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Fast, broad-range disinfection of bacteria, fungi, viruses and prions

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Effective disinfectants are of key importance for the safe handling and reprocessing of surgical instruments. This study tested whether new formulations containing SDS, NaOH and 1-propanol (*n*-propanol) are simultaneously active against a broad range of pathogens including bacteria, fungi, non-enveloped viruses and prions. Inactivation and disinfection were examined in suspension and on carriers, using coagulated blood or brain homogenate as an organic contaminant. Coomassie blue staining was used to assess whether the formulations undesirably fixed proteins to rough surfaces. A mixture of 0.2 % SDS and 0.3 % NaOH in 20 % *n*-propanol achieved potent decontamination of steel carriers contaminated with PrP^{TSE}, the biochemical marker for prion infectivity, from 263K scrapie hamsters or from patients with sporadic or variant Creutzfeldt–Jakob disease. 263K scrapie infectivity on carriers was decreased by $\geqslant$5.5 logs. Furthermore, the formulation effectively inactivated poliovirus, hepatitis A virus and caliciviruses (including murine norovirus) in suspension tests. It also yielded significant titre reductions of bacteria (*Enterococcus faecium*, *Mycobacterium avium*; >6 logs), fungi (spores of *Aspergillus niger*; $\geqslant$5 logs) and poliovirus (>4 logs) embedded in coagulated blood on carriers. The formulation was not found to fix proteins more than was observed with water as the cleaning reagent. In conclusion, SDS, NaOH and *n*-propanol can synergistically achieve fast, broad-range disinfection.

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INTRODUCTION

Effective disinfection is of the utmost importance in the maintenance of reusable surgical instruments. In this context, an ideal disinfectant that is also applicable to heat-sensitive devices should have a fast and broad-range activity against bacteria, viruses and fungi, as well as prions, while being easy to use and free of effects that fix (i.e. bind) organic contaminants containing proteins to the surface of instruments. Such requirements cannot readily be met, as a variety of pathogens such as mycobacteria, fungal spores, non-enveloped viruses [such as poliovirus (PV)] and, last but not least, prions are known for their unusually high tolerance to inactivation (Beekes *et al.*, 2004; Fernie *et al.*, 2007; Rutala *et al.*, 2008; Taylor, 2004).

Prions, or 'proteinaceous infectious particles' (Prusiner, 1982), are considered to be the causative agents of transmissible spongiform encephalopathies (TSEs) such as scrapie in sheep, bovine spongiform encephalopathy in cattle and Creutzfeldt–Jakob disease (CJD) and its variant form (vCJD) in humans. According to the prion hypothesis, these agents are composed essentially – if not entirely – of misfolded prion protein (PrP), which is derived from a host-encoded cellular precursor (PrP^{C}) (Prusiner, 1998). The deposition in the central nervous system of disease-associated PrP, referred to as PrP^{Sc} (Prusiner, 1998) or PrP^{TSE} (Brown & Cervenakova, 2005), with an abnormal folding and/or aggregation structure is a pathological hallmark of TSEs, and PrP^{TSE} has been used in many studies as a molecular marker for prion infectivity (Beekes & McBride, 2007).

The study described in this report emanated from previous work in which we tested various formulations for their ability to detach, destabilize or degrade PrP^{TSE} attached to steel wires and to inactivate prion infectivity on such carriers. Similar steel wire models for prion disinfection of surgical instruments (Flechsig *et al.*, 2001; Zobeley *et al.*,

1999) have also been employed by other research groups (Fichet *et al.*, 2004, 2007; Yan *et al.*, 2004). In our previous studies, we identified a formulation of 0.2 % SDS and 0.3 % NaOH (hereafter referred to as SDS/NaOH) as a highly potent disinfectant against 263K scrapie agent from hamsters (Lemmer *et al.*, 2004, 2008). We have now explored whether, by adding different concentrations of ethanol or *n*-propanol to this formulation, its protein-destabilizing and prion inactivating activity may be extended to disinfection of bacteria, viruses and fungi without causing effects that would undesirably fix proteins.

RESULTS

Efficacy of the tested formulations against bacteria, fungi and viruses

The efficacy of candidate formulations was tested using a broad range of conventional pathogens known to be highly tolerant to commonly used disinfectants, such as enterococci, *Mycobacterium avium*, norovirus, PV, hepatitis A virus (HAV), and spores of *Aspergillus niger*. As the activity against infective agents embedded in proteinacous matrices was of utmost interest, tests were performed using carriers contaminated with pathogens in coagulated blood. Additional suspension tests [some samples of which contained an additional organic burden of 10 % fetal calf serum (FCS)] were carried out to corroborate the findings obtained with carrier assays.

Efficacy against enterococci and mycobacteria. The efficacy against mycobacteria and enterococci was examined in a quantitative carrier test using coagulated blood as the test contaminant (Table 1). Whilst SDS/NaOH without alcohol showed no relevant inactivating effect, the addition of 50 % ethanol or 20 % *n*-propanol resulted in reduction factors (RFs) of >6 $\log_{10}$ units (logs). All formulations found to be effective in the carrier test were also at least as active when assessed in quantitative suspension tests (data not shown).

Efficacy against non-enveloped viruses. The virucidal activities of the various formulations were initially assessed for a broad range of viruses including vaccinia virus, simian virus 40, adenovirus, caliciviruses [feline calicivirus (FCV) and murine norovirus (MNV)] and PV in quantitative suspension tests. MNV, PV and HAV were identified as the most tolerant viruses in these assays (data not shown for other viruses), and PV was subsequently also used in quantitative carrier tests with coagulated blood as the test contaminant. The findings with PV, HAV and caliciviruses are summarized in Table 2.

