Testing, characterization and regulations of antimicrobial textiles

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Abstract

Nowadays, antimicrobial textile has been widely applied in several sectors, including hospitals and healthcare centres, food industry, clothing industry and in domestic environment. Antimicrobial textiles are particularly used in active patches and dressings for wound healing, infection prevention and control (IPC) articles, deodorization and anti-fungi clothing, among other applications. This chapter reviews the characterization, standard testing methods as well as existing regulations in Europe and the United States for antimicrobial textiles. Antimicrobial textiles were characterized based on their application area. A summary of the efficacy testing standards on antimicrobial textiles was presented and critically discussed. Safety evaluation, comprising the risk assessment was also introduced. The increasing use of antimicrobial textiles is in need of further development of regulations and international testing standards for safety and efficacy evaluation *in vitro* including preclinical testing if applicable. Moreover, particular attention was given to the development of durability test standards.

Keywords: Antimicrobial tests, Standards, Cytotoxicity, Durability, Regulation, Biocide, Biofilm.

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1 Introduction

Nowadays, antimicrobial textile has been widely applied in several sectors, including hospitals and healthcare centres, food industry, clothing industry and in domestic environment (Espitia et al., 2012; Li et al., 2006; Page et al., 2009). The function of antimicrobial treatment may differ in diverse areas (Kramer et al., 2006). Generally, when antimicrobial agents are incorporated into a textile substrate, two purposes are intended. One, to protect the textile itself against bio-deterioration from microbial corrosion for longevity; the other one, which represents most of the cases, to provide new properties to protect humans and animals in the name of public health as antimicrobial textiles, and odour control (Gutarowska and Michalski, 2012; Yuan and Cranston, 2008).

In the critical nosocomial environment, surgical suture has been already incorporated with antimicrobial agents, namely triclosan, as a commercial available product decreasing postoperative wound complications (i.e. excessive inflammatory response) (Rasic et al., 2011). In the surgical practice, there are studies about incorporation of antimicrobial agents in articles such as surgical drapes, scrubs, masks, to reduce surgical site infections (SSI) (Li et al., 2006; Rozman et al., 2017). In addition, antimicrobial textiles have been extensively applied in wound dressing, reducing wound infection due to bacteria colonisation and thereby stimulating healing process (Silver et al., 2007; Simões et al., 2018). Numerous wound dressing products containing silver ions or silver nanoparticles (AgNPs) can be readily found in the market (Thomas and McCubbin, 2003). It is important to denote that healthcare textiles play a sizable role in the acquisition and transmission of healthcare-associated pathogens (Mitchell et al., 2015). Logically, there is no doubt that antimicrobial textiles can help prevent healthcare-associated infections (HCAIs) (Borkow and Gabbay, 2008). As an example, healthcare workers (HCWs) uniforms, patients bedsheets, privacy curtains etc. are also gradually being functionalized with antimicrobial compounds (Han and Yang, 2005; Schweizer et al., 2015). In the clothing

industry, antimicrobial textiles are mostly applied for deodorization and anti-fungi action (Akira, 1995; Islam et al., 2012). Therefore, they are often found in sport clothes, socks, shoe lining, underwear etc.

Antimicrobial textiles are often achieved by adding antimicrobial agents/substances on textile substrates by various chemical or physical means (i.e. build in, after-treatment, or grafting) (Liao et al., 2019). The term "antimicrobial" refers to microorganism repellent or reduction of microorganism load on the textiles or its surroundings. Therefore, their efficacy is normally evaluated by the microorganism reduction through antimicrobial tests. In the development of antimicrobial textiles, generally efficacy of both antimicrobial agents and antimicrobial textiles as a whole are tested. Several international recognized standards organizations, such as European Committee for Normalization (CEN - *Comité Européen de Normalisation* in French), American Society for Testing and Materials (ASTM), International, and American Association of Textile Chemists and Colorists (AATCC), Association of Official Agricultural Chemists (AOAC), International Japanese Industrial Standards (JIS), International Organization for Standardization (ISO) have issued standards for the efficacy test of antimicrobial agents and antimicrobial textiles. This chapter encompasses the reviews of both test standards for antimicrobial agents and antimicrobial textiles following critical discussion. Most used testing standards available in the literature were also summarized and discussed. It is denoted that the use of different standards in the evaluation of the antimicrobial efficacy leads to incomparable results (Pinho et al., 2010). Also, inadequate selection of antimicrobial test method *in vitro* results in performance discrepancy for similar biocides and textiles (Anderson et al., 2017). Since most of the antimicrobial textiles applications are or may be in contact with human body, essential safety requirements are need (Seong et al., 1999). Generally, the biocompatibility, cytotoxicity, irritation potential, and sensitization are evaluated to fulfil the requirement in the regulation. This chapter also takes a look at the regulation side of antimicrobial textile labelling in Europe and the United States of America (USA).

Overall, the chapter mainly answers the following questions regarding antimicrobial textiles:

1) What are the available testing standards for the antimicrobial efficacy evaluation?
2) What are the other tests required when considering the safe use of antimicrobial textiles?
3) What is the current regulation for antimicrobial textiles in Europe and the USA?

This chapter will guide the researchers and manufacturers selecting the appropriate testing methods for their products, ensure sufficient antimicrobial efficacy *in situ*, while fulfilling the regulatory compliance.

2 Antimicrobial efficacy testing protocols for antimicrobial textiles

2.1 Classification and characterization

Before discussing the antimicrobial testing and regulation, it is important to clarify the category of antimicrobial textiles. As discussed in the introduction, antimicrobial textiles are defined as textiles functionalized with antimicrobials capable of microbial growth inhibition or/and biocide activity. A brief mention to microorganism repellent (anti-fouling) will be performed. However, the antimicrobial textiles targeting at protecting textile itself are not in the scope of this chapter. Such consideration is based on the claim made from the regulatory body (discussed elsewhere in the chapter). Based on their antimicrobial mechanisms of action, antimicrobial textiles can be divided into the following categories (Sjollema et al., 2018):

i. Textile capable of control release of antimicrobials;
ii. Textiles that kill adhering microorganisms directly by contact, without antimicrobial compound release (contact killing);
iii. Textiles that prevent microbial adhesion (anti-fouling).

