ENGINEERING OF
BIOMATERIALS
INŻYNIERIA BIOMATERIAŁÓW
JOURNAL OF POLISH SOCIETY FOR BIOMATERIALS AND FACULTY OF MATERIALS SCIENCE AND CERAMICS AGH-UST
CZASOPISMO POLSKIEGO STOWARZYSZENIA BIOMATERIAŁÓW I WYDZIAŁU INŻYNIERII MATERIAŁOWEJ I CERAMIKI AGH

Number 159
Numer 159
Volume XXIV
Rok XXIV

JANUARY 2021
STYCZEŃ 2021

ISSN 1429-7248

PUBLISHER:
WYDAWCA:

Polish Society for Biomaterials in Krakow
Polskie Stowarzyszenie Biomateriałów w Krakowie

ADDRESS OF EDITORIAL OFFICE:
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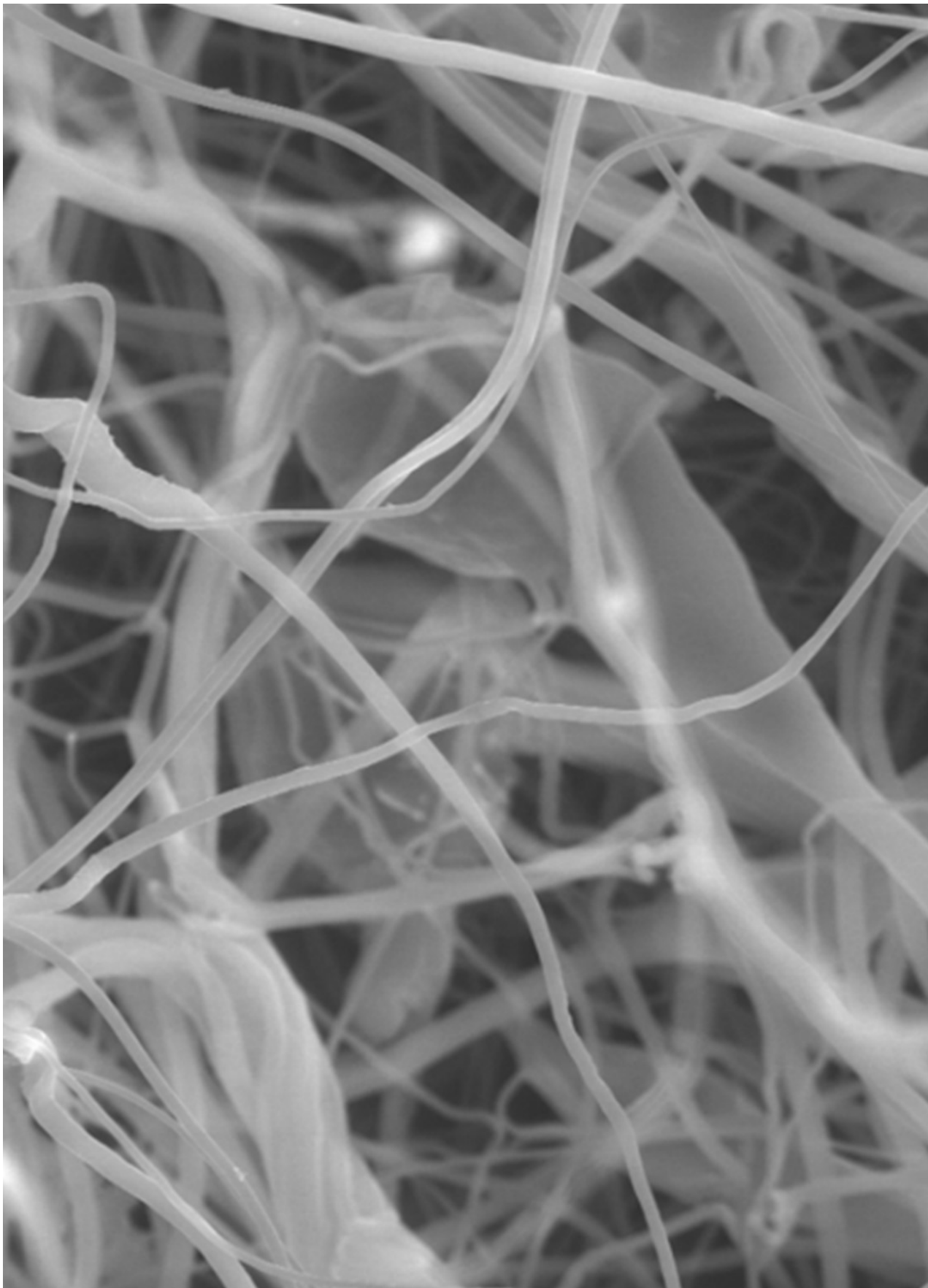
Issue: 250 copies
Nakład: 250 egz.