In contrast to the results with bacteria, ethanol or propanol alone did not exert a relevant inactivating effect on these viruses. However, when 20 % *n*-propanol was used in a mixture with SDS/NaOH, RFs of 4 logs or more were observed in quantitative suspension tests for caliciviruses, HAV (with 10 % FCS as an additional organic burden) or PV (with or without 10 % FCS as additional organic burden), and in the quantitative carrier test with coagulated blood for PV.

Efficacy against spores of *A. niger*. SDS/NaOH, 50 % ethanol and 20 % *n*-propanol alone were not able to achieve a significant disinfection of carriers contaminated with *A. niger* spores (Table 3). However, strong synergistic effects in terms of disinfection were observed, yielding RFs of $\geqslant 5$ logs, when formulations of SDS/NaOH in 50 % ethanol or 20 % *n*-propanol were applied.

Efficacy of the tested formulations against prions

Alcoholic mixtures of SDS/NaOH were tested *in vitro* for their potential to decontaminate steel carriers from PrP^{TSE} that had been coated with brain homogenates from scrapie hamsters or patients with sporadic CJD (sCJD) or vCJD. The disinfection of steel wires contaminated with 263K scrapie agent by these formulations was monitored in hamster bioassays.

Decontamination of steel wires from PrP^{TSE} of 263K scrapie hamsters. As described previously (Lemmer *et al.*,

Table 1. Efficacy of the tested disinfectant formulations on bacteria in a quantitative carrier test with coagulated blood

RF values are indicated in logs and were calculated from the difference between the bacterial load on carriers after processing in the tested formulations and the bacterial load on carriers after similar processing in dH_2O only. All incubations were carried out for 20 min at 20 °C.

Formulation	Concentration	*E. faecium* RF	*M. avium* RF
SDS/NaOH	0.2 %/0.3 %	0	0
Ethanol	50 %	>6	>6
SDS/NaOH in ethanol	0.2 %/0.3 % in 50 %	>6	>6
n-Propanol	20 %	>6	>6
SDS/NaOH in *n*-propanol	0.2 %/0.3 % in 20 %	>6	>6

Table 2. Efficacy of the tested disinfectant formulations on viruses as determined by different test formats

RF values are indicated and were calculated as specified for Table 1. ND, Not determined. All incubations were carried out for 20 min at 20 °C.

Formulation	Concentration	PV		HAV		Caliciviruses
		RF*†	RF‡	RF*	RF†	RF*
SDS/NaOH	0.2 %/0.3 %	>4	⩽4	⩽3	ND	>4
Ethanol	50 %	<1	<1.5	<1	ND	<2§
SDS/NaOH in ethanol	0.2 %/0.3 % in 50 %	>4	>4	⩾4	>4	>5
n-Propanol	30 %	<0.5	ND	<1	ND	>4
	20 %	<0.5	<1.5	<1	ND	<1§
SDS/NaOH in *n*-propanol	0.2 %/0.3 % in 30 %	>4	ND	⩾4	ND	>5
	0.2 %/0.3 % in 20 %	>4	>4	>3 to <4	>4	>5

*The disinfecting activities of examined formulations were determined for PV, HAV and caliciviruses by quantitative suspension tests.
†The virucidal activity against PV and HAV in the suspension test was also monitored with an additional organic burden of 10 % FCS.
‡Inactivation of PV was additionally measured in a quantitative carrier test with coagulated blood.
§The data for caliciviruses refer to both FCV and MNV; however, where specifically indicated, values refer to MNV because of the higher observed tolerance.

2004), the efficacy of the decontamination of steel wires from PrP^{TSE} was assessed *in vitro* by comparing the initial load of contamination on the wires with the amount of total PrP and PrP27–30 [the proteinase K (PK)-resistant core of PrP^{TSE}] residually attached to the carriers after processing in the test solutions. According to the established sensitivity of our assay, the complete disappearance of PrP^{TSE} staining in eluates from the wires with or without PK treatment indicated a 500–1000-fold reduction in PK-resistant PrP or total PrP.

When contaminated steel wires were processed in a mixture of SDS/NaOH containing 50 % ethanol, a strong signal for residual PrP was observed after incubation for 5 min (Fig. 1a, lane 1), and after 20 min of incubation, weak staining for PrP was still visible without PK digestion (Fig. 1a, lane 3). After PK digestion, complete disappearance of residual PrP^{TSE} could be observed (Fig. 1a, lanes 2 and 4). When 50 % *n*-propanol was used instead of 50 % ethanol in the mixture, residual staining of PrP was less intense after 5 min (Fig. 1b, lane 1) and was nearly absent after 20 min of incubation (Fig. 1b, lane 3). After digestion with PK, immunostaining of residual PrP^{TSE} completely disappeared (Fig. 1b, lanes 2 and 4). These findings indicated that 50 % *n*-propanol and particularly 50 % ethanol in the solution caused effects that fixed PrP^{TSE} compared with SDS/NaOH alone (Lemmer *et al.*, 2004).

We therefore focused on *n*-propanol and lowered its concentration in SDS/NaOH to 20 or 30 %. This led to the disappearance of specific PrP immunostaining both with and without PK treatment after incubation for 5 or 10 min (Fig. 1c, d). Obviously, lowering the concentration of *n*-propanol in SDS/NaOH to 20 or 30 % avoided the effects of the alcoholic component that fixed proteins and restored the efficacy of wire decontamination to the level previously observed for SDS/NaOH without alcohol (Lemmer *et al.*, 2004). Thus, in the next step, we validated the prion disinfecting activity of SDS/NaOH in 20 or 30 % *n*-propanol by steel wire bioassays.