The first two referred mechanisms are a proactive approach, being commonly applied in the clinical environment and clothing industry due to their ability to actively eliminate or inhibit the growth of microbes avoiding their proliferation. In the case of disposable textile products, there is no preference between these two mechanisms. However, in the case of reusable textile products, the release of antimicrobials from textile material is rather impractical due to the laundry process. Therefore, for reusable textile products, immobilization of antimicrobials is required. Anti-fouling textiles (category iii) act passively, repelling microorganisms from the textile surface through surface modifications, which is not the main focus of this chapter.

The antimicrobial efficacy testing method in a large extent depends on their antimicrobial mechanisms of action and concentration. Furthermore, the regulation differs with the intended applications of antimicrobial textiles, as a brief example, wound dressings that will be applied in contact with damaged skin tissue possess different requirements than a textile that will contact with healthy skin. Thereby, the antimicrobial textile can be divided into three categories based on their field of application:

a. Medical textiles
b. Hospital textiles
c. Clothing textiles

Medical textiles (category a.), are medical devices that come in contact with class 1 sterile tissue or vascular system, class 2 mucous membranes or non-intact skin, and class 3 intact skin. Typical examples are surgical sutures, surgical drapes, surgical meshes in class 1, wound dressing in class 2, and surgical mask, surgical scrubs in class 3.

Hospital textiles (category b.), comprise healthcare workers' uniforms, bedlinens, privacy curtains in non-critical situations in clinical settings.

Clothing textiles (category c.), normally refers to antimicrobial textiles application in the clothing industry, aiming to reduce the bio-deterioration, malodour, or fungi corrosion. They are commonly found in sportswear, underwear, socks, etc.

2.2 Antimicrobial efficacy testing standards

Antimicrobial textiles consist of active textiles which require efficacy tests of antimicrobial substances, fabric and their combination, are often required during their development. CEN, ASTM, AATCC, AOAC, ISO, Clinical and Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and Organisation of Economic Co-operation and Development (OECD) are international recognized standard organizations providing different testing methods based on the intended application and context. Other national associations such as JIS and Canadian Standards Association (CSA) also stipulate relevant standards for antimicrobial efficacy tests.

There are numerous variables affecting the antimicrobial efficacy in a testing procedure, namely: sample size, inoculum concentration, culture medium or buffer formulation (nutrients availability), and these factors vary according different test methods. It is hard to imagine a comparable result among all the available test methods (Deshpande et al., 2016; Pinho et al., 2010). An adequate selection of testing methods plays an important role in leading to a successful application for the intended purpose. Many of the testing standards were noted to provide a "wet" condition (with a droplet of bacteria inoculum) in the tests, which is rather in favour of the antimicrobial efficacy of some antimicrobials (e.g. silver ions) (Liao et al., 2019). However, this method does not encompass all real life situations. Humidity, temperature, and organic load of the surroundings are critical factors affecting the antimicrobial efficacy of antimicrobial textiles.

In this section, both antimicrobial efficacy testing standards for active substances and antimicrobial textiles are introduced.

2.2.1 Testing standards for antimicrobial active substances

Antimicrobial active substances, are chemicals with antimicrobial properties against various bacteria, fungi, mycobacterial spores, virus etc. The most commonly used active substances in antimicrobial textile application are metal-based antimicrobials composed of metal ions or metal nanoparticles (NPs), being the most common: silver, gold, copper, zinc oxide; polymer-based antimicrobials such as chitosan, quaternary ammonium compounds (QACs); natural-based antimicrobials, i.e. antimicrobial peptides (AMPs) etc. (Jain et al., 2014; Lemire et al., 2013; Morais et al., 2016).

The minimum inhibitory concentration (MIC) test can be used for an initial screening of the antimicrobial activity of the active substances (CLSI, 2017; CLSI, 2018a; Watanabe et al., 2019; I. Wiegand et al., 2008). There is a high probability that the followed methodology and inoculum concentration can significantly influence the result of MIC (Arikan, 2007). For biocidal activity assessment, minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) is often adopted (CLSI, 1999). Zone of inhibition (ZoI) (also known as Kirby−Bauer radial disc diffusion, agar disk diffusion test) is another commonly used screen method as a qualitative assessment of the antimicrobial susceptibility (against bacteria and fungi) with direct active substances liquid (also known as agar well diffusion method) or filter paper disk inoculated with active substances (CLSI, 2018b; CLSI, 2018c) . This method is simple to implement, inexpensive, relatively quick and the results are easily visualized (Barnard, 2019). However, it is accurately difficult to distinguish the effect between growth inhibition or microorganism killing. Therefore, additional tests should be performed.

When it comes to the antimicrobial efficacy test, the active substances can also be treated as disinfectant. CEN Technical Committee (TC) 216 – Chemical disinfectants and antiseptics – provides test methods for antimicrobial efficacy evaluation of disinfectants. CEN classifies testing standards into 2 phases. Phase 1 refers to suspension tests, giving basic antimicrobial evaluation without organic load (bovine albumin fraction V are normally used in the test) for generalized use. Phase 2 consists of a 2 step assessment. Phase 2 step 1 is either suspension-based or carrier-based tests providing options of clean and dirty conditions targeting a more specific sector (food, industrial, domestic, institutional areas, medical field, or veterinary areas). While Phase 2 step 2 is simulating the practical use of disinfectant and antiseptics in the proposed field, such as disinfectant for a hand rub (EN 1500:2013) or disinfectant used with mechanical action (EN 16615:2015). Phase 2 step 2 test methods tests the antimicrobial efficacy

of active substances integrated in other forms. Table 1 below exhibits a summary of CEN testing standards appropriate for active substances testing.

Table 1 Testing standards for active substances of antimicrobial textiles from CEN.

