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RESEARCH ARTICLE

Hydrogel Based Skin Contacting Medical Devices and Cytotoxicity: An Overview of Challenges and Recommendations from a Regulatory Perspective

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Abstract

Background/Objectives : Hydrogels are highly water-swollen polymeric materials with diverse properties and excellent biocompatibility. Despite their immense potential, hydrogel-based medical devices often exhibit a cytotoxic response, which raises major concerns from a regulatory perspective. The focus of this review is on possible reasons that cause such cytotoxicity and furthermore offers recommendations from a regulatory point of view for a successful biocompatibility assessment of medical devices. **Methods:** Hydrogel based medical devices intended for clinical use should demonstrate their biocompatibility in a series of preclinical studies based on their type and duration of body contact, as defined in the ISO 10993-Part 1:2018 standard. As a seminal investigation in the biocompatibility assessment, the devices should be tested for cytotoxicity according to ISO 10993-Part 5:2009 using various methods including tests on extracts, direct and indirect contact methods. **Findings:** This review has summarized various factors that may lead to a cytotoxic response, including sterilization, change in pH, and medium absorption. In this regard, we proposed a stepwise strategy to assess the reason for such a cytotoxic response rather than justifying it with literature and *in vivo* study results. **Novelty:** The factors contributing to a positive cytotoxic response are of paramount importance in the field of hydrogel based medical devices. Understanding these factors is crucial for ensuring the safety and efficacy of such technologies. This review serves as a valuable resource in shedding light on these critical aspects from a regulatory perspective.

Keywords: Medical Device; Hydrogel; Biocompatibility; Cytotoxicity; Risk Assessment

1 Introduction

Hydrogels are a class of highly hydrated three-dimensional polymer network that offers tunable physicochemical and functional characteristics including microarchitecture, porosity, viscoelasticity, degradability and stimulus-responsiveness which are demanded for specific applications. In addition, the hydrogels tend to mimic the natural cellular environment and many tissues. To date, a wide variety of synthetic and biopolymers for the fabrication of hydrogels have been explored by different approaches. The major synthetic polymers used in the fabrication of hydrogels include poly(ethylene glycol), poly(acrylamide), poly(vinyl alcohol) and poly(acrylic acid). On the other hand, a variety of natural polymers including hyaluronic acid, cellulose, collagen, chitosan and alginate have been extensively used in the fabrication of hydrogels[1–3]. Inspired by the excellent features, the hydrogels have emerged as promising candidates for widespread biomedical applications, including drug delivery, wound healing, tissue engineering, cell therapy, surgical tools, device coatings, contact lenses, and biosensors. The schematic representation illustrating the properties, regulated cell activities, the interaction of the hydrogel matrix with cells and signaling cascades, and various biomedical applications of hydrogels can be accessed elsewhere[4].

Despite concrete research efforts, various hydrogels have been approved for clinical applications by the US Food and Drug Administration (FDA) and/or the European Medical Agency (EMA) due to their excellent adjustable characteristics and biocompatibility. In this context, Aswathy et al., Øvrebø et al., and Mandal et al., have reviewed available commercial hydrogels intended for different biomedical applications including contact lenses, wound dressings, drug delivery, cancer care and regenerative medicine[5–7]. Biocompatibility is considered a key feature for all materials intended for biomedical applications to demonstrate their successful function *in vivo* without immune rejection. The biocompatibility of hydrogels is strongly influenced by various factors including surface properties, size and chemical composition. Since the hydrogels are used as medical devices, the biocompatibility of the hydrogels must be demonstrated according to the standard "Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process (ISO 10993-Part 1: 2018)" and applicable endpoint specific standards[8].