Table 3. Efficacy of the tested disinfectant formulations on spores of *A. niger* in a quantitative carrier test with coagulated blood

Reduction factors (RF) are indicated and were calculated as specified for Table 1. All incubations were carried out for 20 min at 20 °C.

Formulation	Concentration	*A. niger* RF
SDS/NaOH	0.2 %/0.3 %	<0.5
Ethanol	50 %	<1
SDS/NaOH in ethanol	0.2 %/0.3 % in 50 %	⩾5
n-Propanol	20 %	<1
SDS/NaOH in *n*-propanol	0.2 %/0.3 % in 20 %	⩾5

Bioassay of the disinfection of steel wires contaminated with 263K scrapie agent. For bioassay validation, we used a recently published *in vivo* carrier assay (Lemmer *et al.*, 2008). Contaminated wires were implanted intracerebrally into hamsters after reprocessing in the test formulations and monitored for their potential to trigger clinical or subclinical (i.e. asymptomatic) infection within an observation period of 500 days. The animals of the positive-control group challenged with implanted contaminated wires that had been rinsed in dH_2O developed terminal scrapie after a survival time of 86 ± 3 (mean $\pm$ SD) days. According to our previously established dose–response relationship (Lemmer *et al.*, 2008), this confirmed an initial infectivity load of $\geqslant 3 \times 10^5$ $LD_{50i.c.imp}$ (50 % lethal dose following intracerebral implantation) per wire. In contrast, all animals that received wires treated with SDS/NaOH containing 20 or 30 % *n*-propanol

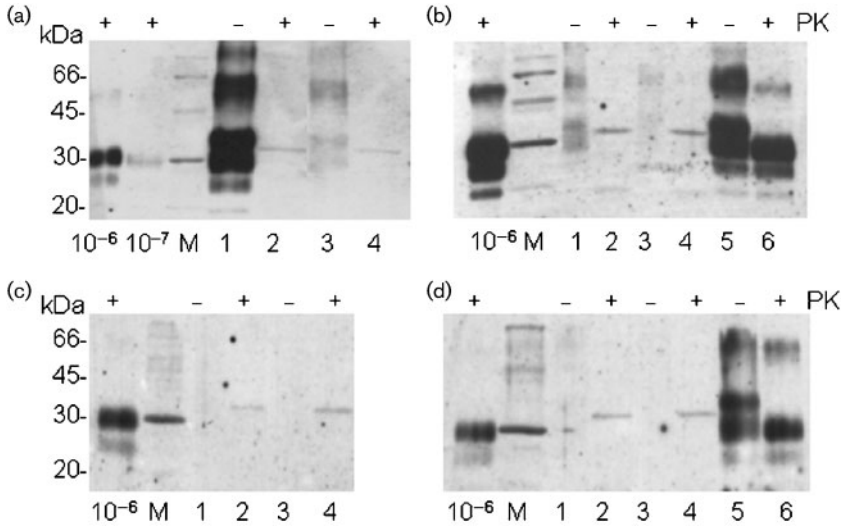

Fig. 1. Efficacy of formulations containing 0.2 % SDS and 0.3 % NaOH in ethanol or *n*-propanol for decontamination of steel surfaces from PrP^{TSE} of 263K scrapie agent. Western blot detection of full-length PrP and PrP27–30, the PK-resistant core of PrP^{TSE}, in eluates from contaminated steel wires after incubation in SDS/NaOH formulations containing 50 % ethanol (a) or 50, 30 or 20 % *n*-propanol (b–d, respectively) with (+) or without (–) PK digestion. (a, b) Lanes 1–4 represent protein eluates from 30 contaminated wires incubated for 5 min (lanes 1 and 2) or 20 min (lanes 3 and 4) at 23 °C in the different formulations. Samples not subjected to PK digestion (lanes 1 and 3) corresponded to 46.2 mm^2 of wire surface, and PK-treated samples (lanes 2 and 4) corresponded to 23.1 mm^2 of wire surface. In (b), lanes 5 and 6 contain eluates (diluted 1 : 10 in sample loading buffer/urea) from 30 contaminated wires incubated in dH_2O for 20 min at 23 °C. These samples corresponded to 4.62 mm^2 (lane 5) and 2.31 mm^2 (lane 6) of wire surface. (c, d) Lanes 1–4 represent protein eluates from 30 contaminated wires incubated for 5 min (lanes 1 and 2) or 10 min (lanes 3 and 4) at 23 °C in the different formulations. Samples not subjected to PK digestion (lanes 1 and 3) corresponded to 46.2 mm^2 of wire surface, and PK-treated samples (lanes 2 and 4) corresponded to 23.1 mm^2 of wire surface. In (d), lanes 5 and 6 contain eluates (diluted 1 : 10 in sample loading buffer/urea) from 30 contaminated wires incubated in dH_2O for 20 min at 23 °C. These samples corresponded to 4.62 mm^2 (lane 5) and 2.31 mm^2 (lane 6) of wire surface. Lanes labelled 10^{-6} or 10^{-7} are internal standards of PK-digested brain homogenate from scrapie hamsters corresponding to 1×10^{-6} g or 1×10^{-7} g brain tissue. Lane M, molecular mass marker.

remained free of clinical scrapie symptoms until termination of the experiment at 503 days after wire implantation (Table 4; a total of $n=5$ animals was excluded from the titration assay due to death unrelated to scrapie). No subclinical infection could be detected by paraffin-embedded tissue blotting for cerebral PrP^{TSE} in any of the animals that survived without scrapie symptoms until 503 days post-implantation (not shown). These findings indicated a reduction in infectivity of $\geqslant$5.5 logs. Thus, the addition of 20 or 30 % *n*-propanol did not compromise the prion disinfecting activity previously observed for SDS/NaOH alone (Lemmer *et al.*, 2008).