Scientific Publishing House AKAPIT
Wydawnictwo Naukowe AKAPIT
e-mail: wn@akapit.krakow.pl



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BIOCIDAL PROPERTIES OF COPPER NANOPARTICLES

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Abstract

Metal nanoparticles (NPs) with antibacterial properties represent a promising alternative approach to antibiotics, whose overuse has led to the appearance of drug-resistant bacteria. This article addresses particularly copper (Cu) nanoparticles since Cu is a structural constituent of many enzymes in living microorganisms. In addition, Cu has a better antibacterial effect and minimal cost compared to silver. The properties of Cu nanoparticles are described here: antibactericide, toxicity mechanisms, oxidation, and copper oxide biocompatibility for medical applications. Along with the advantages of Cu nanoparticles, the nanotoxicity still remains to take into consideration such as in targetting different bacteria strains, bacteria's resistance, the effect of size, the effect of NP chemical composition, the effect of oxidation, and the corona phenomenon effect. The methodology of Cu nanoparticles synthesis, related to the biocidal effect, is illustrated by some limitations and some breakthrough such as chitosan stabilizer (CS), laser ablation, plasma induction, and flow-levitation method (FL). Although Cu nanoparticles are beneficial for bacterial elimination, these nanoparticles are graded harmful to the human body and the environment because of their toxic effects. Thus, it requires further improvement and further investigation to create super antibacterial Cu nanoparticles, to develop some interesting research work around this subject, and to reveal some promising medical findings.

Keywords: *antibacterial properties, nanotoxicity, biocompatibility, copper nanoparticles*

[Engineering of Biomaterials 159 (2021) 2-17]

doi:10.34821/eng.biomat.159.2021.2-17

Introduction

In this day and age, nanoparticles (NPs) applied to biology and medicine are found in various applications such as fluorescent biological labels, drug and gene delivery, biodetection of pathogens and proteins, probing of DNA structure, tissue engineering, tumour destruction via heating (hyperthermia), separation and purification of biological molecules and cells, MRI contrast enhancement and on a variety of surfaces to make them water repellent, anti-glare, self-cleaning, ultraviolet light resistant, scratch resistant, and even anti-microbial, antiseptic or disinfectant [1-4]. Several classes of antimicrobial NPs and nanosized carriers for antibiotics delivery have proven their effectiveness for treating infectious diseases, including antibiotic-resistant ones, *in vitro* as well as in animal models [5]. The application of nanomaterials as new antimicrobials should provide new modes of action and/or different cellular targets, compared with existing antibiotics which are somehow promoting multiple drug resistance microbes [6].

To date, the impact of NPs on various microbes (bacteria, biofilm, fungi, and viruses) has not yet been fully studied and their mechanisms of action are not thoroughly known [7–9]. In short, a biofilm is composed of bacterial cells attached to a surface, mixed in extracellular polymeric substances (EPS) produced by the bacteria [10]. It was reported that biofilms are more resistant than planktonic bacterial cells to various stress factors, including heavy metals, toxins, and bactericidal agents. However, the properties of materials at the nanoscale are different from those of the same materials at the micro and the macro scale [11–13]. Since some of these properties are not yet fully understood, researchers are occupied with investigating the possible toxic impact of NPs on microorganisms and endeavouring to pinpoint the toxicity mechanisms involved [14–17].

In this paper, first, we will review some socio-economic data on nosocomial infections and the importance of preventing biofilm formation in the current problematic of bacterial resistance against antibiotics. Second, we will examine how copper nanoparticles can be a promising alternative approach in terms of toxicity for microbes and biocompatibility for human cells. Finally, we will go over the main synthesis methods available nowadays and some strategies to overcome the oxidation layer in order to maintain the integrity of the NPs' properties.

