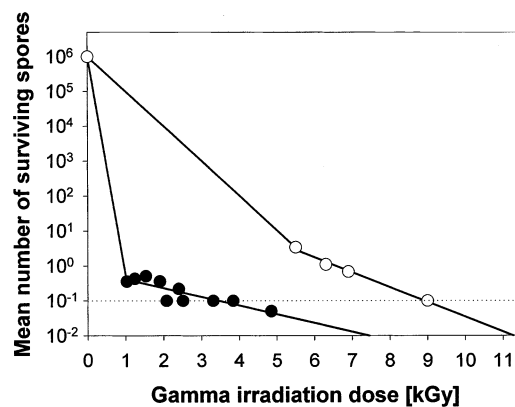


Fig. 2. *B. subtilis* spore inactivation by gamma irradiation without (open circles) and with a paralleled action of 0.6% hydrogen peroxide solution over 4 days (filled circles).

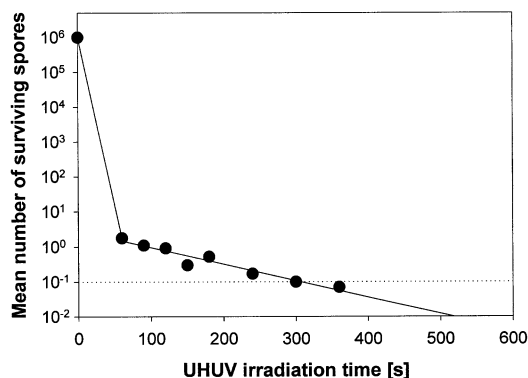


Fig. 3. *B. subtilis* spore inactivation by 0.3 mW/cm² universal homogeneous ultraviolet (UHUV) irradiation.

during several weeks of storage after the irradiation as observed in our study have been characterized a general risk involved in the use of ionizing irradiation. If such long-term material changes were found, it must be suspected that minimum but hardly to detect material changes, as well as a possible release of low molecular decomposition products may start immediately after the irradiation already. Due to the various factors which determine the radiation resistance of polymeric materials, it is nearly impossible to predict material changes in general. Moreover, such changes are often results of long-term secondary processes coming off in polymeric products, which appear unaffected immediately after the sterilization procedure. Therefore, short-term, as well as long-term effects of irradiation sterilization must be checked individually for the special materials and products which should be treated (Cesar and Schultz, 1987; Bruck and Mueller, 1988; Szycher, 1991; Jacobs, 1995; Nair, 1995).

As alternatives for products, which cannot be sterilized by conventional procedures, mostly liquid chemicals are recommended. But, if such methods are used for biosensor treatment the main challenge is to verify the antimicrobial efficacy in close connection to the maintenance of the sensor function.

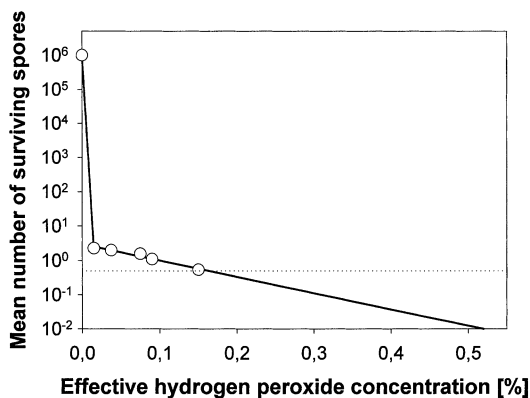


Fig. 4. *B. subtilis* spore inactivation by solutions of an inclusion compound of hydrogen peroxide with lauryl sorbitan ester in urea.