STANDARD	PRINCIPLE	TARGET MICROORGANISM	APPLIED AREA
EN 1040:2005	Phase 1	Bacteria	/
EN 1275:2005	Phase 1	Fungi or yeast	/
EN 14347:2005	Phase 1	Spores	/
EN 1276:2019	Phase 2 step 1	Bacteria	Food, industrial, domestic and institutional
EN 13704:2018	Phase 2 step 1	Spores	Food, industrial, domestic and institutional
EN 13610:2002	Phase 2 step 1	Virus	Food, industrial, domestic and institutional
EN 1650:2019	Phase 2 step 1	Fungi or yeast	Food, industrial, domestic and institutional
EN 13727:2012+A2:2015	Phase 2 step 1	Bacteria	Medical
EN 14348:2005	Phase 2 step 1	Mycobacteria	Medical (including instrument disinfectants)
EN 17126:2018	Phase 2, step 1	Spores	Medical
EN 14476:2013+A2:2019	Phase 2 step 1	Virus	Medical
EN 13624:2013	Phase 2 step 1	Fungi or yeast	Medical
EN 1656:2019	Phase 2 step 1	Bacteria	Veterinary
EN 14204:2012	Phase 2 step 1	Mycobacteria	Veterinary
EN 14675:2015	Phase 2 step 1	Virus	Veterinary
EN 1657:2016	Phase 2, step 1	Fungi or yeast	Veterinary
EN 13623:2010	Phase 2 step 1	Legionella	Aqueous systems

2.2.2 Testing standards for antimicrobial textiles

In CEN, (TC) 248 has the responsibility of standardization of textiles, textile products and textile components of products in the European Union. Minimum requirements for textiles

products such as dimension stability, safety design, colour fastness, tensile properties, resistance to liquid depending on the final purpose of the textile products etc. are listed in the published standards under TC 248. However, in terms of other expected behaviours in a specific product, standardization may also be required by other CEN TC. For instance, TC 205 non-active medical devices working on identifying, adopting, adapting or preparing standards supporting applicable European regulations for non-active medical devices such as surgical clothing and drapes (EN 13795-1:2019), medical face masks (EN 14683:2019+AC:2019), and wound dressing (EN 13726-1/2/3/4) etc. ISO TC 38 is in charge of standardization of textiles. However, antimicrobial property is an extra function added to existing textile products. Therefore, it is necessary to evaluate the fulfilment of the requirements of both the products and antimicrobial efficacy. It is worth to mention that, ASTM has published standard guide for the use of standard test methods and practices for evaluating antibacterial activity on textiles (ASTM E2922 – 15), which identifies some existing ASTM and other industry standard test methods applicable for testing the antibacterial performance on textiles and discusses options within each method that have been used to address specific end-use performance expectations (2015). There are principally two types of testing methods: qualitative and quantitative. The discussed testing methods are listed in Table 2 and 3.

Table 2 Qualitative antimicrobial tests methods for antimicrobial textiles.

Standard Code	Standards/Methods
AATCC TM 147:2004 (accredited ISO/IEC 17025)	Antibacterial activity assessment of textile materials: parallel streak method.
AATCC TM 90	Antibacterial activity assessment of textile materials: agar plate method
ASTM E2722	Test method for using seeded-agar for the screening assessment of antimicrobial activity in fabric and air filter media
JIS L 1902:2008 (Halo method)	Testing for antibacterial activity and efficacy on textile products
SNV 195920	Examination of the antimicrobial effect of impregnated textiles by the agar diffusion test

*Swiss Association for Standardization (SNV).

Table 3 Quantitative antimicrobial tests methods for antimicrobial textiles.

Standard Code	Standards/Methods
AATCC TM 100:2004	Antibacterial finishes on fabrics, evaluation of. (accredited ISO/IEC 17025)
ASTM E2149-13a	Test method for determining the antimicrobial activity of antimicrobial agents under dynamic contact conditions
ASTM E2180	Test method for determining the activity of incorporated antimicrobial agent(s) in polymeric or hydrophobic materials
ISO 20743	Textiles - determination of antibacterial activity of antibacterial finished products
ISO 22196	Plastics - measurement of antibacterial activity on plastics surfaces
JIS Z 2801:2000	Antimicrobial products - test for antimicrobial activity and efficacy
IBRG* TEX13/005/1.0	Quantitative method for evaluating bactericidal

*International Biodeterioration Research Group (IBRG).

The above-mentioned testing methods were well discussed in ASTM E 2922 – 15, therefore, to avoid repetition, only the remaining are presently further discussed.

Qualitative antimicrobial testing methods

ZoI, previously mentioned, was not only used for active substances testing, but also applied for testing fully developed antimicrobial textiles (Hudzicki, 2009). It is one of the most frequently used qualitative (or semi-quantitative in specific situations) method for the first step screen of antimicrobial activity of antimicrobial textiles. In fact, many aforementioned methods such as AATCC TM 147:2004, AATCC TM 90, SNV 195920, and JIS L1902 incorporated the principle of ZoI. AATCC TM 30-2004 (Antifungal activity, assessment on textile materials: Mildew and rot resistance of textiles) is similar test method but used to test against fungi. AATCC TM 174 Part I adapting from AATCC TM 147 against bacteria and Part III adapting from AATCC TM 30 against fungi is another test method based on ZoI. ISO 20645:2004 Textile fabrics — Determination of antibacterial activity — Agar diffusion plate test is another example. ZoI-based standards are easy to operate when the specimen is flat (without crimping). However, ZoI-based test standards require that antimicrobial active substances are able to diffuse from the textile substrate into the agar, which means that it is not suitable for immobilized antimicrobial substances. Also, it detects only grow inhibition but not biocidal

effect. Finally, it should be highlighted that this method is not appropriate for active substances that react with the agar or culture medium ingredients.

Quantitative antimicrobial testing methods

The OECD has published in its series on biocides and testing and assessment a guideline document for quantitative method for evaluation antimicrobial activity of porous and non-porous materials (Ashworth et al., 2014). Porous materials are often referring to textile materials. The guideline details the requirements for test methodology, comprising the description of test bacteria, preparation of test materials, preparation of the test inoculum, inoculation of test materials, incubation, recovery of bacteria from the test samples and measurement of colony forming units (CFU), results and test report layout (OECD, 2014). Therefore, this chapter will solely refer its establishment and highlight the adequacy of this guideline than preforming its copy. In addition, the OECD member countries are encouraged to perform the test methods described in the guideline documents for evaluation of antimicrobial activity of materials.

Besides the quantitative methods mentioned in the guideline ASTM 2922-15, another test standard ASTM E3160 entitled: quantitative evaluation of the antibacterial properties of porous antibacterial treated articles was developed in 2018 (ASTM, 2018). This new test standard is able to determine both bactericidal and bacteriostatic activity.