Nosocomial infections (NIs): A Hospital-Acquired Infection

Nosocomial infections (NIs) are an important focus of infection prevention in all countries, but in developing countries, they are a major cause of preventable disease and death [18]. For example, NIs rates range from as low as 1-7% in several European countries and the Americas, to more than 40% in parts of Asia, Latin America, and sub-Saharan Africa [19]. In 1987, a prevalence survey, involving 55 hospitals in 14 developing countries in 4 World Health Organization Regions (Europe, Eastern Mediterranean, South-East Asia and Western Pacific), found an average of 8.7% of all hospital patients had Nis [20]. Thus, at any time, over 1.4 million patients worldwide will have infectious complications acquired in the hospital. According to the European Centre for Disease Prevention and Control, about 4.1 million patients are affected by healthcare-associated infections in the EU every year, including approximately 37 000 lethal cases. The most frequent are related to urinary tract infections (UTIs) (28%), followed by respiratory tract infections (RTIs) (25%) and bloodstream infections (BSIs) (10%) [21].

Many infections are associated with medical devices, such as catheters (urinary, vascular), surgical implants (e.g., vascular grafts, cardiac pacemakers), and mechanical ventilation devices [22,23]. The most significant of them (also shown in FIG. 1) are: i) infections following surgery or invasive medical procedures; ii) UTIs, pneumonia, diarrhoea, and skin lesions; iii) maternal and new-born infections. Overall, implant-associated infections contribute to increased patient morbidity and cost. The adhesion of serum proteins to the implant and the low vascularity in the area of the trauma, create an ideal environment for bacterial adherence [24].

The organisms causing most NIs usually come from the patient's own body (endogenous flora). They may also come from contact with staff (cross-contamination), contaminated instruments and needles, and the environment (exogenous flora) [25,26]. Virtually, every pathogen has the potential to cause infection in hospitalized patients, but only a limited number of both Gram-positive (Gr+) and Gram-negative (Gr-) bacteria are responsible for the majority of NIs. The most common pathogens that cause NIs are *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Escherichia coli* (*E. Coli*) [27]. The most prominent pathogens include methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus*, that are two of the main causes of pneumonia and surgical wound BSIs, [28,29] followed by *E. coli*, a remarkable food-borne pathogen. *E. coli* is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteraemia, cholangitis, UTIs, traveller's diarrhoea, and other clinical infections, such as neonatal meningitis and pneumonia. In the US, *E. coli* is the leading cause of both community acquired and nosocomial UTIs. As many as 50% of women have had at least one episode of UTIs in their lifetime. *E. coli* causes 12-50% of NIs, and 4% of diarrhoeal disease [30].

Additionally, both *Klebsiella pneumonia* (*K. pneumonia*) and *P. aeruginosa* are opportunistic pathogens of pronounced multidrug resistance in individuals with impaired immune systems. Other important candidates include penicillin non-susceptible *Streptococcus pneumonia*, *Enterococcus faecalis,* and vancomycin resistant *Enterococci* [21].

The origin of implant-associated infections can be tracked back to the implantation site, where bacterial colonization and subsequent biofilm formation generate a surface layer highly inert to current medical treatment. Consequently, in many cases, implant removal and/or amputation represent the only alternatives [31]. Upon implantation into the body, a "race for the surface" is initiated between tissue cell integration and microbial colonization, which is generally decided within several hours. Since most of the patients are immunocompromised, the host defence usually fails to prevent colonization prior to tissue integration [32]. Most of the pathogens are found ubiquitously on the patient's skin or in the patient's body and, thus, can freely access the implantation site. In the first 2 h after implantation, bacterial colonization is governed by reversible physical adsorption. After 2-3 h, chemical bridging reactions ensure a much stronger adhesion between bacteria and substrate. Subsequently, within a period of 24 h or longer, the excretion of extracellular substances ensures both microbial multiplication and resistance to mechanical, immunological and medical removal attempts [33]. This biofilm formation is highly dependent on the bacterial species and some colonized devices may not become clinically infected at all, or the infection can emerge only after several months after implantation [22].