Since any sterility test is a destructive procedure, sterilization methods must include a sufficient security addition as the sterility assurance level (SAL) of 10^{-6} is to guarantee the sterility of a large number of products based on the investigation of a small random sample. However, it is impossible to prove the very low degree of microbial contamination of 10^{-6} after sterilization. Therefore, the definition of SAL is based on the general assumption that the inactivation of microorganisms by physical or chemical means follows a first-order exponential law. To find sufficient sterilization process conditions which will meet the SAL requirements usually test objects with a known bioburden (i.e. microbial contamination) are exposed to fractions of the sterilization process in that way that a mean number of surviving germs can be estimated experimentally. These experimental results then are extrapolated to the SAL range to find the final parameters for a sterilization treatment under the general assumption that inactivation kinetics are the same both in the experimentally controllable range of relatively high germ contamination and in the range of decreasing probability of microbial survivors up to the SAL range of 10^{-6} (Baird, 1999; Hoxey and Thomas, 1999).

Even if it is not yet part of any pharmacopoeial rule alkalized glutaraldehyde solution is a widely established 'chemosterilizing' agent (Russell, 1994). The common use-dilution of 2% glutaraldehyde is said to produce sterility if the exposure is long enough whereas the recommended times vary between minutes and several hours (Scott and Gorman, 1991; Russell, 1994; see also Table 1). In our study, spore reductions by about 6 log₁₀ cycles after 0.5 h using 3% glutaraldehyde solution, as well as 1 h using 2% glutaraldehyde solution were measured starting from a mean bioburden as high as 10^6 spores per test object. An extrapolation of these data to the SAL range would result in final treatment times of about 1 h (3% glutaraldehyde), as well as 2 h (2% glutaraldehyde), respectively, to obtain a mean number of surviving germs of about 10^{-6} . But, in our experiments, a prolongation of the glutaraldehyde action time resulted in a final mean number of surviving spores still around 10^{-1} per test strip even after 5 h. This indicated that the glutaraldehyde induced spore inactivation obviously does not follow a homogeneous first order kinetics. With these findings, any extrapolation of the whole inactivation curve, as well as parts of it exceeding the range covered by experimental data is very problematic. However, based on our experimental data, an extrapolation over not more than one additional log₁₀ cycle seems to be acceptable to get a reliable security addition. Consequently, a treatment with 2–3% alkalized glutaraldehyde solution over about 9 h will result in a mean degree of contamination of 10^{-2} spores per test object. Any additional sporicidal effect by further pro-

longation of the glutaraldehyde action time could not be proved experimentally.

The influence on the sensitivity of enzyme glucose sensors was negligible under these conditions. But, a drastic increase of the sensor's response time T₉₅ was measured after an action of 2% glutaraldehyde over 0.5 h already. Especially considerable was the fact that the response time increase still continued 72 h, as well as 144 h after the sensors were removed from the glutaraldehyde solution and rinsed intensively and repeatedly in distilled water. Various fields of glutaraldehyde usage, e.g. as fixative, in leather tanning, for enzyme immobilization and as antimicrobial effective agent, respectively, are mainly attributed to its cross-linking reactivity because of two free aldehyde groups (Scott and Gorman, 1991; Russell, 1994). From our point of view, the observed response time increase which must be attributed to modified membrane diffusion properties could be explained by membrane cross-linking reactions caused by reactive glutaraldehyde residues which remained in the sensor membrane despite an intensive sensor rinsing even after the end of glutaraldehyde treatment. The possible absorption of glutaraldehyde by polymeric materials is reported in the literature just as its toxic, as well as sensitizing potential to patients exposed to equipment treated with glutaraldehyde (Scott and Gorman, 1991). Consequently, if glutaraldehyde is used for the antimicrobial treatment of biosensors assigned for medical or biological applications, problems with the desorption of the active compound have to be taken into consideration similar to that known from ethylene oxide gas sterilization.

Generally, material damaging or functionality influencing effects of sterilization procedures, as well as the degree of potentially toxic residues are dependent on the intensity (i.e. dose, concentration, action time etc.) of the physical or chemical treatment. To reach a reduction of the intensity of the respective single procedure without a loss of antimicrobial efficacy, we have tested a strategy, which is based on the combination of different antimicrobial principles.