Besides all the antimicrobial efficacy tests against planktonic microorganisms, it is also of great interest of antimicrobial textiles the ability to act against sessile bacteria (biofilm). Biofilm comprises accretions of microorganisms enclosed in a self-produced matrix of extracellular polymers attached on a surface, representing a robust mode of microbial growth (Hall-Stoodley et al., 2004). Increasingly more evidences show the correlation between the existence of biofilm and HCAIs, especially in wound infection (Black and Costerton, 2010; Percival et al., 2015). It is thereby of great importance for antimicrobial textiles to evaluate their antimicrobial efficacy against biofilm. The assessment of biofilm presence and growth in a consistent way is highly challenging. Currently, there are five methods developed for biofilm testing with standard procedures to evaluate biofilm growth. The methods developed are presented in Figure 1 (according to their publication dates) (ASTM International, 2013; ASTM International, 2017a; ASTM International, 2017b; ASTM International, 2017c; ASTM International, 2019).

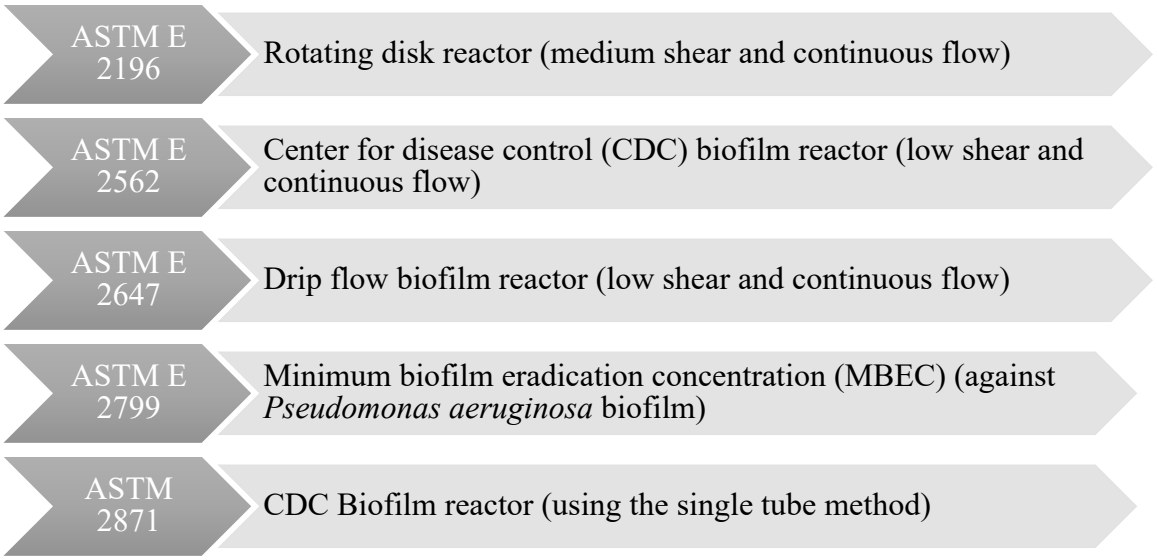

Figure 1. Test methods of antimicrobial efficacy against biofilms.

The standard methods allow a consistent biofilm growth and ensure the repeatability and reproducibility of the test against biofilm. By modifying the test methods, researchers can study factors of interest (e.g. testing surface) (Harrison et al., 2009; Pérez-Díaz et al., 2016). However, the test methods involving continuous flow system required expensive lab techniques, more complicated to executed unlike the assays with static biofilm systems (Merritt et al., 2011).

Table 4 depicts antimicrobial efficacy testing method for antimicrobial textiles in literature studies. It is noticed that the most often used testing methods are ZoI based protocols, static contact-killing test (AATCC TM100 and JISL 2801) and dynamic contact-killing test (ASTM E 2149).

Table 4 Antimicrobial efficacy test methods in literature studies, describing textile substrate (TS), antimicrobial substances (AMS), and coating methods (CM) investigated.

Testing methods	Textile substrate	Antimicrobial substances	Coating methods	Application areas	Reference
MIC (AMS) AATCC TM 100 (AMT)	Spun-bond polypropylene (PP) (outer layer of N95mask)	Nanoparticles (containing silver nitrate and titanium dioxide)	Mathis 2-Roll Type HF-350 textile finishing machine (Padding machine)	Surgical masks	(Li et al., 2006)
AATCC TM 100	Melt-blown PP nonwovens	Gemini surfactant (GS) compounds: GS-12-6-12	Applying a set of porous biocidal structures (SPBS) to the melt-blown nonwovens	Respiratory protective devices (RPDs)	(Majchrzycka et al., 2017)
AATCC TM 100; EN ISO 20743:2007	Cotton fibres	Silver nanoparticles (AgNPs)	Sol-gel coating with a reactive organic-inorganic binder	N.S.	(Tomšič et al., 2008)
AATCC TM 100	50 % Polyester (PET) / 50 %cotton fabric	Silane quaternary ammonium compounds (Si-QAC)	Create covalent bonding form in the finishing process of the fabric	HCWs uniforms	(Rozman et al., 2017)
Modified AATCC TM 100	Cotton fabric	Monomer 3-(4 – vinylbenzyl)-5,5-dimethylhydantoin (VBDMH)	Admicellar polymerization using a cationic surfactant	N.S.	(Ren et al., 2008)
Modified AATCC TM 100	Wool/acrylic blended yarns	Rose Bengal (RB) photosensitizer	Specified dyeing process	Limited use-garments in hospital	(Chen et al., 2019)

