

Original Article

Protein residuals on reusable medical devices and patient safety impact

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Abstract

Cleaning is an essential step to ensure the safe processing of reusable medical devices. In recent years, national and international standards and guidelines have matured to define scientifically sound analytical requirements to define endpoints for cleaning. Although these requirements can be based on what is practically achievable in clinical practice, it is important to ensure that cleaning endpoints are scientifically based to ensure patient safety. The impact of protein cleaning endpoint concentrations at or greater than $6.4 \mu\text{g}/\text{cm}^2$, the current recommended cleaning level, in posing a toxicity or immune risk to patients has not previously been evaluated. This study investigated the impact of residual protein levels on patient toxicity and concluded that an acceptable level of protein in cleaning efficacy studies to be in the 3-6.4 $\mu\text{g}/\text{cm}^2$ range.

Introduction

Reusable device processing can be defined as activities to prepare a new or used device for its intended use. Processing steps can include cleaning, disinfection, and/or sterilization, depending on the device criticality. The types and levels of residual clinical soil (e.g., blood, tissue, bone, mucus) found on these devices following patient procedures can vary considerably depending on their clinical and surgical use (Table 1). To better characterize the challenge for reprocessing these devices an assessment of the biochemical levels on devices was first studied in detail by Alfa et al (2), which focused on flexible endoscopes before and after detailed manual cleaning of the instrument suction channels of various endoscopes. This, and other similar studies (1, 3) have subsequently guided those interested in validating methods for processing reusable medical devices to choose protein as the most common biochemical marker, and the re-

sidual protein level acceptance criteria has been established in certain standards and guidance documents to be $<6.4 \mu\text{g}/\text{cm}^2$ regardless of clinical use (2, 4). However, in some cases, more stringent levels of protein have been recommended based on practical experience in clinical practice ($\leq 100 \mu\text{g}/\text{device}$, depending on the device side; 5, 6) or based on reducing the risk of prion contamination ($5 \mu\text{g}$ BSA equivalent per instrument side; 7).

Keywords

- protein
- cytotoxicity
- residuals
- cleaning
- reusable device
- endpoints
- tests
- acceptance criteria
- toxicity

Although such requirements are useful, they should consider overall patient safety. Residual protein remaining on a device before patient use presents several patient safety concerns. First, residual protein buildup may prevent the effectiveness of the disinfection or sterilization process. Second, the patient may experience an adverse immune response to residual protein. A further consideration is the risk of prion contamination and transmission potential on surgical devices, but this may not be assumed to be achieved by focusing on detectable protein reduction alone (8).

Studies have shown that various chemical and physical methods used for the disinfection or sterilization processing of reusable medical devices are effective even under visual soil conditions, so the first safety concern for residual protein has been well characterized. For example, disinfectants and sterilization processes are often tested in the presence

of visual soil in accordance with various standards, such as 5-10% serum or BSA (9) or 3g/L BSA + red blood cells (10). In both cases, these levels of visual soil would be greater than $\sim 50 \mu\text{g}/\text{cm}^2$ (the approximate visual detection level of protein on device surfaces). It is, however, important to note that disinfection or sterilization cannot be assumed to be achieved on devices with visual clinically soiled surfaces and soil build up in certain areas of devices can lead to ineffective penetration of these antimicrobial processes.

To address the second patient safety concern, a study was conducted to investigate the impact of residual protein levels on patient toxicity. There has been a lack of consideration in this area to understand the risk of pro-

tein concentrations at or greater than $6.4 \mu\text{g}/\text{cm}^2$ initiating an immune response caused by acute toxicity. A sensitive test system (cytotoxicity using mouse L-929 cells) was used to evaluate patient safety under various protein exposure conditions. Within the study, proteins were selected that would be considered clinically relevant to patient safety.

Materials & Methods

Protein selection and preparation.