The ISO 10993-Part 1:2018 standard categorizes medical devices based on the nature and duration of body contact, which plays a key role in requiring biocompatibility testing to address various *in vitro* and *in vivo* endpoints. The category of medical devices based on the type of body contact includes non-contact, surface contacting, externally communicating and implant medical devices. In addition, based on the duration of body contact, medical devices are classified into devices with limited exposure ($\leq$ 24 hours), devices with prolonged exposure (> 24 hours to 30 days) and devices with a long-term exposure (> 30 days). The hydrogels are extensively used as surface-contacting medical devices in various clinical applications. According to the ISO 10993-Part 1:2018 standard, the surface contacting medical devices are further sub-categorized based on the type of skin contact including intact skin (e.g., electrodes, external prosthesis, fixation tapes, compression bandages and monitoring of various types), mucosal membranes (e.g., contact lenses, urinary catheters, intra vaginal and intra- intestinal devices (stomach tubes, sigmoidoscope, colonoscope, gastroscope), endotracheal tubes, some dental prostheses and orthodontic devices), breached or compromised surfaces (e.g., dressing or healing devices and occlusive patches for ulcers, burns and granulation tissue).

It should be emphasized that the vast amount of surface contacting medical devices uses hydrogels as part of their components that are likely to come into contact with intact skin. For instance, hydrogels are used as sensors or patches on healthy skin to track or identify a patient's health status. In addition, hydrogel based non-invasive blood glucose sensors are used in the field of diabetic diagnosis because the blood glucose level fluctuates abnormally in diabetics due to inadequate insulin secretion or poor insulin action. Consequently, this patch also helps in regular blood glucose monitoring[9]. Furthermore, hydrogel ionic skin sensors are used to measure blood pressure, human movements and complex muscle movements during speech[10]. In addition, the hydrogels are also used in the electronics of epidermal patches electronics to monitor vital signs, body temperature and blood pressure, as well as for the detection of analytes in bodily fluids[11]. Therefore, our review mainly concerns on the hydrogels in surface contacting medical devices that likely to come in contact with intact skin.

As these hydrogel devices are intended to be in contact with intact skin for different time duration, the ISO 10993-Part 1:2018 standard requires devices to be tested for cytotoxicity, skin sensitization, and skin irritation (including intracutaneous reactivity) to determine their biocompatibility demonstrate nature as mandatory requirements for the regulatory process. The cytotoxicity studies in accordance with the standard "Biological evaluation of medical devices — Part 5: Tests for *in vitro* cytotoxicity (ISO 10993-Part 5:2009)" must be conducted as part of the testing requirement, which is considered as a preliminary and sensitive study in the biocompatibility testing[12]. Although the hydrogels have been well proven to have excellent biocompatibility, they also showed severe cytotoxicity when the medical devices were tested as finished devices where the hydrogels are also part of the component that triggers great concerns on the regulatory perspective among the authorities and notified bodies.

The main focus of this review is to summarize the possible factors that induce cytotoxic response of the hydrogel based medical devices and provide some recommendations to overcome the issues. Furthermore, we propose a strategy to assess the

cytotoxicity of the hydrogel-based devices from a regulatory point of view. We believe the review would be of great benefit to the scientific community and researchers interested in developing viable hydrogel based biomedical applications. Since various highlighted factors that can contribute to a positive cytotoxic response, this review could help to better understand the safety and efficacy of hydrogels and provide guidance for the development of safer and more effective hydrogel-based technologies in future.

2 Methodology

In vitro cytotoxicity is the most important endpoint study that ideally needs to be performed to understand the cytocompatibility of the medical devices before starting *in vivo* studies. In 1990, the United States Pharmacopeia (USP) 22 first included the cytotoxicity testing for the purpose of regulatory process[13]. The cytotoxicity could be defined as an adverse effect caused by the medical device that leads to cell death. There are many factors that could induce the cell death including chemical stimuli (when certain materials are exposure to cells [fibroblasts, NK or T cells]) or physical /environmental conditions (radiation exposure, temperature or pressure extremes, etc.)[14]. The cytotoxicity of the medical devices needs to be demonstrated in accordance with the ISO 10993-Part 5:2009 standard which offers three different methods including extraction, direct contact and indirect contact. The different available methods of conducting cytotoxicity are summarized as follows,

2.1 Test on extracts

The main scope of the test is to mimic the clinical application. The test materials are extracted in medium containing 5% serum (as this extraction medium can extract both polar and non-polar compounds). Duration of test item extraction varies based on patient contact duration and the ratio for extraction depends on thickness of the test material[15]. Cell viability following exposure is assessed by proper quantitative assays [NRU assay (detects lysosome function), MTT assay (detects mitochondrial enzyme activity) or XTT assay (measures viability of cells via mitochondria dehydrogenase)] and compared with untreated control. There is no risk of cellular damage (as direct contact) or heat shock (as agar diffusion method)[16].