Modified AATCC TM 100	Poly(methyl methacrylate-co-methacrylic acid) polymer composite nanofibers doped with montmorillonite (MMT)	Cationic photosensitizer methylene blue (MB)	Immersion in MB for six days following desorption equilibrium with phosphate-buffered saline solution for around three days	N.S.	(Wang et al., 2018)
Modified AATCC TM 100	Para-aramide and PET fabric	Copper (II)	Copper coating after poly-pyrrole (PPy) coating	Hospital textiles	(Irene et al., 2016)
Modified JISL 2801	100 % PET plain weave fabric	PPy nanoparticles	Ultrasound-assisted coating process	N.S.	(Sanchez (Ramirez et al., 2019)
Modified ASTM E2149-01	Cotton fabric	Silver nanoparticles (AgNPs)	AgNPs deposition by immersion, coated with γ-methacryloxypropyl trimethoxysilane (MPS)	N.S.	(Kurajica et al., 2012)
ASTM E2149-01 (Shaking-Flask Test)	Cotton fabric	Chitosan derivatives	Using citric acid (CA) as the crosslinking agent (between the synthesized chitosan and cotton fabric)	N.S.	(Fu et al., 2011)
Modified ASTM e 2149-01	50 % PET/ 50 % cotton fabric	Silver ions	Immersion in a ceramic carrier	N.S.	(Condo et al., 2015)
JISL1902:2002	Viscose fabric	Silver nanoparticles (AgNPs)	Sol-gel process following dip coating method	N.S.	(Mahltig et al., 2011)
JIS L 1902: 2002	Alginate	Ionic Ag and AgNPs	Commercial available	Wound dressing	(Wiegand et al., 2009)

JIS L 1902: 2002	Cotton fabric	β-cyclodextrin-antiseptic-complex	Covalent boding with a reactive anchor	Wound dressing	(Reddersen et al., 2016)
Modified ZoI Modified ASTM E2149-01	50 % PET / 50% cotton fabric	Eugenol-loaded human serum albumin (HSA)/silk fibroin (SF) nanocapsules	Crosslinking reaction using EDC/NHS system	Wound dressing	(Quartinello et al., 2019)
FZ/T 73023-2006 standard method	Cotton fabric	Silicone quaternary ammonium salt based nanocomposite (OQAS/(Ag/ZnO))	Immersion in the nanocomposite dispersion, following padding and drying process	N.S.	(Gao et al., 2019)
ZoI and growth-inhibition assays (grow in liquid broth)	Silk fibron	AgNPs	Dropping	Wound dressing	(Uttayarat et al., 2012)
ZoI	Cotton fabric	Chitosan–silver hydrogels	Padding–squeezing–drying method	N.S.	(Kozicki et al., 2016)
ZoI, long-term antimicrobial activity assessment	Gelatin nanofiber mats	Antibiotics	Incorporation of antibiotics in the electrospinning process	Wound dressing	(Dhand et al., 2017)
ZoI	Cotton fabric	Chitosan	Immersion in a blend of chitosan (CS), polyethylene glycol (PEG) and polyvinyl pyrolidone (PVP)	Wound dressing	(Anjum et al., 2016)

Tissue compatibility studies Wound healing studies			overnight, padding, PVP coating outside, Freeze drying (-80)		
Bacterial growth was monitored under light microscope	Bacterial cellulose	Antimicrobial peptides (AMP) ε-poly-L-Lysine	Carbodiimide chemistry	Wound dressing	(Fürsatz et al., 2018)
Preclinical tests with murine diabetic model	Viscose/rayon	Copper oxide particles	PP fibres impregnated with Copper oxide particles	Wound dressing	(Borkow et al., 2010)

Note: GS-12-6-12 Hexamethylene-1,6-bis(**N**,**N**-dimethyl-**N**-dodecylammonium bromide); N.S. Not specified; MMA Methyl methacrylate; MAA Methacrylic acid; MAA1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide EDC/N-hydroxysuccinimide (NHS).

2.3 Safety test

The antimicrobial textile market has increased considerably during the last decade mainly due to the advances in biomaterials and nanotechnology (Agnihotri and Dhiman, 2017). This fact raised several concerns about the safety of these materials, promoting the development of new procedures in biocompatibility tests (Morais et al., 2016). Biocompatibility is an extremely important element to ensure that the materials will not cause unwanted biological reactions when in contact with human/animal tissues and, consequently, induce the reaction of the host immune system (Shah and Dobrovolskaia, 2018). Several methods have been designed to determine local and systemic reactions that may present potential toxicological effect and objectively evaluate the biological safety of the products and ensure that there are no associated health risks (Williams, 2016). The use of antimicrobial materials may cause adverse effects owed to chemical and physical reactions associated with the properties of textile surface. Therefore, the biocompatibility tests may: i. indicate the chemical and physical interactions between the material and the eukaryotic biological tissue and also the eukaryotic biological response to these reactions; ii. pin point harmful components of the materials and avoid significant adverse effects; iii. establish the potential risk of the material may pose to the user (Gad, 2019). In addition, the biocompatibility tests should be performed in the final product and not just in the individual components. Even if the individual components do not present cytotoxicity, their interaction including any addition during the manufacturing process may result in unacceptable biocompatibility results (Tan et al., 2019). Processes like sterilization, washing, anodization/passivation and rising may also influence the biocompatibility. Therefore, the biocompatibility tests should be performed to the final product, after all the processes are adopted during the production (Escudero-Castellanos et al., 2016).

After a brief explanation about pre-testing, the most common biocompatibility tests will be discussed in this section. They are mentioned as "the big three" and include cytotoxicity, irritation and sensitization testing. The evaluation of these three biological effects are mandatory on medical applications and strongly recommended in other antimicrobial textiles applications. However, there are numerous other tests to evaluate the biological effects of antimicrobial textiles namely systemic toxicity, genotoxicity, hemocompatibility and carcinogenicity (De Jong et al., 2020). The available standards for biocompatibility are recommended for the testing of medical devices but they are also applied for the testing of antimicrobial textiles in general (Hilgenberg et al., 2016).

2.3.1 Pre-testing and risk assessment

Currently, the biocompatibility testing demands a meticulous planning in order to obtain the required results within the shortest time span. The ISO 10993 presents a series of guidelines for the biological evaluation of materials to manage the risks of the products for human health and safety. The ISO 10993-1 standard (Biological evaluation of medical devices, Part 1: Evaluation and testing within a risk management process) was updated in 2018 and provides the pretesting considerations about how to plan the biocompatibility tests for materials depending on the contact site, contact time and host tissues particularities. It is presented a systematic approach to perform a biological evaluation, select the most appropriate methods and propose the risk assessment of a product. The standards ISO 10993-18:2020 (Biological evaluation of medical devices, Part 18: Chemical characterization of medical device materials within a risk management process) and ISO 10993-17:2020 (Biological evaluation of medical devices, Part 19: Physico-chemical, morphological and topographical characterization of materials) display the guidelines for a complete chemical and material characterization to identify and quantify the leachable compounds released from the material and understand the basic mechanisms to assess the potential cytotoxicological risks. The initial characterization using chemical, physical, morphological and topographical methods provide relevant information for risk assessment and can support the biocompatibility testing in order to minimize the need for *in vivo* testing, due to its associated costs, time, and animal welfare risks (Brown, 2020; Qin, 2016; ISO, 2018; ISO 2020a; ISO, 2020b).