Four (clinically relevant for surgical devices) human blood proteins were selected with various cell toxicity profiles (summarized in Table 2). A commercially available albumin suspension (*ThermoScientific*), not containing preservatives, was used directly. 5mg

of Horseradish Peroxidase (HRP; *Fisher Scientific*) was dissolved in 1 mL of 0.1 M potassium phosphate to give a $5000 \mu\text{g}/\text{ml}$ stock solution. Human Neutrophil Cathepsin G (*Fisher Scientific*) was prepared by dissolving $50 \mu\text{g}$ in 100 mL 0.88% w/v sodium chloride and 0.42% w/v sodium acetate, to yield a $500 \mu\text{g}/\text{ml}$ stock solution. These protein solutions were supplemented with 5% human serum. The serum was harvested from fresh human donor blood, by allowing it to clot and centrifuging (at 2000G) to recover. The protein/serum solution was then diluted in commercially available minimal essential media (MEM), to give concentration curves for each protein ranging from 190 to $1.9 \mu\text{g}/\text{ml}$.

Table 1: Estimation of residual soil levels on reusable medical device surfaces after clinical use with recommended cleaning endpoints.

Device contamination following clinical use ¹	Analyte and average contamination/cm ² of the test devices			
	Bacteria (log ¹⁰ /cm ²)	TOC (μg/cm ²)	Protein (μg/cm ²)	Hemoglobin (μg/cm ²)
Surgical devices	-0.5	52	244	18
Flexible colonoscopes	4.9	40	37	6
Recommended Cleaning Endpoints²	N/A	12	6.4	2.2

¹ Based on Cloutman-Green et al (1) and Alfa et al (2), ² Action level based on draft ISO 15883-5 (2017).

Table 2: Proteins evaluated in this study.

Protein	Toxicity Characteristics
Albumin (or bovine serum albumin, BSA)	Albumin is one of the main proteins found in blood plasma and is designed to bind water (among other substances) to regulate the oncotic pressure of blood. the reference protein used in protein detection assays. It is representative of a benign protein and characterized to not have any toxic or harmful effects to humans, due to lack of biochemical reactions, but can be harmful to cells by causing dehydration (22).
Horseradish Peroxidase (HRP)	Horseradish peroxidase is a protein characterized as an eosinophil peroxidiser. Eosinophils are a type of white blood cell, part of the body's immune system. When presented to the body in elevated concentrations these proteins are known to cause tissue toxicity (22).
Cathepsin G, Human Neutrophil	Neutrophils are found in the white blood cells in humans and are another essential part of the immune system. These proteins are linked to acute inflammation at the injury site and are first responders for targeting pathogens. They are also implicated in certain connective tissue diseases (22).
Cobra Venom Factor (CVF)	Complement proteins are prevalent in blood, and an essential part of the human immune system. CVF is comprised of a three-chain protein which resembles the complement protein C3b functionally. It is used as the catalyst for initiating the complement activation cascade in hemocompatibility assays and combined with IgG or IgM can cause severe toxicity (23).

During initial testing using an albumin stock with a preservative (e.g., sodium azide), some cytotoxicity was observed but this was entirely due to the presence of the preservative. Without the preservative, albumin, a benign protein, would need to be present at extremely high concentrations to demonstrate a cytotoxic result.

As cobra venom factor mimics the protein C3b, the catalyst protein to initiate the human immune system, it was evaluated to show the probability of residual protein resulting in complement activation in a patient. For a cytotoxicity response in a patient to occur, all other proteins needed in the complement activation cascade would also have to be present to yield a toxic reaction. During this study, despite supplementing with 5% human serum as a source of other proteins, only in 20% human serum and high concentrations of cobra venom factor yield a cell toxicity reaction (Table 3). It was therefore, concluded, a complement protein such as Cobra Venom Factor should not be evaluated for toxicity levels using the concentration curve bracketing the protein residual acceptance criteria for reusable medical devices.

Cytotoxicity testing.