Hydrogen peroxide (H₂O₂) is well known as an effective antimicrobial agent with a broad activity spectrum and a very low toxicity (Block, 1991). Despite the well known inactivation of glucose oxidase by H₂O₂ (Mallikides and Weiland, 1982) 0.6% H₂O₂ had no influence on the calibration characteristics of the sensors if the maximum treatment time was not longer than 4 days. Combined with such kind of H₂O₂ treatment, the gamma irradiation dose which was necessary to reduce the mean contamination from 10⁶ to 10⁻¹ spores per test strip could be lowered from 9 to 3 kGy. Obviously, the combined H₂O₂ action supported above all the initial reduction of the high experimental bioburden. The irradiation dose dependent spore reduction curve was similarly flat as it was found with glutaraldehyde

already. Consequently, also with this combination, an extrapolation of the experimental data to the SAL range seems to be not possible in general. From our experiments, it may be concluded that a mean number of surviving spores of 10⁻² can be reached with 7–8 kGy gamma irradiation paralleled by a 4-day action of 0.6% H₂O₂. Using this combination to treat enzyme glucose sensors, the functionality was kept in principle but a sensitivity reduction by about 40% must be accepted. However, any damage of polymeric materials caused by gamma irradiation did not occur even after several weeks storage time.

But, because of its enzyme inactivating effect, the scope between antimicrobial effective and functionality influencing H₂O₂ action parameters remained very small. That means that any further support of the antimicrobial activity by higher concentrations or longer action times of H₂O₂ will be impossible. Newly-developed inclusion compounds of H₂O₂ with tensides in urea have an increased antimicrobial efficacy compared with pure hydrogen peroxide (Jülich et al., 1999). Consequently, if lower hydrogen peroxide concentrations are needed for the same antimicrobial activity, a lower influence on the sensor functionality must be expected. After a 3 day action of 1% of the H₂O₂ inclusion compound which corresponded to an effective H₂O₂ concentration of 0.15%, a *B. subtilis* spore reduction by more than 6 log₁₀ cycles was reached. On the other hand, a three-fold higher concentration of the inclusion compound (3.1%, corresponding to 0.46% H₂O₂) acting over 4 days was without any influence on the sensor calibration characteristics.

Also with a reduced dose as it is feasible in combination with H₂O₂ gamma irradiation induced material irritations cannot be fully excluded. Recent investigations gave first references that gamma irradiation may influence the biocompatibility of sensor materials more than assumed up to now (Urban et al., 1998). Moreover, gamma irradiation demands special security measures making a decentralized use impossible. Consequently, an optimization of that part of the combination treatment seemed to be essential, too.

The intensive antimicrobial activity of ultraviolet (UV) radiation which includes sporicidal, as well as virucidal efficacy is well known. But, UV radiation does not penetrate solids and is extensively absorbed by glass and plastics. Therefore, the traditional application fields are in the disinfection of drinking water, as well as in air and surface disinfection (Szycher, 1991; Russell, 1999). We have tested a newly designed device where UV radiation is generated by a gas discharging plasma circulating around the biosensor or the spore test strip which should be treated. With this technique called universal homogeneous ultraviolet (UHUV) irradiation a multidirectional irradiation of the product can be realized (Jülich et al., 1998). By this means, the shadow