2.3.2 Cytotoxicity

Cytotoxicity testing is a primary method for establishing the safety of a material. It allows an early assessment of the material destiny, determining if the material can continue further testing, or if it requires any modifications, or even, if the martial must be abandoned, all at the initial stages of development (Srivastava et al., 2018). The cytotoxicity evaluation of materials described in ISO 10993-5:2009 is based in *in vitro* tests and expresses the toxicological effect of the leachable compounds in the material after the incubation of cultured cells in contact with the material either directly or through diffusion (De Jong et al., 2020; ISO, 2009). Three different methodologies are presented: i. test on extract, ii. test by direct contact and iii. test by indirect contact. The first type is the most commonly used technique, where the material is immersed in a culture medium, and the fluid extracts are seeded with cells, and after an

incubation period, the cytotoxicity is assessed. It is extremely useful for soluble substances and the results are consistent with the *in vivo* tests. The extraction solutions (polar and nonpolar) should simulate or exaggerate the final use situations to determine any potential toxicity (Przekora, 2019). The second method, the direct contact, is highly sensitive, able to detect weak cytotoxicity as the samples are directly deposited over cell cultures (Srivastava et al., 2018). As for the indirect method, the agar overlay assay, is suitable for material with large toxicity, comprising the use of a bulk filter (Li et al., 2015). ISO 10993-12:2012 also regulates the samples to test, the control samples (at least one negative and one positive, noncytotoxic or cytotoxic response, respectively) and the extraction methodology and preparation. This standard also includes a testing plan to guide the operators to the most appropriate test for the material to be evaluated (Przekora, 2019). These methods are designed to determine the biological response of mammalian cells *in vitro* using appropriate biological parameters and several cell lines are accessible for cytotoxicity testing. Nevertheless, the American Type Culture Collection (ATCC) methodology is preferred (De Jong et al., 2020).

The cytotoxicity can be assessed by the evaluation of cell morphology, cell damage, cell growth or by measuring the cellular activity, via quantitative and qualitative methods. Quantitative methods include the tetrazolium salt assay (e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), water-soluble tetrazolium salt (WST-1)), the colony formation cytotoxicity test, trypan blue, neutral red uptake and lactate dehydrogenase LDH assay. Qualitative analyses include direct contact, morphological grading of cytotoxicity of extracts. The combination of the LDH assay to indicate the membrane damage and a metabolic activity assay (e.g., tetrazolium salt) is frequently used (Liu et al., 2018; Iqbal and Keshavarz, 2017; Sampaio et al., 2016).

2.3.3 Irritation and sensitization potential

Assessment of potential allergic reactions, namely irritation and sensitization, belongs to the basic set of toxicology tests of antimicrobial textiles. An irritation reaction occurs immediately after the first exposure and is a non-immunologic local inflammatory reaction caused by external stimuli. The sensitization reaction only takes place after repeated or prolonged exposure and is independent of the dose. A slight exposure may cause a severe or even possibly lethal toxic reaction, inducing vesiculation or necrosis, and may be systemic. These characteristics may hinder the perception of the toxic reaction (Park et al., 2018). ISO 10993-

10:2010 describes the *in vitro*, *in silico* and *in vivo* methods for the assessment of materials with regard to their potential to produce irritation and skin sensitization. It evaluates the possible contact hazards from chemicals released from textile materials that may produce irritation of the skin, mucosal and eye or skin sensitization. The initial *in vitro* methods are recommended for the initial screening prior to animal testing. Despite the numerous information extracted from *in vitro* testing results, an animal test is usually required prior to human testing (ISO, 2010). According to ISO 10993-10, there are *in vitro* and *in vivo* methods available for irritation evaluation. However, the *in vitro* test for skin irritation has been validated just for neat chemicals. Therefore, antimicrobial textiles have to be tested using *in vivo* methods. The active and control samples are deposited directly in the skin of healthy rabbits and the appearance of each application site (redness and swelling) is evaluated in terms of erythema and eschar formation after 1, 24, 48, and 72 h. The skin irritation degree (from negligible to severe) of the material is determined by hematoxylin and eosin (H&E) stained images (Gu et al., 2018). After this, human studies can be carried out due to the discrepancies from animal and human skin irritation reactions. The human tests are only permitted if the material had no negative effects in previous animal tests (Hilgenberg et al., 2016; Qin, 2016).

Sensitization testing, also presented in ISO 10993-10:2010, is based on *in vivo* tests to assess the ability of leachable compounds to prompt skin hazards. To help to investigate whether a material contains chemicals that cause antagonistic effects after repeated or prolonged exposure four methods are commonly used, namely: murine local lymph node assay (LLNA), Guinea pig assay, Guinea pig maximization test (GPMT) and closed-patch test (Buehler test) (ISO, 2010). The LLNA was the first alternative method to experience formal validation for skin sensitization hazard tests. It is a useful tool to measure the relative potency of skin sensitizing chemicals and presented extreme utility in terms of driving improvements in risk assessment, risk management and protection of human health (Basketter et al., 2017). The other models (Guinea pig assay, GPMT and Buehler test) are animal models with invaluable relevance to the study of allergic and toxicological reactivity. Guinea pig models are among the most frequently used methods. The GPMT is usually considered the most sensitive procedure to detect the capacity of a substance to induce contact hypersensitivity, and is among the best methods to extrapolate the results to humans and may also be used to elucidate dose-response relationships. However, due to the ethical issues and concerns about animal well-being, a multi-phase program is required to develop a non-animal method with regulatory acceptance to predict skin sensitization (Hoffmann et al., 2018; Modjtahedi et al., 2011).