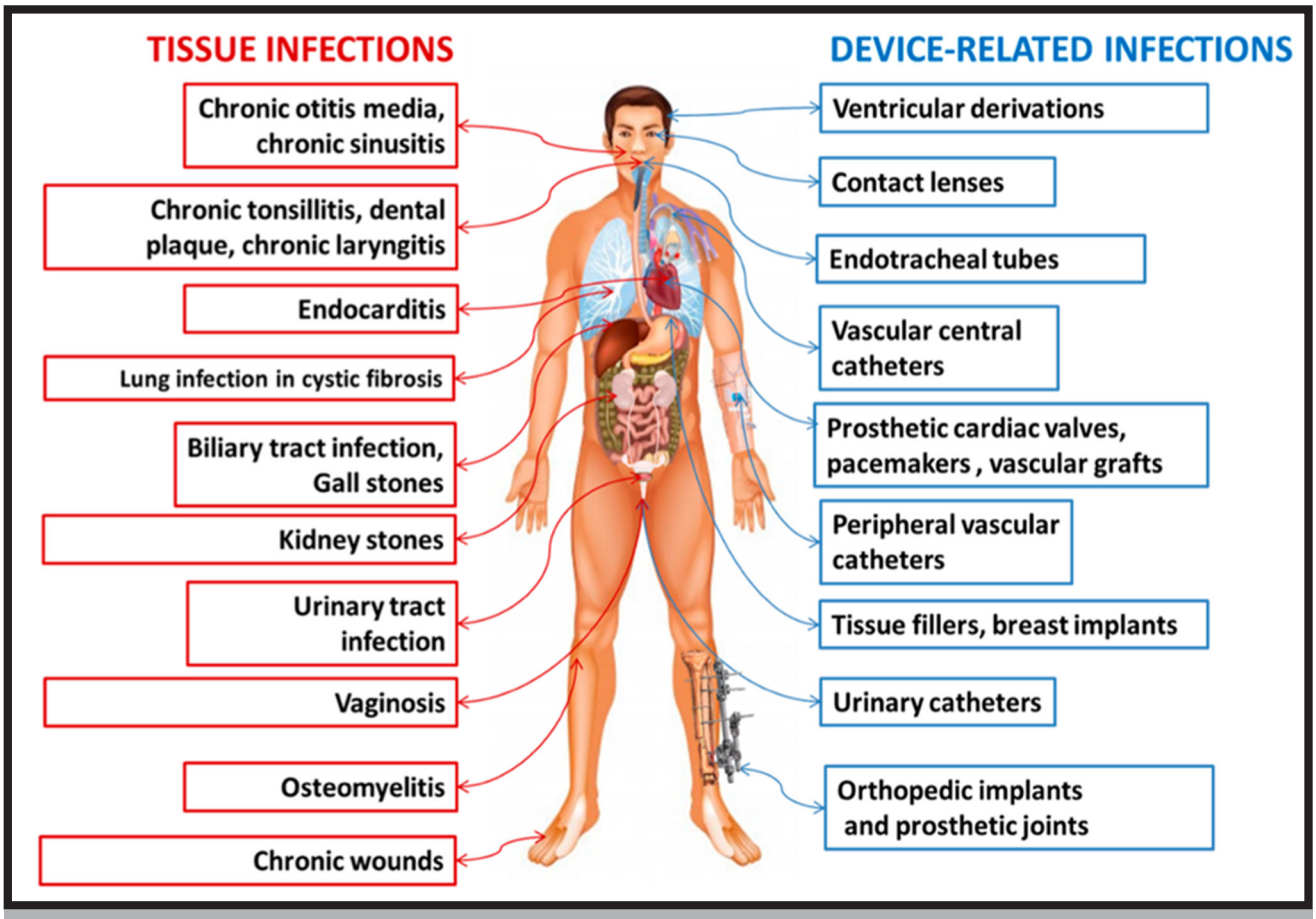

FIG. 1. Typical bacterial infections and their respective biomedical devices [25].