Mouse L-929 cells were seeded on a 6 well plate (Corning) and allowed to set for 24 hours under condition (37°C in-

cupation). The protein dilutions were transferred to the L-929 mouse cell pre-seeded wells with 0.5ml of dilution per well and incubated at 37°C. All tests were conducted at minimum in duplicate, with negative (media) and positive (latex) controls. Cells were evaluated after incubation of 48 hours at 37°C and scored for percentage of cell death via acceptance criteria outlined in ISO 10993-5 (11; see Figure 1).

Estimation of protein concentration on device surfaces.

By directly inoculating the test system with a known amount of protein (in $\mu\text{g}/\text{ml}$) above and below the protein acceptance level of $6.4 \mu\text{g}/\text{cm}^2$ the toxicity profile of each protein was challenged. The unit conversion from $\mu\text{g}/\text{cm}^2$ to the delivered units of $\mu\text{g}/\text{ml}$ was calculated using the assumption that most devices will be extracted in a typical volume of $3\text{cm}^2/\text{ml}$. For example, to achieve the delivered dose of $20 \mu\text{g}/\text{cm}^2$ the test sample would be spiked with 1mL of protein at a concentration of $60 \mu\text{g}/\text{ml}$. The estimated levels of protein tested as well as extrapolated protein levels per device surface area are compared in Figure 2.

Results

Four proteins were evaluated over a range of concentrations using the ac-

ceptance criteria outlined in ISO 10993-5 for cytotoxicity (11). In accordance with this standard, any test yielding a cytotoxicity result of 3 or greater (> 50% of cell death) would be considered a toxic result and be considered a risk to patient safety. The results of this study are summarized in Table 3.

The protein albumin showed no reaction with the cells as all scores for the cytotoxicity assay were zero. Horseradish peroxidase, used to mimic eosinophils (types of white blood cells), showed some cytotoxic behavior at $20 \mu\text{g}/\text{cm}^2$ (two test sample yielding a cytotoxicity score of 1 with the third sample yielding a score of 2) with severe toxicity presenting at approximately $60 \mu\text{g}/\text{cm}^2$. The concentration at which the cytotoxicity test should start to see a failing score of 3 was estimated to be $\sim 48 \mu\text{g}/\text{cm}^2$ calculated by using a linear regression analysis. Cathepsin G was hypothesized to be more cytotoxic than other proteins common in a blood soil, as it is a neutrophil-based protein (protease). This hypothesis was proved to be correct as severe toxicity (cytotoxicity score of 4) at $10 \mu\text{g}/\text{cm}^2$ was observed. Toxicity was not observed in concentrations of $4.0 \mu\text{g}/\text{cm}^2$ or below. Although this protein is considered a greater risk, using a linear regression of the curve it can be estimated that the concentration

Table 3: Cytotoxicity of various proteins evaluated. Cytotoxicity scoring is summarized in Figure 1, where <2 is considered non-cytotoxic.

Estimated Protein Concentration ($\mu\text{g}/\text{cm}^2$)	Observed Cytotoxicity Score for Proteins Tested						
	$63\mu\text{g}/\text{cm}^2$	$20\mu\text{g}/\text{cm}^2$	$10\mu\text{g}/\text{cm}^2$	$6\mu\text{g}/\text{cm}^2$	$4\mu\text{g}/\text{cm}^2$	$1.9\mu\text{g}/\text{cm}^2$	$0.63\mu\text{g}/\text{cm}^2$
Albumin (3 replicates)	0	0	ND ¹	0	0	0	0
Horseradish Peroxidase (3 replicates)	4	1-2 ²	ND	0	ND	0	0
Cathepsin G Human Neutrophil (2 replicates)	ND	ND	4	ND	0	0	ND
Cobra Venom Factor (6 replicates)	0 ³	0	ND	0	ND	0	ND

¹ ND - Not Determined

² Two test samples yielding a cytotoxicity score of 1 with the third sample yielding a score of 2

³ Cytotoxicity (score = 3) observed when supplemented with 20% serum only.

at which the cytotoxicity test would fail to be $\sim 8 \mu\text{g}/\text{cm}^2$, which is above current acceptance criteria for protein residual in a cleaning efficacy test.