2.4 Durability test

Durability of antimicrobial textiles refers to the desired physical durability and chemical stability over a specific time of use. It is especially important for reusable textiles, such as uniforms, bedlinen, privacy curtains, towels, etc. that still maintain sufficient antimicrobial efficacy after laundry (exposure to detergent and high temperature). Physical durability, namely resistance to tear, abrasion etc. is identical comparable requirements to other textile products. Giving as an example, CEN/TC 248 Working Group 16 Textiles in healthcare system issued the technical specification for textile products used for healthcare and social services facilities (Ref. No. CEN/TS 14237:2015) indicating characteristics, test method and minimum performance properties of textile products intended to be used after industrial laundering (CEN, 2015). However, in this chapter, the durability of the antimicrobial textiles will focus on the antimicrobial efficacy performance.

Many studies started paying attention to the durability of antimicrobial efficacy in the development of new antimicrobial textiles (Fu et al., 2011; Gao et al., 2019; Shahid-Ul-Islam and Butola, 2019). The durability study of antimicrobial textiles normally combines the simulation of the laundering process with an antimicrobial test. Existing standards/methods simulating home or industrial laundry process for textile products were implemented for reproducibility and consistency of the research work.

AATCC has established monography of Standardization of hand laundering for fabrics and apparel and standardization of home laundry test conditions for test methods utilizing laundry procedures (such as AATCC TM 124, 135, 143, 150) in the technical manual. The guideline listed detailed parameter settings of type of machine, temperature, water level etc. of laundering, drying, and restoring. Also, "1993 AATCC Standard reference detergent and laundry detergents in general" and "2003 AATCC Standard reference liquid laundry detergent" were developed, listing the comparable reference detergent to powder and liquid laundry detergent in the market. However, the specimen size required in the monography is relatively large, which is not favourable for the testing of antimicrobial textiles which generally comprise small specimens. Therefore, accelerated washing procedure developed in AATCC test methods can be an alternative, AATCC TM 61 Colorfastness to laundering: accelerated, where five typical home laundering processes are recommended. This test method is similar to EN ISO 105-C06, Textiles - tests for colour fastness - Part C06: Colour fastness to domestic and commercial laundering (accredited from ISO 105-C06). Unfortunately, industrial laundering procedure is

not covered in AATCC since TM 87-1965 Colorfastness to washing, industrial laundering: accelerated is discontinued.

ISO/TC 38/SC 2 Cleansing, finishing and water resistance tests cover the standards providing exacting laboratory settings of textile domestic and industrial laundry procedures under standardized conditions. ISO 6330, Textiles - domestic washing and drying procedures for textile testing and ISO 15797:2017 Textiles - industrial washing and finishing procedures for testing of workwear (labelling workwear to be industrially laundered) are the given examples. CEN has also adopted the ISO standards previously mentioned for domestic and industrial washing testing in the laboratory setting. Other national standards, such as CSA Z314. 10-03 (selection use, maintenance and laundering or reusable textile wrappers, surgical gowns, and drapes for heal care facilities) and JIS L 1930 Textiles - domestic washing and drying procedures for textile testing can also be used as a reference laundry procedure.

There is also a new developed protocol from ASTM E3162-18, Standard practice measuring the durability of antibacterial agents applied to textiles under simulated home laundering conditions, which can determine the durability of standard antimicrobial treatment on textiles undergoing multiple home laundering cycles (ASTM, 2018). Table 5 exhibits the durability tests of antimicrobial textiles performed in the literature. Notably, many studies developed their simulation of washing process in the study and some are even poorly described (Xing et al., 2007). It is suggested using standard washing procedure while evaluating the durability of antimicrobial textiles in research, to ensure consistent and comparable result with the others. The standard washing process developed by AATCC, ISO etc. can in a large extent simulate the laundry process in reality (either domestic or industrial) with laboratory settings, which is supportive in understanding or predicting the performance of antimicrobial textiles in field/practice.

Table 5 Durability tests of antimicrobial textiles in literature, summary of TS, AMS, CD, wash protocols and wash cycles.

Antimicrobial test	Textile substrate	Antimicrobial substances	Coating methods	Wash protocol	Wash cycles	Application areas	Ref.
AATCC TM 100	Cotton Woven Fabric	*Penicillium amestolkiae* elv609 extract	Dyeing process	AATCC TM 147	30/50	Wound dressing	(Rozman et al., 2018)
ISO 22196 (JIS 2801)	13 textile products in German market	One AEGIS* coated, 10 silver coated, and two untreated	/	DIN EN ISO 6330	30/70/100/150/200	Atopic dermatitis treatment	(Srour et al., 2019)
ASTME2149-01	Bleached woven 100% cotton fabric	Hybrid ZnO NPs/ Chitosan	Ultrasound-assisted coating	Simulation of hospital laundering regimes	10	Hospital textiles	(Petkova et al., 2014)
AATCC TM 100	Viscose fabric	Micro-needles of Cu2O	*In situ* synthesis	AATCC Technical Manuel for home laundry	5	N.S.	(Emam et al., 2017)
MIC AATCC TM 147	Cotton fabric	Biogenic silver nanoparticles	Immersion, padding	Home developed wash cycle	10	In medical environment and agriculture clothing	(Ballottin et al., 2017)

GB/T 20944.3-2008 (Shaking Flask Method)	Woven, bleached, and scoured cotton fabric	AgNPs	Ag NPs grafted oxidized cotton fabric (Ag-GOCF) by immersion	AATCC TM 61-1996	10/30/50	N.S.	(Zhang et al., 2013)
Shaking Flask Method	Cotton fabric	Nanocrystalline TiO_2 hydrosol	Sol-gel synthesis	GOST 9733.4-83	5	Biomedical	(Galkina et al., 2014)
Shaking Flask Method	Desized and bleached polyester fabric	TiO_2 NPs	Pre-treated with alginate and immersion	Home developed wash cycle	5	Garments	(Mihailović et al., 2010)
AATCC TM 100	Cotton fabric	$AgNO_3$	Sol-gel method aided by water glass with padding method	Home developed wash cycle (Not well described)	1/5/10/20/50	N.S.	(Xing et al., 2007)

*AEGIS: 3-(trihydroxysilyl) propyldimethyloctadecyl ammonium chloride.