Biofilms are More Resistant than Planktonic Bacteria [34]

A biofilm is composed of bacterial cells attached to a surface, mixed in Extracellular Polymeric Substances (EPS) produced by the bacteria [10]. It is a natural tendency of microorganisms to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of EPS that they produce, forming a biofilm. The National Institutes of Health revealed that among all microbial and chronic infections, 65% and 80%, respectively, are associated with biofilm formation [35]. There is good evidence indicating that the biofilm mode of life leads to increased resistance to antimicrobial products [36,37]. Biofilm-associated bacteria are more resistant to antimicrobials, compared to planktonic cells, and this makes their elimination a great challenge [38,36]. Moreover, the emergence of bacteria resistant to conventional antimicrobials clearly shows that new biofilm control strategies are required [38,37]. As biofilm formation mechanisms will only be discussed briefly, the reader is directed to several excellent comprehensive reviews in this area [10,39,40].

The attachment of microorganisms to surfaces and subsequent biofilm development are complex processes, affected by numerous variables including heavy metals, toxins, and bactericidal agents. Several recent studies [41–45] have shown a role of cell-to-cell chemical signalling (quorum sensing) in biofilm formation [41], where this system determines extracellular matrix fine-structure by controlling its secretion through the bacterial cell wall [42]. Biofilms form a gel phase, within which microorganisms live [43]. The EPS matrix acts as a barrier, in which diffusive transport prevails over convective transport [43]. A function frequently attributed to EPS is their general protective effect on biofilm microorganisms against adverse conditions. As an example, it has frequently been observed that biofilm cells can tolerate high concentrations of biocides [36,44,45]. The EPS matrix delays or prevents antimicrobials from reaching target microorganisms within the biofilm by diffusion limitation and/or chemical interaction with the extracellular proteins and polysaccharides [44]. Moreover, within the EPS matrix, the molecules required for cell–cell communication and community behaviour may accumulate at concentrations high enough to be effective [43]. Ideally, preventing biofilm formation would be a more logical option than treating it. However, there is presently no known technique that can successfully prevent or control the formation of unwanted biofilms without causing adverse side effects.

The main strategy in preventing biofilm formation is to clean and disinfect regularly, before bacteria attach firmly to surfaces [46,47]. Several attempts have been made to avoid biofilm formation by the incorporation of antimicrobial products into surface materials, by coating surfaces with antimicrobials [48] or by modifying the surface physicochemical properties [49]. Other authors reported biofilm formation inhibition by coating surfaces with silver [50]. It was observed that planktonic organisms are more susceptible to environmental stress and bactericidal agents, compared to biofilms [51].

In the environment, bacteria can attach to various surfaces and live as an organized biological system, in a secreted glycocalyx matrix [52,53]. This biofilm provides additional protection for bacteria from environmental stress, toxins, and bactericidal agents. Costerton et al. (1999) [54] hypothesized that the ionic bonds between the bactericidal agent and the glycocalyx matrix caused the depletion of bactericidal agents with a depth of the biofilm matrix. It was reported that bacteria in biofilm produce more EPS under environmental stress or exposure to toxic compounds [55]. Interestingly, the chemical and physical properties of EPS may vary among bacterial types and strains [56].