Overall, of the 4 different types of proteins evaluated, all required a level above $8 \mu\text{g}/\text{cm}^2$ to show a cytotoxic result. The results of this study suggest the level of protein required to initiate a cytotoxic reaction in a patient would be greater than the recommended cleaning level of $\leq 6.4 \text{ mg}/\text{cm}^2$.

Discussion

Cleaning is an essential step to ensure the safe processing of reusable medical devices. In recent years, national / international standards and guidelines have matured to define analytical acceptance criteria to define endpoints for cleaning. Although these requirements can be based on what is practically achievable in clinical practice, it is important to ensure that cleaning endpoints are scientifically based to ensure patient safety. For this purpose, cleaning processes and the levels of acceptable residuals on a device should meet at least two patient safety criteria: levels of residuals should not interfere with subsequent disinfection or sterilization processes being used, and any residuals should not pose a toxicity risk to patients.

With very few (if any) exceptions, reusable medical devices will encounter protein during clinical use (1-3), and protein has been described as being present at significant levels on reusable medical devices after patient (in particular surgical) use (1). Even devices such as lower gastrointestinal endoscopes that are found to be contaminated with high concentrations of microorganisms also present with high concentrations of protein after clinical use (noting that such microorganisms are a significant source of protein themselves; 2, 3). For this reason, protein and other indicators such as total organic carbon (TOC) that can detect available carbon in proteins as well as other macromolecules present in surgical soils, are ideal biochemical markers to evaluate device cleanliness. But the level of protein that should be acceptable as being safe for subsequent processing steps continues to be an area of some debate.

A detectable level of protein residual at $\leq 6.4 \text{ mg}/\text{cm}^2$ of device surface has

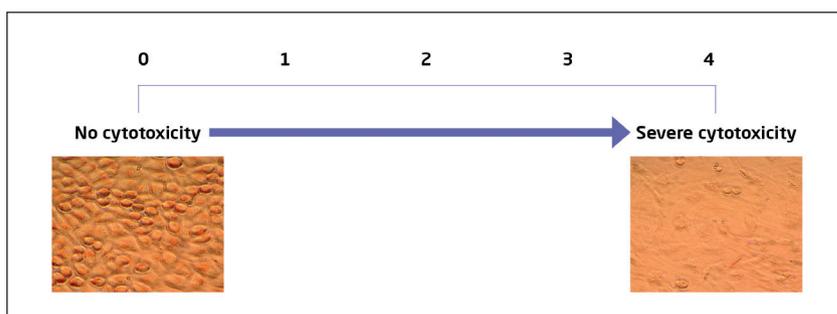


Figure 1: A summary of the cytotoxicity scale, based on the test methodology defined in ISO 10993-5. Note that a score of <2 is typically considered non-cytotoxic (11).