2.2 Test by direct contact

In this method, the tested materials are placed in direct touch with the cell culture in order to identify any potential toxicity even from weak toxic materials[17]. The direct contact method is highly sensitive and able to detect weak cytotoxicity. The direct contact method ensures that the cells are exposed directly to the agent being tested. It is more likely to provide meaningful data regarding the potential toxicity of the test item. The direct contact method allows both qualitative and quantitative assessment of cytotoxicity. A qualitative assessment of cytotoxicity is made by microscope observation of changes in cell morphology and reactivity zones. The direct contact test involves placing the specimen of the biomaterial on the cell layer in the center of the vessel. Reduction of cell viability by more than 30 % in the highest concentration of Test Item extract-treated culture compared to concurrent vehicle control-treated culture is considered.

2.3 Test by indirect contact

2.3.1 Agar diffusion method

Agar diffusion tests are beneficial for assessing the cytotoxicity of elastomeric closures. In these tests, the agar layer acts as a cushion. The cells are plated onto the culture dishes in a known aliquot. Test material is spread over the solidified agar (covering 10% surface area of the culture flask) after the agar has set. As stated in the previous methods culture vessels are incubated for 24 h to 72 h. The agar protects the cells from any mechanical damage and allows leachable chemicals to diffuse from the product or packaging samples. The cells are then evaluated to determine the toxicity of the samples.

2.3.2 Filter diffusion method

Filter diffusion tests are beneficial for assessing the cytotoxicity of Dental materials. The Millipore (cellulose acetate) filter method modifies the oral contact situation in that primary cells are grown on one side of the filter, and the test material is placed in contact with the opposite surface of the filter. Thus, any leachable substance must diffuse through the 0.45μm filter pores to exert any cytotoxic effects on the cells. Qualitative evaluations of the cells are evaluated after 2 and 24 hours of exposure. A suitable staining process (succinate dehydrogenase staining for specific staining and fluorescein diacetate staining for non-specific staining) is used to make a qualitative assessment of the cytotoxic effects.

2.4 Colony formation assay

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay which is based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. Clonogenic assay is the method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents. Only a fraction of seeded cells retains the capacity to produce colonies. Before or after treatment, cells are seeded out in appropriate dilutions to form colonies in 6- 7 days. Colonies are fixed with methanol, stained with Gimesa Solution and counted using a stereomicroscope.

However, selection of appropriate method plays critical role in the estimation of the toxic effects [18,19].

3 Overview of challenges faced and factors inducing cytotoxicity of hydrogels

Most hydrogel materials that come into contact with intact skin are used non-sterile in patients (e.g. patches or electrodes) and their exposure duration greatly varies depends on the application. There are different factors that trigger the challenge over cytotoxicity evaluation of the hydrogels as given below.

a) Sterility

The first challenge in performing the cytotoxicity experiment is sterility issues. As these patches are non-sterile, they can contaminate the extract and render the extract unusable for the study. The guideline denotes that testing of non-sterile devices must be performed exactly as supplied and handled aseptically until the end of the study. The sterilization of the test material may be warranted to prevent microbial contamination of the cell culture, but the sterilization process must not alter the properties of the test material. If non-sterile test materials are used, testing for bacterial contamination is required since contamination may result in an inaccurate assessment of cytotoxicity.

b) Change in pH

The second challenge is the change in pH, which had a profound impact on the cytotoxicity assessment. The optimum pH of the culture medium is 7.2 to 7.4. The hydrogel can swell and absorb more water/medium, which can change the pH of the medium. The swelling process involves the absorption of liquid by an immersed material. The medium penetrates inside the hydrogel network and interacts with hydrophilic groups, resulting in their dissociation. Therefore, the formation of ions with numerous electrostatic interactions, such as repulsion, increases the distance between the polymer chains and consequently leads to a loosening of the polymer network [20].

c) Manufacturing contaminants

The third challenge is the impurities from the manufacturing of the hydrogels containing medical devices. Certain materials used in the manufacture of medical devices or as processing aids may contain chemicals that could leads to the cytotoxic effects. For example, skin-contacting electrodes with adhesives containing detergents can be expected to have greater than grade 2 cytotoxicity to L929 cells, which might be acceptable if the manufacturer can confirm that there are no other chemical constituents causing the adverse cytotoxic response.