Decontamination of steel wires from PrP^{TSE} of patients with sCJD and vCJD. In order to examine the effect of SDS/NaOH containing 20 or 30 % *n*-propanol on human PrP^{TSE} *in vitro*, we used steel wire grids coated with brain homogenates from patients with sCJD or vCJD as test carriers. For contamination of grids with sCJD material, brain homogenate from a patient with the most frequent subtype of sCJD (MM1, i.e. PrP^{TSE} type 1, homozygous genotype for methionine at codon 129 of the prion protein gene; Heinemann *et al.*, 2007) was used. By using steel wire grids (which provided a larger surface for PrP^{TSE} binding than steel wires) as test carriers, we were able to monitor depletion of PrP^{TSE} over a range of 3.3 logs (Fig. 2a, lanes 1–5 and Fig. 2b, lanes 1–4). With this modified *in vitro* assay, no residual immunostaining for PrP^{TSE} could be detected after processing in the SDS/NaOH solution containing 20 % *n*-propanol (Fig. 2a, lane 6 and Fig. 2b, lane 5) for 20 min (sCJD) or even just 5 min (vCJD). This indicated at least a 2000-fold reduction in the PrP^{TSE} load on the carriers by this formulation. However, when the SDS/NaOH mixture used for decontamination contained 30 % instead of 20 % *n*-propanol, a residual signal of sCJD-associated PrP^{TSE} was observed (Fig. 2a, lane 7), indicating binding or stabilization of this specific form of PrP^{TSE} by the higher concentrated alcohol, which did not occur with vCJD material (Fig. 2b, lane 6). This binding/stabilizing effect could be omitted by incubation of the sCJD brain homogenate on the grids for 20 min in SDS/NaOH alone (Fig. 2a, lane 8), with (or without) subsequent treatment, again for 20 min, in SDS/NaOH containing 30 % *n*-propanol (Fig. 2a, lane 9).

Table 4. Bioassay for prion infectivity on wires coated with 263K scrapie brain homogenate: findings after reprocessing in formulations of 0.2 % SDS/0.3 % NaOH containing 20 or 30 % *n*-propanol

For contamination, wires were incubated in 150 µl 10 % 263K scrapie brain homogenate, providing an initial infectivity load of $\geqslant 3 \times 10^5$ $LD_{50i.c.imp}$ per wire. Survival times until the development of terminal scrapie are provided in days post-implantation (p.imp.; mean ± SD), and survival times in bold (>500 days p.imp.) refer to hamsters that were sacrificed at the indicated time points without having developed clinical signs of scrapie. Residual wire infectivity was deduced from attack rates and survival times using a dose–response relationship previously established in an end-point titration experiment (Lemmer *et al.*, 2008). Titre reductions were calculated by comparing the residual infectivity with the contamination load prior to decontamination. Bioassays were carried out in duplicate (bioassay groups 1 and 2). All incubations were carried out for 10 min. ND, Not determined; Res. inf., residual infectivity; RT, room temperature; UD, undetectable.

Formulation	Concentration	Temperature (°C)	Bioassay group 1				Bioassay group 2			
			Attack rate	Survival time (days p.imp.)	Res. inf. per wire ($LD_{50i.c.imp}$)	RF	Attack rate	Survival time (days p.imp.)	Res. inf. per wire ($LD_{50i.c.imp}$)	RF
None: rinsed in dH_2O only	–	RT	3/3	86 ± 3	$\geqslant 3 \times 10^5$	–*	ND	ND	ND	ND
SDS/NaOH in *n*-propanol	0.2 %/0.3 % in 20 %	23	0/4†	**503**	UD	⩾5.5	0/6	**503**	UD	⩾5.5
SDS/NaOH in *n*-propanol	0.2 %/0.3 % in 30 %	23	0/5‡	**503**	UD	⩾5.5	0/4†	**503**	UD	⩾5.5

*A reduction due to rinsing in dH_2O could not be quantified in our experimental setup.
†Two out of six challenged animals died for reasons unrelated to scrapie; accordingly, the number of animals in the respective groups was set to $n=4$.
‡One out of six challenged animals died for reasons unrelated to scrapie; accordingly, the number of animals in the group was set to $n=5$.