casting which is inevitable using conventional UV lamps can be reduced very effectively. A mean reduction from 10^6 to 10^{-1} spores per test strip was obtained within 300 s irradiation time. However, even if this UHUV irradiation technique makes it possible to irradiate surfaces effectively which are not completely smooth the lack of an antimicrobial depth effect is persisting now as before. This was demonstrated by the fact that a direct UHUV irradiation of glucose oxidase resulted in a rapid loss of enzyme activity on the one hand. On the other hand, an irradiation of enzyme glucose sensors where the enzyme is covered by a membrane was without any influence on the sensor functionality (Abel et al., 1999). Although it should be discussed generally if such a depth effect is really necessary for biosensor sterilization the use of UHUV irradiation within a combination procedure will reduce this problem from the first. A combination of 300 s UHUV irradiation with an action of 3.1% H_2O_2 inclusion compound (corresponding to 0.15% H_2O_2) resulted in a spore reduction by at least 8 \log_{10} cycles which could be proved experimentally. Any influence on the sensor functionality has not to be anticipated. Consequently, a wide variability of the action parameters of the H_2O_2 inclusion compound, as well as of the UHUV irradiation dose for further optimization of the efficacy of this sterilization procedure will be possible without consequences to the sensor functionality.

5. Conclusions

Now as before it has to be stated that 'there is no general agreement about sterilization procedures' for enzyme glucose sensors (Pickup and Thévenot, 1993). There are highly effective thermal methods, which cannot be used for heat sensitive products as biosensors. Consequently, the main problem can be focussed on the fact that there is no sufficient method which can guarantee both a sterility within the meaning of a SAL of 10^{-6} and a sufficient sensor functionality after sterilization. As it was pointed out in detail elsewhere it is very questionable in general if non-thermal antimicrobial procedures can guarantee the sterility assurance level as it is demanded by the pharmacopoeias (von Woedtke et al., 2001).

It could be demonstrated in this study by highly resistant bacterial spores that it is possible to prove a spore reduction by about 8 \log_{10} cycles from a mean number of 10^6 to 10^{-2} spores per test object experimentally. From our point of view, a much more higher level of assurance of an antimicrobially active procedure should not be demanded for irritable goods as biosensors which cannot be sterilized by standard thermal procedures. Any extreme intensification of an antimicrobial treatment using physical or chemical means,

respectively, must lead without fail to massive material, as well as functional disturbances.

Using gamma irradiation, as well as alkalized glutaraldehyde solution such influences on the polymer material integrity, as well as the sensor functionality could not be fully excluded even after treatments resulting in a spore reduction by 8 \log_{10} cycles only. However, if the antimicrobial activity is determined by one parameter only the chances of variations are very limited. Therefore, we have introduced a new concept to combine different antimicrobial principles. The combination of low-dose gamma irradiation with a treatment with 0.6% H_2O_2 solution over 4 days was proved to be relatively useful. However, the sporicidal effective combination of universal homogeneous ultraviolet irradiation over 300 s only combined with a solution of an inclusion compound with an effective H_2O_2 concentration of 0.15% over 3 days as it was tested in this study had the lowest influence on glucose sensor function. Moreover, there are still possibilities to optimize this combination procedure to get much more security addition. Consequently, this combination is recommended as the preferred sterilization method for glucose biosensors at that time.

Nevertheless, it is not possible from our point of view to give a general recommendation for 'that final method for glucose sensor sterilization' because the several possible influences on sensor functionality may vary considerably dependent on the sensor materials, enzyme immobilization, preparation technique etc.

The main lack of all concepts for sensor sterilization recommended up to now including the present study can be focussed on the fact that any sterilization procedure has to be adapted to a biosensor whose development is finished under the aspect of technical functionality only. For the future, to realize an optimized coordination between sterility assurance and all aspects of functionality, as well as material stability, an adequate sterilization procedure should be part of the product development as early as possible. This includes that the selection of special materials or other functional elements, respectively, should be made not only under the aspect of the sensor function but also with regard to its sterilizability. Due to various general problems connected with the use of traditional procedures for the sterilization of irritable goods as biosensors integrated sterilization concepts must be developed and established in the future. Such concepts should be based not only on final antimicrobial treatments of the sensors but must include the realization and validation of germ reducing measures in every step of the product manufacturing to guarantee a 'functional biostability' of the biosensors including all aspects of sensor function in vitro, as well as in vivo.

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