4 Recommendations for overcoming the challenges

According to the FDA guidance, the risk assessment should not only evaluate the materials used in the device, but also the processing of the materials, the manufacturing methods (including the sterilization process) and any residues of manufacturing aids used during the process [21].

The hydrogel synthesis involves addition of different additives that can increase the risk of inducing cytotoxicity. In the past five years, experimental attempts have been made to overcome the sterility problems of the hydrogel. For instance, a pre-treatment experiment on hydrogel using different types of sterilization techniques like exposure to gamma irradiation, and disinfection with 70% ethanol was conducted and found that hydrogel treated with steam sterilization (autoclave) suffered evident morphological degeneration by the effect of the high temperature and pressure and therefore not advisable. Other sterilization techniques such as disinfection in 70% ethanol and gamma irradiation of hydrogel showed no major difference in the cytotoxicity outcome [22].

To date, several proven terminal sterilization methods are available, including moist or dry heat, gamma irradiation, and gas sterilization (e.g. ethylene oxide). These methods can result in changes in the chemical, physical and mechanical properties of the materials, eventually leading to the formation of toxic residues. In addition, ozone gas is a potential agent for use as a sterilant for medical devices because of its known strong oxidative effects [23].

Furthermore, few experimental trials were performed prior to cell culture experiments. The hydrogels were sterilized overnight with 70% v/v ethanol/water, then collapsed at 37°C, washed with PBS, re-sterilized with antibiotic/antimycotic, and washed three times to remove the antibiotic/antimycotic in sterile PBS. The hydrogel was then swollen in the culture medium for subsequent cell seeding [24].

If non-sterile hydrogel extract has been contaminated during sterility testing, this can be communicated to the manufacturer and a repeat experiment performed with filter sterilization using a 0.22 μm filter, stating that the process did not alter the properties of the test material.

During the extraction process of the hydrogel there may be a change in pH which in turn will affect the cytotoxicity results. The positive cytotoxicity result can be due to acidic or alkaline pH. When pH is the only source of positive results, simultaneous testing by changing the pH to a range compatible with the cell line (pH 7.2 to 7.4) can be performed and confirm the result. The result of the study can be reported with both experiments.

5 Strategy for cytotoxicity evaluation of hydrogel based medical devices - a regulatory perspective

An establishment of the testing strategy is extremely essential to meet the regulatory requirements in biocompatibility evaluation of medical devices with hydrogels as the finished devices often exhibit cytotoxic response. In this context, the valuable contributions of service providers in cytotoxicity testing strategy making are extremely valuable as they very often deal with such medical devices and confront failures to successfully meet the regulatory requirements. Accordingly, a stepwise approach to deal with *in vitro* cytotoxicity failures has been described a recently published white paper [25]. In principle, these stepwise approaches could be also be taken in to account when investigating the cytotoxic response shown by the hydrogel based medical devices. A total of six steps needs to be considered from a regulatory perspective as described below.

a) Verification of the accuracy of the test results

As a first step, it is highly important to verify the accuracy of the cytotoxicity test results. In particular, the extraction conditions used in the study, the result of the negative and positive control, the expiration date of the chemicals used and the variances between the technical replicates must be carefully evaluated. Furthermore, it is critical to check whether any special preparations have been forgotten during the extraction process. For instance, the silver/silver chloride (Ag/AgCl) stud portion of the electrocardiogram (ECG) electrode patches was excluded from the extraction as elution of these components could trigger the cytotoxic response. In addition, a deep insight into the post-extraction state needs to be verified, particularly in terms of the presence of particles and a change in pH.

b) Assessing the test parameters

The next step should be to ensure that the test parameters, including extraction vehicle, variations in extraction conditions and method which need to be evaluated for the reasons for the observed cytotoxicity.