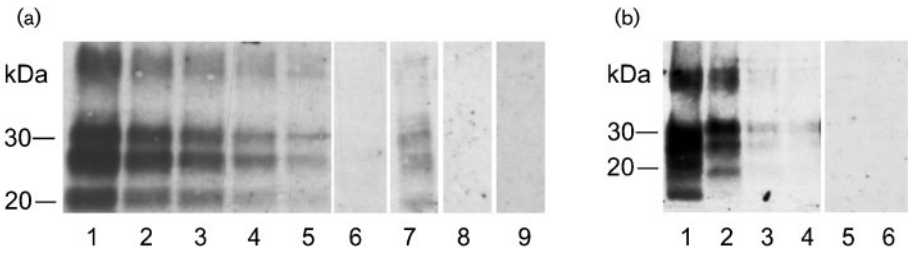

Fig. 2. Efficacy of formulations containing 0.2 % SDS, 0.3 % NaOH and *n*-propanol for the decontamination of steel surfaces from PrP^{TSE} of CJD or vCJD agent. Western blot detection of PrP27–30, the PK-resistant core of PrP^{TSE}, in eluates from contaminated steel wire grids after incubation in the formulations and PK digestion. (a) Findings for PrP^{TSE} from sCJD (type MM1) brain homogenate. Lanes 1–5, dilution series of protein eluate from a contaminated steel wire grid (25 µl out of a total sample volume of 400 µl per grid was diluted 1 : 100, 1 : 300, 1 : 500, 1 : 1000 and 1 : 2000, respectively). Lanes 6–8, protein eluates from contaminated grids incubated for 20 min at 23 °C in the following formulations: SDS/NaOH in 20 % *n*-propanol (lane 6), SDS/NaOH in 30 % *n*-propanol (lane 7) and SDS/NaOH without alcohol (lane 8). Lane 9, protein eluate from a grid incubated initially for 20 min at 23 °C in SDS/NaOH without alcohol, and subsequently for a further 20 min at 23 °C in SDS/NaOH in 30 % *n*-propanol. Samples were digested with PK (20 µg ml^{-1}) for 20 min, and 25 µl of the different dilutions and test samples were loaded in lanes 1–9. (b) Findings for PrP^{TSE} from vCJD brain homogenate. Lanes 1–4, dilution series of protein eluate from a contaminated steel wire grid (25 µl out of a total sample volume of 400 µl per grid was diluted 1 : 40, 1 : 100, 1 : 1000 and 1 : 2000, respectively). Lanes 5–6, protein eluates from contaminated grids incubated for 5 min at 23 °C in the following formulations: SDS/NaOH in 20 % (lane 5) or 30 % (lane 6) *n*-propanol. Samples were digested with PK (20 µg ml^{-1}) for 10 min, and 4×25 µl of the different dilutions and test samples was successively loaded in lanes 1–6 (with intermittent electrophoresis for concentrating applied material in the stacking gel prior to subsequent loading).

Analysis of fixation of proteins to rough carrier surfaces by the formulations used for disinfection

The formulations were further assessed in a qualitative assay for effects that fix proteins to rough test surfaces. For this purpose, residual protein from hamster brain homogenate or coagulated sheep blood on frosted glass strips was stained with Coomassie blue after processing in the disinfectant formulations (Fig. 3).

For the blood samples, a visual examination of our assay results revealed that incubation in glutardialdehyde (Fig. 3, lower panels, lanes 1 and 2) or peracetic acid (Fig. 3, lower panels, lanes 3 and 4) resulted in the detection of more coloured organic material on the carrier surfaces than found for water (Fig. 3, lower panels, lanes 11 and 12). The blood sample treated with glutardialdehyde did not show a blue staining, but a red/black staining. NaOCl produced only a very weak blue staining (Fig. 3, lower panels, lanes 5 and 6), and blood-contaminated carriers that were incubated in SDS/NaOH (Fig. 3, lower panels, lanes 7 and 8) or SDS/NaOH containing 20 % *n*-propanol (Fig. 3, lower panels, lanes 9 and 10) did not show a stronger Coomassie blue staining than that observed after incubation with water (Fig. 3, lower panels, lanes 11 and 12).

When hamster brain homogenate was used as the organic contaminant instead of blood, only a faint blue staining was found after treatment in glutardialdehyde (Fig. 3, upper panels, lanes 1 and 2), whilst incubation in peracetic acid (Fig. 3, upper panels, lanes 3 and 4) again produced a strong blue staining signal. With NaOCl or SDS/NaOH, no residual staining was observed (Fig. 3, upper panels, lanes 5 and 6, and 7 and 8, respectively). A weak blue staining of glass carriers after incubation in the formulation of 0.2 % SDS, 0.3 % NaOH and 20 % *n*-propanol (Fig. 3, upper panels, lanes 9 and 10) indicated residual binding of brain homogenate proteins to the carrier surface, but, as for blood, this formulation did not produce a stronger Coomassie blue staining than that observed with water (Fig. 3, upper panels, lanes 11 and 12). Similarly processed non-contaminated glass strips consistently produced negative results (Fig. 3, upper and lower panels, lane 13).

DISCUSSION

Efficacy of tested formulations for broad-range disinfection

We found that a formulation of 0.2 % SDS and 0.3 % NaOH in 20 % *n*-propanol (pH 13.0 ± 0.05) exerted a strong disinfecting activity against bacteria (including mycobacteria), fungal spores, non-enveloped viruses and prions. This formulation was also effective in the presence of challenging substrates for disinfection such as dried brain homogenate or coagulated blood.

Whilst SDS/NaOH (pH 12.8) alone (Lemmer *et al.*, 2004) was virtually inactive on *Enterococcus faecium* and *M. avium*, these pathogens were efficiently inactivated by the