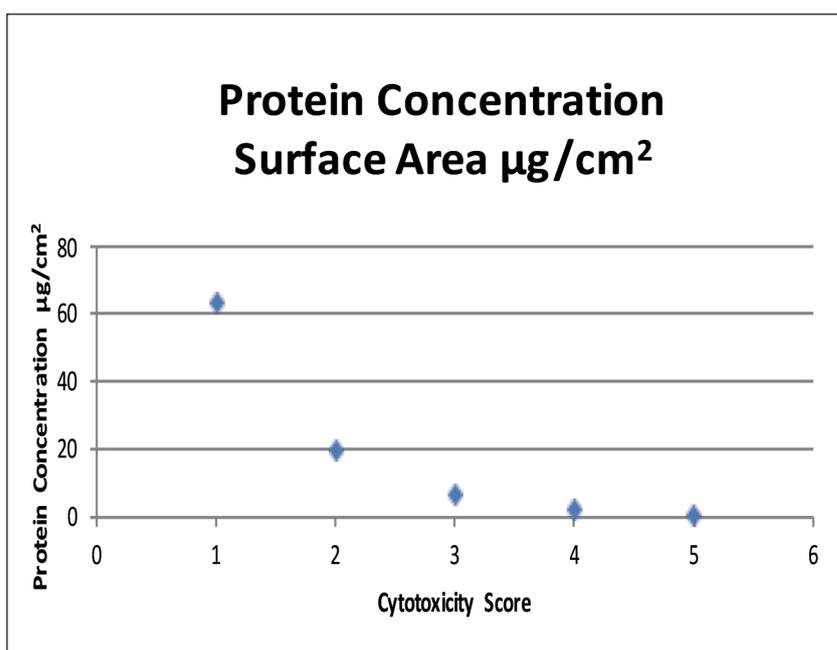
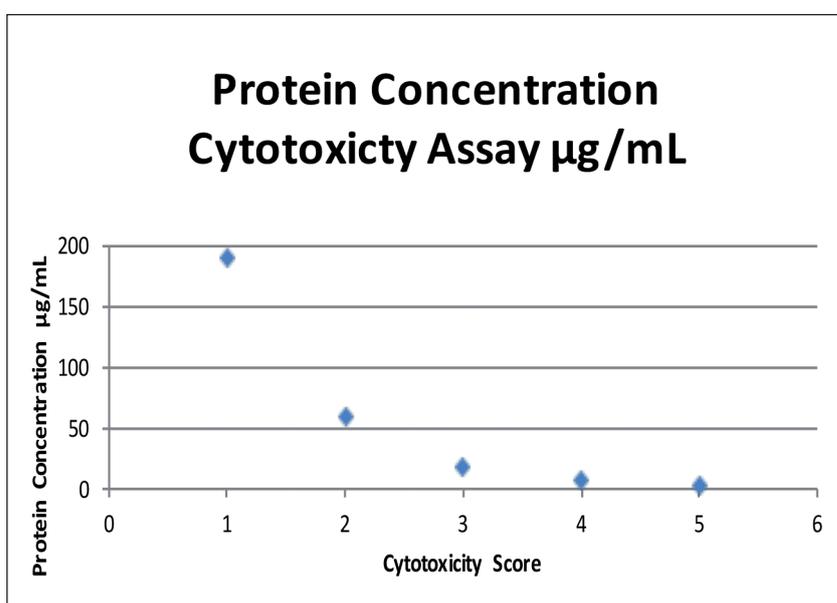


Figure 2: Relationship between the levels of proteins tested in the cytotoxicity assay ($\mu\text{g}/\text{mL}$) and estimated levels on a device surface area ($\mu\text{g}/\text{cm}^2$).

been used for many years as an acceptable level to validate reusable device cleaning efficacy (4,16). This level of protein is significantly below the visibly detectable level of proteins on surfaces ($\sim 50 \text{ mg/cm}^2$) and is expected to have little to no impact on the successful disinfection or sterilization of a reusable device (9,10). For example, in studies testing antimicrobial efficacy of a variety of low temperature sterilization processes in the presence of higher levels of visually detectable soils (10% serum and 0.65% salt) $>6 \log_{10}$ reductions of a variety of test organisms, including bacterial spores, were observed (17). Sterilization processes (including steam-based) have a significant level of overkill built into their validated cycles to overcome low levels of soils that may remain after cleaning. There are some important points to consider in this conclusion. First, it is assumed that the level of protein detected is not all located at one location in or within a device, which could lead to a much greater, localized challenge to the antimicrobial process. It is for this reason that certain country guidelines recommend the determination of the total protein detected per device and define endpoints for such tests (5,6). At a minimum, the likelihood of certain device features being at a high risk of soil retention should be considered in the evaluation of device design for cleanliness and in cleaning validations. Second, it is also assumed that the level of soil does not accumulate over time (e.g., in the device design features discussed above) and therefore does not pose a risk of failed disinfection/sterilization over repeated use of the device.