c) Examination of the components and materials used in the device

In the third step, an in-depth evaluation of the types of materials and components used in hydrogel based medical devices should be performed, as these factors have significant impacts on the outcome of the cytotoxicity study. It has been well demonstrated in the past that variety of materials, metals and plasticizers possesses cytotoxic potentials. Therefore, if a device consists of suspected components, the cytotoxicity of the hydrogels must be checked by excluding these components or by parallel dilutions with the commercially available comparative device of similar composition.

d) Evaluation of tests and process adjustments

This assessment step mainly focuses on the assessment of the current cytotoxicity data compared to the historical data to understand if such potential effects have been observed in the past and what are the reasons responsible for causing such a cytotoxic response. This assessment also helps to figure out the strategy to overcome or justify such cytotoxic response in biocompatibility assessment in a similar way as in the past. Furthermore, it should also be noted that a historically non-cytotoxic medical devices could also turn out to exhibit cytotoxic response because of change in manufacturing process including packaging and supplier.

e) Experimental tests

If the cause of the cytotoxic response cannot be identified in the previous investigations, further a stepwise experimental trials are needed to progress toward understanding the cause of cytotoxicity. In this aspect, the hydrogel based medical devices could be tested for the cytotoxicity at various stages of the manufacturing and in different batches to gain further insights. In addition, it would be also useful to test different types of hydrogels.

f) Addressing the cytotoxicity failure in a biological risk assessment

In the last step, the cytotoxicity response of the hydrogel based medical devices can be addressed through biological risk assessment if the observed cytotoxic potential does not correlate with any of the previous steps. This biological risk assessment can be written by the experts using supporting information, including discussions of literature, *in vivo*, chemical characterization and post-market surveillance data, showing that no adverse effects are expected if these hydrogels based medical devices are used in clinical applications.

GLR Laboratories Pvt. Ltd. is a pioneer in providing biocompatibility testing for medical devices in India over the past decade. Therefore, the laboratory has extensively evaluated the cytotoxicity potentials of various hydrogel based medical devices over the years according to ISO 10993-Part 5:2009 as a part of the biocompatibility testing process. The laboratory has performed a total of 46 cytotoxicity tests on hydrogel-based medical devices intended for various clinical applications since 2015. To provide an overview of the tests performed in our laboratory, a total of 21 cytotoxicity studies were conducted using Neutral Red Uptake (NRU) assay using BALB/c 3T3 cells as an *in vitro* test system, while 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to determine the cytotoxicity using L929 cells as a test system for 23 studies. Furthermore, each one colony formation assay and agar diffusion assay was performed using V79 and BALB/c 3T3 cells respectively. An overview of the studies conducted at GLR Laboratories Pvt. Ltd. is graphically illustrated in Figure 1. Furthermore, the ISO 10993-Part 12:2021 standard and corresponding previous version has been adopted to choose the sample ratio and extraction condition for cytotoxicity testing[15]. An overview of different sample ratios used for conducting the cytotoxicity studies is shown in Figure 2. Although majority of the cytotoxicity studies performed on the hydrogel based medical devices did not show any cytotoxic potential, in some cases we also had a positive cytotoxic response. However, the failures in the cytotoxicity has been systematically addressed as described previously in the stepwise process for successful biocompatibility evaluation. An example of negative and positive cytotoxicity responses of hydrogel extracts treated in elution method and colony formation assay are shown in Figures 3 and 4.

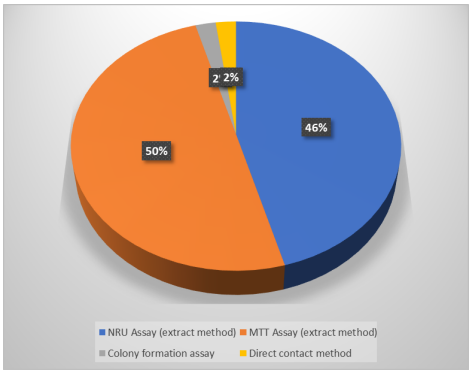

Fig 1. An overview of different types of cytotoxicity studies conducted since 2015 at GLR Laboratories Pvt. Ltd. on various hydrogel based medical devices