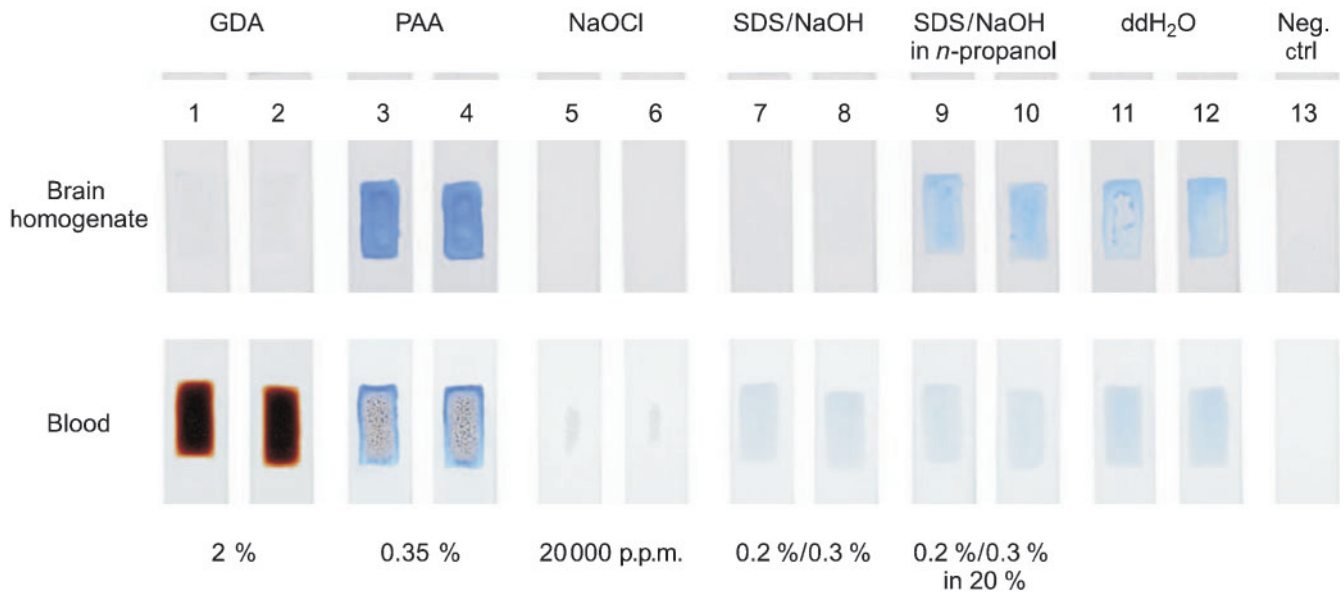

Fig. 3. Protein binding to rough carrier surfaces by various formulations used for disinfection applied to 10 % (w/v) dried hamster brain homogenate (upper panels) or coagulated sheep blood (lower panels). Contaminated frosted glass strips were tested in duplicate for residual protein by Coomassie blue staining after treatment for 30 min at 20 °C in 2 % glutardialdehyde (GDA, lanes 1 and 2), 0.35 % peracetic acid (PAA, lanes 3 and 4), sodium hypochlorite (NaOCl) with 20 000 p.p.m. available chlorine (lanes 5 and 6), formulations of SDS/NaOH (lanes 7 and 8) and SDS/NaOH in 20 % *n*-propanol (lanes 9 and 10) and ddH_2O (lanes 11 and 12). Lane 13, non-contaminated glass strip incubated for 30 min in ddH_2O (negative control).

formulation containing 20 % *n*-propanol. In contrast, 20 % *n*-propanol alone did not exert a relevant inactivating effect on PV, HAV or caliciviruses (including MNV), whereas in combination with SDS/NaOH effective inactivation of these viruses was observed. Although vegetative forms of fungi are usually quite sensitive to conventional disinfectants, spores of *A. niger* are more tolerant (Rutala *et al.*, 2008). However, we observed a pronounced synergistic effect of SDS/NaOH and 20 % *n*-propanol against spores of *A. niger*.

The presence of 20 % *n*-propanol did not compromise the activity against 263K scrapie prions previously established for SDS/NaOH alone (Lemmer *et al.*, 2004, 2008). The latter finding is of particular importance, as alcohols may potentially bind proteins to metal surfaces (Prior *et al.*, 2004), stabilize PrP^{TSE} and enhance the tolerance of prions to inactivation (Taylor, 1999). In cases where higher alcohol concentrations would be required in disinfectant formulations for specific purposes, our findings suggest that adverse effects that fix or stabilize PrP^{TSE} could be avoided by a two-step procedure: initial treatment in SDS/NaOH alone and subsequent incubation in formulations containing SDS/NaOH as well as the higher concentrated alcohol.

By assessing the decontamination of steel wire grids contaminated with human brain homogenates from a patient with the most frequent subtype of sCJD (PrP^{TSE} type 1, homozygous for methionine at codon 129 of the PrP gene), which accounts for about 63 % of the sCJD cases in Germany (Heinemann *et al.*, 2007), or from a patient with vCJD, a reduction of least 2000-fold in the PrP^{TSE} load was demonstrated for SDS/NaOH in 20 % *n*-propanol. This is consistent with the data observed in our study for the disinfection of steel wires from PrP^{TSE} of 263K scrapie agent. Based on recent findings, it has been recommended that any prion inactivation procedures should be validated by bioassay against the prion strain for which they are intended to be used (Giles *et al.*, 2008). This would require comprehensive experiments in animals. Furthermore, even the best bioassay models commonly available for vCJD, familial CJD or the six different forms of sCJD (Heinemann *et al.*, 2007) appear suboptimal for this purpose in terms of their sensitivity and the rather limited ranges of titre reduction they would allow to be demonstrated. In order to promote the further development of effective disinfectants, it might therefore be helpful to compare and validate candidate formulations against human prions with a focus on the most relevant human TSEs such as sCJD/subtype MM1 or vCJD. Additionally, alternative techniques such as protein misfolding cyclic amplification (Castilla *et al.*, 2006; Jones *et al.*, 2007) or novel cell culture assays may be considered as potential surrogate methods for bioassays in animals.

Protein binding to surfaces by formulations used for disinfection

We performed a qualitative assessment of whether our new disinfectant formulation fixed organic contaminants containing proteins to carrier surfaces, and whether the formulation did this more, or no more, than water or conventional disinfectants such as peracetic acid. In order to mimic challenging conditions in terms of protein retention, frosted glass strips with a rough surface were used as test carriers in this assay.

By visual examination, our Coomassie blue assay indicated that glutardialdehyde and peracetic acid fixed blood proteins to carrier surfaces, compared with cleaning by water alone, which is in accordance with previously reported findings using metal carriers (Kampf *et al.*, 2004). In contrast, blood-contaminated carriers that were incubated in the formulation of 0.2 % SDS, 0.3 % NaOH and 20 % *n*-propanol did not show a stronger Coomassie blue staining than that observed after cleaning with water.