Bacterial Resistance to Antibiotics is an Increasing Threat

Another major limitation of antimicrobial therapy is the development of bacterial resistance to antibiotics. The resistance of bacterial pathogens to traditional antibiotic therapy is an increasing threat. The resistance of microorganisms to antibiotics is steadily rising, with reports showing that quite a number of the presently recognized antimicrobial agents are less effective and resisted by one species of microorganisms or another, so it appears to be no single antimicrobial agent, available for human and animal use, which is not resisted by some microorganisms. Gram-negative bacteria include pathogenic strains of *E. coli*, *Acinetobacter baumannii* (*A. baumannii*), *K. pneumonia* and *P. aeruginosa*, all of which, are increasingly resistant to most available antibiotics. More than 70% of bacteria causing infections are now resistant to at least one of the drugs most commonly used for treatment. A global medical challenge in the 21st century is the treatment of vancomycin-resistant microbes, because vancomycin is the latest generation of antibiotics and is assumed most effective against *S. aureus* infection [42]. One of the most recent new wave of “super super bugs” came with the emergence of mutant New Delhi metallo beta-lactamase NMD-1, which is an enzyme that confers bacterial multiple drug resistance [57]. It first emerged in New Delhi and has now spread worldwide from Britain to New Zealand. In 2009, *K. pneumoniae* was the first bacterium identified to produce NMD-1 in a patient with an infection that did not respond to many antibiotics [58]. Two classes of antibiotic-resistant pathogens are emerging as major threats to public health. First, MRSA is estimated to cause ~19,000 deaths per year in the U.S. [59]. Apart from their high mortality rate, MRSA infections lead to an estimated $3-4 billion US dollars of additional health care costs per year. Further, the rising prevalence of MRSA increases the likelihood that vancomycin resistant *S. aureus* [60]—just as deadly as MRSA, but more challenging to treat—will become a new scourge in hospitals. Pathogens from the second class, multidrug-resistant (MDR) and pan-drug-resistant (PDR) Gr- bacteria, are less prevalent than MRSA, but they pose the grave threat of infections that are truly untreatable [61]. These strains of *A. baumannii*, *E. Coli*, *K. pneumoniae*, and *P. aeruginosa* are resistant to some MDR or all PDR of the antibiotic classes commonly used to treat Gr- bacteria such as penicillin, cephalosporins, carbapenems, monobactams, quinolones, aminoglycosides, tetracyclines, and polymyxins [61]. Prospects for finding new antibiotics for Gr- pathogens are especially poor: their outer membranes block the entry of some antibiotics, and efflux pumps expel many of the remainder. Despite the rise of resistant pathogens, the rate of new antibiotic approvals is dropping. Where will new antibiotics come from? In the past, this question was generally answered through the synthetic tailoring of a small group of “scaffolds.” Novel therapeutic strategies to tackle bacterial infection are imperative to maintain the ability to rapidly evolve antibiotic treatment regimens.

Nanotechnology approach

In comparison to conventional antibiotics, nanostructured antimicrobial agents help in reducing toxicity, overcoming resistance, and lowering the cost. Metal and metal oxide NPs offer a new line of research in combating infectious diseases due to resistance developed by several pathogenic bacteria against antibiotics. An advantage of these nano-antibiotics is that naturally occurring microbes have so far not developed a known resistance against them.

Moreover, they use multiple biological pathways to exert their antimicrobial mechanisms such as disruption of the cell wall, inhibition of DNA, protein, or enzyme synthesis, generating photocatalytic reactive oxygen species (ROS), and some unexpected activities which are absent in previously used antibiotics and disinfectants. In addition, the preparations of these NPs are more cost-effective than antibiotic syntheses, they are also more stable during long-term storage and, unlike antibiotics, can withstand harsh processing conditions, such as high pH and temperature, without being inactivated [62].

Copper (Cu) and Copper Oxide (CuO) as Antibacterial Candidates

There are two main reasons for inspecting the biological activity of copper NPs (Cu-NPs). Firstly, as Cu-NPs are released into a natural ecosystem and human environment when applied as catalyst components, anticorrosive coatings, and conducting materials, it is of utmost importance to consider the risks caused by them [63,64]. Cu is a structural constituent of many enzymes in living organisms and it can generate toxic effects at high concentration, when in free ionic form, by generating ROS that disrupt the DNA and amino acid synthesis [65]. Indeed, Cu-NPs could then present some risks, since an excess of Cu in the human body leads to the generation of the most damaging free radicals, such as the hydroxyl radical (•OH) [66]. Secondly, Cu-NPs could play important roles in developing a new generation of drugs for medicine and agriculture thanks to their known antibacterial potential [67,68]. Therefore, it becomes necessary and of interest to identify the underlying mechanism(s) of its biological activity.

The antimicrobial activity of Cu-NPs against several microorganisms has already been reported [69–73]. One of us (Deryabin et al., 2016) [74], for instance, showed in a comparative study with 8 metal and metal oxide NPs, and 10 carbon-based NPs, that the Cu- and CuO-NPs had the highest antibacterial activity against *B. subtilis* and *E. coli* but were less effective against the marine bacterium *Photobacterium phosphoreum*. A study investigating the toxicity of Cu-NPs on microorganisms by Yoon et al. [71] reported their preliminary studies on the toxicity of Cu-NPs on *E. coli* and *B. subtilis* using agar plate assay. Their assay showed the antimicrobial characteristics of the particles on both microbes tested. Moreover, Cu-NPs are very reactive because of their high surface-to-volume ratio and can easily interact with other particles and increase their antimicrobial efficiency. For instance, Cu monodispersed NPs (2-5 nm) have revealed a strong antibacterial activity and were able to decrease the microorganism concentration by 99.9% [69].