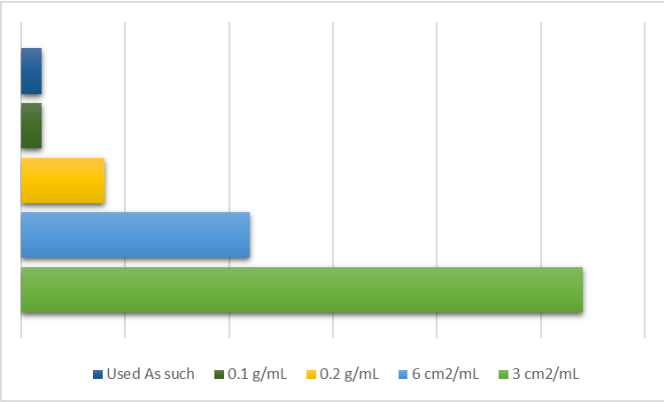

Fig 2. An overview of different sample ratios used for conducting the cytotoxicity studies in accordance with ISO 10993-Part 12:2021 standard

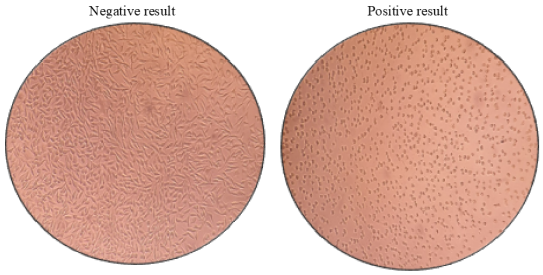

Fig 3. Comparison of hydrogel extract-treated L929 cells that were cytotoxically negative (left image) and positive (right image) after performing extract method. More than 80% viability was observed in negative control image and complete lysis of cells were observed in positive control image

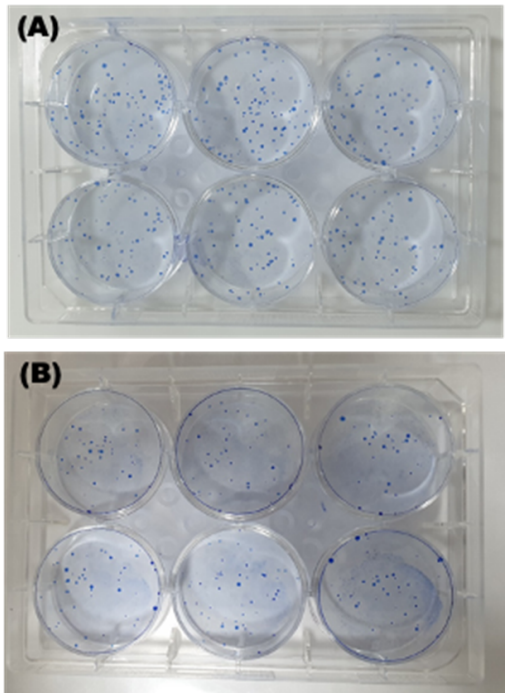

Fig 4. Comparison of hydrogel extract-treated V79 cells that were cytotoxically negative (A) and positive (B) using colony formation method. More than 70% of stained colonies are visible in Negative results plate and reduction in colony count was observed in positive plate

Therefore, it is evident that the stepwise strategy proposed from regulatory perspective is advantageous in addressing the cytotoxicity complications associated with the testing of hydrogel based medical devices. We believe that the proposed strategies would be of benefit to the scientific community addressing such issues in their biocompatibility assessment and manufacturing such hydrogel based medical devices. Furthermore, these strategies could be used to investigate the real reasons for the cytotoxicity instead of merely defending the response based on *in vivo* study results.

6 Conclusion

This review describes the rationale behind the positive cytotoxic response obtained from intact skin contacting hydrogel based medical devices during biocompatibility evaluation and further provides a detailed stepwise strategy to encounter such response from a regulatory perspective. If the cytotoxicity studies performed with known biocompatible hydrogels turned out to show a positive cytotoxic response, this is generally justified compared to *in vivo* studies. However, instead of justifying the cytotoxic response from the *in vivo* studies, a detailed investigation is required as proposed in the review that would help in figuring out

rationale behind the cytotoxic response. The proposed strategy would be beneficial to the scientific community and researchers in developing such hydrogel based medical devices to meet regulatory requirements in biocompatibility assessment.

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