When brain homogenate was used as the organic contaminant instead of blood, incubation in peracetic acid again produced a strong staining signal with Coomassie blue, whilst no staining was found after treatment in glutardialdehyde. As glutardialdehyde is well known for its protein-fixing properties (Kampf *et al.*, 2004), this finding may indicate that glutardialdehyde interferes with the staining reaction by masking binding sites for Coomassie blue. Such potentially interfering effects, which may also explain why the blood samples treated with glutardialdehyde did not show a blue Coomassie stain but rather their original reddish staining, have to be taken into account when interpreting the findings of our assay. However, they do not seem to account for the absence of Coomassie blue staining after processing of brain homogenate on glass strips with NaOCl or SDS/NaOH, as, for these formulations, efficient cleaning of steel wire carriers contaminated with 263K scrapie brain homogenate has already been demonstrated by electron microscopy (Lemmer *et al.*, 2008). Thus, with respect to the observed weak Coomassie blue staining of glass carriers after incubation in the formulation of 0.2 % SDS, 0.3 % NaOH and 20 % *n*-propanol, the most obvious conclusion would be that – compared with SDS/NaOH alone – some protein was fixed to the carrier surface due to the presence of alcohol. It has been reported previously that alcohols can potentially bind protein to metal surfaces (Prior *et al.*, 2004). However, despite this potential adverse effect of alcohols, the formulation of 0.2 % SDS and 0.3 % NaOH in 20 % *n*-propanol was also not found to fix proteins more to carrier surfaces than was observed with water as cleaning reagent when using brain homogenate as organic contaminant.

Practical considerations and outlook

Our findings suggest a mixture of 0.2 % SDS and 0.3 % NaOH in 20 % *n*-propanol as a potent candidate formulation for the general elimination of infectious agents in the routine maintenance of surgical steel instruments (including drills used in dentistry). The components of our

formulation are inexpensive and the mixture is simple to prepare. The solution was found to be effective within 20 min (the uniform exposure time tested on the examined bacteria, viruses and fungi) or even shorter exposure times tested on prions. Compared with standard formulations commonly recommended for prion disinfection (e.g. 1–2 M NaOH or 2.5–5 % NaOCl), our mixture appears as a rather mild reagent with much higher compatibility with steel surfaces, although the alkaline nature of the formulation would still be corrosive in some situations. *n*-Propanol is easily flammable at higher concentrations and is not odourless, but in light of the widespread use of alcohol-based hand and instrument disinfectants in hospitals, these factors do not appear to be as critical. SDS, NaOH and *n*-propanol are potentially toxic; however, our formulation can be handled easily and safely under appropriate conditions of use.

Addressing the disinfection of TSE agents not only in its own right but in combination with conventional pathogens as recently also reported by others (Lehmann *et al.*, 2009) may open new avenues for the effective broad-range disinfection of surgical instruments and heat-sensitive medical devices.

METHODS

Efficacy testing of formulations against bacteria, fungi and viruses

Test organisms and nutrient media. *E. faecium* DSM 2146 (grown in brain heart infusion agar), *M. avium* DSM 44156 (grown in Middlebrook 7H10 agar with oleic acid, albumin fraction V/bovine, glucose, catalase/beef enrichment), *A. niger* ATCC 16404 (grown in malt extract agar); FCV strain F-9 in Crandell Rees feline kidney (CrFK) cells, PV type I LSc-2ab in L20B cells, HAV strain HM 175cyt in RHK cells (all grown in minimum essential medium with Hanks' salt solution), MNV strain S99 in RAW 264.7 cells (grown in modified Dulbecco's minimum essential medium; Park *et al.*, 2007).

Formulations used for disinfection. The formulations used were as follows: (a) 0.2 % (w/v) SDS, 0.3 % (w/v) NaOH; (b) 0.2 % (w/v) SDS, 0.3 % (w/v) NaOH, 50 % (v/v) ethanol; (c) 0.2 % (w/v) SDS, 0.3 % (w/v) NaOH, 30 % (v/v) *n*-propanol; (d) 0.2 % (w/v) SDS, 0.3 % (w/v) NaOH, 20 % (v/v) *n*-propanol; (e) 50 % (v/v) ethanol; (f) 20 % (v/v) *n*-propanol; (g) 2 % (v/v) glutardialdehyde; (h) 0.35 % (v/v) peracetic acid; (i) sodium hypochlorite (NaOCl) solution with 20 000 p.p.m. available chlorine. All formulations were prepared in ddH_2O.

Microbial test suspensions. Subcultured preparations of *E. faecium* were pelleted, washed and diluted in ddH_2O to achieve suspensions containing at least 10^9 c.f.u. ml^{-1}. Subcultured preparations of *M. avium* were pelleted and washed in 0.1 % (v/v) Tween 80 in ddH_2O. After resuspension using a glass/Teflon homogenizer the bacterial solution was adjusted to a concentration of at least 10^9 c.f.u. ml^{-1}.

For *A. niger*, after sporulation, spores were harvested with 0.05 % (v/v) Tween 80 in dH_2O. The fungal suspensions were adjusted to 10^8–10^9 c.f.u. ml^{-1}.

For virus preparations, confluent monolayers of CrFK, L20B, RHK or RAW 264.7 cells in culture flasks were infected with FCV, PV, HAV or MNV, respectively. Supernatants were harvested after 5 days (PV, MNV and FCV) or 14 days (HAV) and concentrated by ultracentrifugation. Infectious titres of virus suspensions were in the range 10^8–10^9 $TCID_{50}$ ml^{-1}.