The level of protein required to initiate a cytotoxic reaction in a patient would be greater than the recommended cleaning level of $\leq 6.4 \text{ mg/cm}^2$, based on the investigations in this report. It is difficult to define and test the many different types of protein that could be considered cytotoxic to humans. The human immune system is designed to protect against a range of foreign contaminants, but it is important to consider the levels of protein defined as a 'clean' endpoint is at least non-toxic. Overall, the demonstration of the lack of significant residuals is a requirement in the demonstration of a successful washer-disinfection process (21), and this can be demonstrated by biocompatibility testing to the requirements of the

requirements in ISO 10993, including cytotoxicity testing (11). This requirement is also proposed in addition to the detection of residual levels of protein and TOC defined in the revised version of ISO 15883-5 (15). This not only covers residual patient tissue material, but also residuals from the cleaning process (e.g., detergents). The cytotoxicity test by MEM elution is arguably one of the most sensitive of the biocompatibility tests. In considering examples of protein toxicity, complement proteins are a part of the human body's immune system and can be activated early upon infection in the absence of antibodies. The activation opsonizes pathogens triggering an inflammatory response in the body, thus fighting infection (6). Since these are not readily digested by the body and are present within the blood, we suspected they would be prevalent on a reusable device after patient use. If introduced into the body from a foreign source, these proteins can become toxic to the host by initiating the body's immune system. In our investigation, the cobra venom factor was used as a surrogate for such proteins and we confirmed that even in this case the levels of residual complement proteins would have to be high to initiate an immune response (Table 3). Although an argument could be made for the toxicity level for each of the proteins used in this study, the results were consistent in that they all showed no cytotoxic effect at or below $6.4 \text{ } \mu\text{g/cm}^2$. Cell death did not start to occur until the concentration of protein reached greater than $8 \text{ } \mu\text{g/cm}^2$, leading to the conclusion that the level of acceptable protein currently defined as $\leq 6.4 \text{ } \mu\text{g/cm}^2$ is an acceptable level.

It is difficult to evaluate the overall impact of the introduction of foreign material into the human body, and particularly into internal tissues. It is known that larger particulates (or visual debris) are difficult for the body's immune system to clear and may lead to adverse patient reaction, such as granuloma or thrombus formation, toxic anterior segment syndrome, and prolonged inflammation (17). In these cases, the residual material is often visual and should require close inspection for detection during inspection and routine monitoring of cleaning processes. But overall, the risks for lower levels of residual soils, including protein, are considered low. Cleaning studies in Germany and other coun-

tries have shown that under well-controlled cleaning protocols that an acceptance level of $\leq 3 \text{ } \mu\text{g/cm}^2$ was routinely achievable, and they proposed acceptable levels for the total detectable levels on specific device types (18). It can therefore be proposed that an acceptable level of protein in cleaning efficacy studies to be in the $3\text{-}6.4 \text{ } \mu\text{g/cm}^2$ range. Some organizations have suggested setting an acceptable level at $\leq 6.4 \text{ } \mu\text{g/cm}^2$ with the expectation to further investigate potential root causes for protein levels between $3\text{-}6.4 \text{ } \mu\text{g/cm}^2$ as protein levels in this range may potentially indicate that features of the device design may be difficult to repeatedly achieve reliable cleaning efficacy over time.

Finally, it may be useful to consider the risk of prions and transmissible spongiform encephalopathies. Prions are transmissible, proteinaceous agents and have been shown to be transmissible on device surfaces (8). Estimates of the levels of prion proteins that would need to be present on surgical devices to allow for disease transmission has been speculated to be several magnitudes below what can be routinely verified by routine protein detection methods (19). But it is well known that the levels of detectable protein do not correlate with prion infectivity (or the ability to transmit disease) (20). So overall, reducing the detectable levels of protein on reusable medical device to below the levels of 6.4 (or even 3) $\text{ } \mu\text{g/cm}^2$ may have little to no impact in reducing the true risks of prion contamination. Indeed, evidence would suggest that specific validated cleaning and sterilization methods that have been shown to reduce the associated risk, even as a universal precaution, is more prudent (8).

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