N.S.: not specified

3. Regulations for antimicrobial textiles

This section of the chapter will mainly introduce the regulatory issue regarding antimicrobial textiles market, distribution, and applications in the EU and USA. It is worth mentioning that antimicrobial textiles have to follow the regulation compliance as a basic requirement in their development. Requirements change depending on the claim of the products documented in the regulatory body.

Antimicrobial textiles based on their final application purposes differs in the claim and labelling. In EU, antimicrobial textiles with the aim of protecting textiles or odour prevention are categorized as treated articles. While antimicrobial textiles with a primary biocidal function, especially with a public healthcare relevance are considered as biocidal products (i.e. antimicrobial textiles applied in hospitals for infection prevention and control) (ECHA, 2018). Both treated articles and biocidal products are covered by the rules and obligations issued by the Biocidal Product Regulation (BPR). However, a biocide product requires an extra step "authorisation" than treated articles before they can be placed in the EU market. The assessment of antimicrobial textiles whether as treated articles or biocidal products should be consulted by treated articles guideline "CA-Sept13-Doc.5.1.e (Rev.1)" (European Commission, 2014). When the antimicrobial textiles contain nanoparticles, specific requirements for nanomaterials are demanded. Those provision defined by BPR apply for active and non-active substances with the following characteristics (ECHA, 2020):

- *50 % or more of the particles have a size between 1-100 nanometres in at least one dimension*
- *Particles are in an unbound state or as an aggregate or agglomerate*

The active substances incorporated in antimicrobial textiles are considered as biocides. In the EU market, biocidal active substances can only be placed in the market with approval or under review since March, 1st 2017. The list of approved active substances supplier is enclosed in Article 95. Further biocidal products legislations can be consulted according to biocidal products directive (Directive 98/8/EC) or Regulation (EU) No 528/2012 of the European Parliament and of the Council (Council of the European Union, 2012).

Additionally, manufacturers and importers of chemicals in the EU market are obliged to fulfil the regulatory framework from Registration, Evaluation, Authorisation and Restriction of

Chemicals (REACH) for each substance (including nanomaterials) manufactured or imported in quantities of 1 tonne or higher per year per company (legal entity) (EPC, 2007).

In the USA, Environmental Protection Agency (EPA) is responsible for antimicrobial textiles regulatory issues under the statutory authority of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Office of Pesticide Programs (OPP) from EPA categorises antimicrobial textiles into two groups, treated articles and antimicrobial pesticides. Treated articles claim indicates that the antimicrobial incorporated into textile is intended to protect textile from microbial deterioration and thereby can be applied to "treated articles exemption" in 40 CFR 152.25(a) (Federal insecticide, fungicide, and rodenticide act, 2000). While the other antimicrobial textiles, categorized as antimicrobial pesticide products, follow the registration process together with risk assessment. Furthermore, antimicrobial pesticide products can be classified as either "public health" or "non-public health" claims. With public health claim, efficacy data to support their intended application must be submitted. Typical antimicrobial hospital textiles with intension of infection control and prevention are normally claimed for "public health". Antimicrobial textiles with odour control is an example of antimicrobial pesticide with "non-public health" claim. However, in the case of microorganism repellent, which controls the microorganism by physical or mechanical actions, does not require EPA registration.

Whereas, regulation becomes more stringent when the antimicrobial textiles are classified as medical devices, such as wound dressings, surgical masks, surgical drapes. For instance, wound dressings combined with drugs (also known as antimicrobial wound dressings) will be regulated as combination products and thereby applies to the rules by USA Food and Drug administration (FDA). The classification of medical devices (wound dressing in the application of antimicrobial textiles) are defined as Class I that are subject only to general controls; Class II subject to general and special controls; and Class III subject to premarket approval) based on their intended use, safety and risk (21 CFR 878.4015) (FDA, 2019). Wound dressing intended to accelerate the wound healing will be considered as Class III; while wound dressing with antimicrobial agents minimizing microbial growth are normally encompassed by Class II (FDA, 2016). To comply with Class II requirements performance standards test, postmarket surveillance, patient registries and/or development of guidelines, and reasonable assurance of safety and effectiveness may be required (FDA, 2009).

It is noticed from the review of the regulation that the legislation of antimicrobial textiles is complex and expensive. Regulation can be more stringent when the antimicrobial textiles are classified as medical devices, such as wound dressings, surgical masks, surgical drapes. It is

one of the reasons hampering the translation of advanced research of antimicrobial textiles downward to the market. However, thinking of the final products in the market, researchers may also take into account the regulation aspect in the development of new antimicrobial textiles, in cooperation with the industry and end users (hospital as an example).

4. Conclusion

The development of novel antimicrobial textiles has obtained great interest due to the growing need to maintain the longevity of textiles, control the odour, wound management, and infection prevention and control. Therefore, the increasing use of antimicrobial textiles is in need of further development of regulations and international testing standards for safety and efficacy evaluation, including preclinical testing if applicable. Reproducibility and simulation of field testing should be the focus of the newly developed testing standards. Tests performed in different facilities or with different method display different results. Also, there is a lack of consistency between the bench test (*in vitro*) and field study result. Antimicrobial textile performance discrepancy was often observed between the research stage and their application *in situ*. Especially in clinical application, the question of how to ensure the clinical success during the application of antimicrobial textiles still remains. Therefore, new *in vitro* testing methods should seek to predict the actual *in situ* performance of antimicrobial textiles. Furthermore, particular attention should be given to the development of durability test standards. In addition, relevant safety tests, namely cytotoxicity, irritation potential and sensitization should be evaluated during the development of antimicrobial textiles. Depending on the envisaged antimicrobial textiles claim, corresponding regulation should be considered and consulted to facilitate their final launch in the market. It should be denoted that the main reasons hindering the development of novel advanced antimicrobial textile are the lack of sufficient testing standards and the complex and expensive regulatory procedures.

The application of antimicrobial textiles in clinical settings have an unquestionable potential to prevent and control nosocomial infections. However, there is still a lack of detailed studies describing if their applications may promote the development of multidrug-resistant organisms (MDROs). Finally, there is still a grievous insufficient development of novel antimicrobial textiles focused on anti-biofilm activity and virus, despite their recognized public health menacing nature. Hopefully, this will swiftly change in a near future.

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