Quantitative suspension tests. Formulations (a)–(f) were applied to preparations of all of test organisms specified above in an Eppendorf Thermomixer at 20 °C. Suspensions of the respective test organisms and disinfectants were mixed at a ratio of 1 : 10. Suspension tests with PV and HAV were also performed with an additional organic burden of 10 % (v/v) FCS, and all solutions were incubated for 20 min under constant shaking (300 r.p.m.). As controls, suspensions of the test organisms were exposed to ddH_2O only.

For the recovery of bacteria and fungi, after incubation, samples were immediately centrifuged (12000 ***g***, 1 min). The pellets were harvested and resuspended in equivalent quantities of 0.1 M phosphate buffer (pH 7) [also containing 3 % (v/v) Tween 80 for mycobcteria and *A. niger*]. Subsequently, samples were serially diluted 1 : 10 in the respective phosphate buffer. From the undiluted test mixtures and their serial dilutions, 0.1 ml aliquots were spread on nutrient medium or, in the case of enterococci, mixed with nutrient medium (1 ml per Petri dish). Incubation was performed at 36 °C for bacteria and at 30 °C for *A. niger*.

The efficacy of disinfection was indicated by the RF: from the mean number of c.f.u. observed after exposure of the test organisms to the respective disinfectant (log n) and the number of c.f.u. observed for the control samples exposed to ddH_2O (log n_0), the RF was calculated as follows: $RF = \log n_0 - \log n$.

For the recovery of viruses, after incubation for 20 min as described above, 1 : 10 serial dilutions of samples were performed using nutrient cell culture medium as the diluent. Inactivation of viruses was determined using $TCID_{50}$ assays on 96-well plates. One hundred microlitres of the various dilutions was added to confluent monolayers and the cytopathic effects were determined after 5 days (PV, FCV and MNV) or 14 days (HAV). Virus titres were calculated according to the methods of Spearman (1908) and Kärber (1931).

Quantitative carrier tests. Quantitative carrier tests were performed according to a method established at the Robert Koch-Institut, Germany (Peters *et al.*, 1995). Briefly, sterilized frosted glass strips were used as carriers. Suspensions of test organisms were mixed with heparinized sheep blood. Immediately before application to the carriers, Protamin 1000 (Roche) was added to the blood suspensions for coagulation. When coagulation of the blood was accomplished, the carriers were incubated in 10 ml of the different disinfectant formulations. After exposure to disinfectant formulations, any residual contamination of test organisms in blood was carefully harvested from the carriers. Recovery of bacteria, fungi and viruses and calculation of RF values were performed as described for the quantitative suspension tests.

Efficacy testing of formulations against prions

***In vitro* carrier assays with PrP^{TSE}.** Decontamination of steel carriers from PrP^{TSE} of 263K scrapie agent was tested in an *in vitro* carrier assay as described previously for SDS/NaOH (Lemmer *et al.*, 2004). Similar *in vitro* studies on the decontamination of steel carriers from PrP^{TSE} of patients with sCJD or vCJD were performed with the following modifications: stainless steel wire grids (DIN 1.4301; Spörl) measuring approximately 100 × 5 mm were contaminated with 25 % (w/v) brain tissue homogenate from a patient with sCJD (type MM1; kindly provided by W. Schulz-Schaeffer, Universitätsklinikum Göttingen, Germany) or from a patient with vCJD (kindly provided by the CJD Surveillance Unit, Edinburgh, UK). After drying, grids were incubated in 45 ml of the disinfectant formulations or in water for 5 min (vCJD) or 20 min (sCJD). Subsequently, grids were washed

and dried (Lemmer *et al.*, 2004), coiled up and treated with 300 µl PK solution (20 µg ml^{-1}) in TBS/Sarkosyl [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % Sarcosyl] for 10 min (vCJD) or 20 min (sCJD) at 37 °C. After PK digestion, samples were mixed with 100 µl 4 × electrophoresis sample loading buffer, boiled for 10 min and analysed by SDS-PAGE and Western blotting for the presence of PrP^{TSE} using the monoclonal antibody 3F4 as described previously (Lemmer *et al.*, 2004). Unless otherwise specified, 25 µl aliquots were used for Western blotting. The studies on tissue samples from human donors were performed in compliance with informed consent and German legal and ethical regulations.

Bioassays with 263K scrapie agent. The disinfection of steel wires contaminated with 263K prion infectivity was examined *in vivo*. For this purpose, contamination of wires, processing for decontamination in SDS/NaOH containing 20 or 30 % *n*-propanol, bioassays in hamsters using a dose–response relationship established by end-point titration, and paraffin-embedded tissue blotting were performed as described previously (Lemmer *et al.*, 2008). The bioassays on the SDS/NaOH/*n*-propanol formulations were carried out in duplicate (bioassay groups 1 and 2). The studies in animals complied with German legal regulations and were approved by the responsible ethics committees and regulatory authorities.

Analysis of fixation of proteins to rough surfaces by the formulations used for disinfection. We examined whether formulations (a), (d), (g), (h) and (i) – as compared with water – additionally fixed proteins to rough test surfaces, using frosted glass strips as carriers. The carriers were contaminated with 10 % (w/v) 263K scrapie brain homogenate or sheep blood capable of coagulation (see Quantitative carrier test above). After contamination, carriers were separately immersed in 10 ml each of the different disinfectant formulations at 20 °C for 30 min. After rinsing with dH_2O, the glass strips were immersed at room temperature for 45 min in 15 ml Coomassie blue solution. Finally, the glass strips were rinsed four to five times in destaining solution [50 % (v/v) methanol, 10 % (v/v) acetic acid in dH_2O]. For each series of stainings, a non-contaminated glass strip was processed similarly as a negative control for comparison.

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