Ren et al. (2009) [75] investigated the antimicrobial potential of CuO-NPs generated by a thermal plasma technology that contained traces of pure Cu- and CuO-NPs, against a range of bacterial pathogens, including MRSA and *E. coli*. Their study revealed that the ability of CuO-NPs to reduce the bacterial populations to zero was enhanced in the presence of a subminimal inhibitory concentration of silver or copper NPs. However, Cu is a component in many vital processes in cells including microorganisms. Therefore, bacteria will want to find an adequate balance in Cu quantity as a cofactor to prevent toxicity from a high amount [76,77]. The minimum inhibitory concentration being the lowest concentration of NPs that completely inhibits the initiation of growth of a bacterium. Ruparelia et al. (2008) [78] showed that Cu-NPs have a greater affinity toward the carboxyl and amine groups at high density on the surface of *B. subtilis*, than that of silver NPs, showing superior antibacterial activity. CuO, being less costly than silver, and easily miscible with polymers, can be an alternative to silver NPs.

Toxicity Mechanisms of Copper-NPs Compared to Copper Microparticles and Bulk Forms

Cu is an essential micronutrient, being part of the structure of several enzymes and other proteins. It is also a redox-active metal, existing in two major oxidation states, Cu^{1+} and Cu^{2+}. The redox cycling of Cu between Cu^{1+} and Cu^{2+} catalyses the intracellular production of hydroxyl radicals, •OH [79,80]. Cu is an important cofactor for many enzymes, although some *in vitro* analysis, using comet assays and Trypan blue staining, suggested that Cu-NPs cause DNA damage and cytotoxicity [81].

Since cytotoxic effects reflect the holistic damage induced by the particles, the cytotoxicity of Cu-NPs, as well as Cu-Microparticles (MPs) and soluble Cu^{2+} were tested against the host strain, *E. coli* RFM443 [81]. It was found that Cu-NPs possess higher biotoxicity to the ecosystem than Cu-MPs and Cu^{2+}, due to their multiple and severe damage effects at much lower toxic concentrations. Moreover, Cu-NPs induce not only oxidative stress in *E. coli*, but also protein, DNA, and cell membrane damage, and ultimately cause cell growth inhibition. This was demonstrated in a study, in which small Cu-NPs (4-5 nm) caused similar dose-dependent degradations of isolated DNA molecules, via the generation of singlet oxygen $^1[O_2]$ in both U937 (from human lung lymphoblast) and HeLa (from cervical cancer cells) cells [82].

Li et al. (2013) [83] used spherical, homogeneous, crystalline Cu-NPs and Cu-MPs, with primary particle sizes of about 90 nm and 10 µm, respectively; when exposed to the ambient atmospheric conditions, both surfaces became partly oxidized into CuO. Compared with Cu-NPs, Cu-MPs showed totally different effects on cells and no obvious responses of any strains were observed, although these two kinds of particles had the same chemical composition. These results imply that the major toxic effects of Cu^{2+} ions are membrane damage and protein damage, which occur only at high concentrations (>140 mg/L), whereas Cu-NPs cause all four kinds of damaging effects (i.e., oxidative stress, and DNA, membrane, and protein damage) at low concentrations (maximum at 80 mg/L). Moreover, the different concentration profiles and damage effects between Cu-NPs and Cu^{2+} ions suggest that the toxic mechanism of Cu-NPs probably does not depend on Cu^{2+} ions.

Cu is an essential nutrient in many organisms, and enzyme-associated Cu is a requirement for aerobic metabolism. On the other hand, excess accumulation of Cu, or intracellular release of free Cu, leads to severe toxicity. Aerobically, Cu readily catalyses reactions that result in the production of hydroxyl radicals •OH through the Fenton and Haber-Weiss reactions ($Cu^{1+} + H_2O_2 \rightarrow$ •OH + HO^- + Cu^{2+}; $•O_2^-$ + $Cu^{2+} \rightarrow Cu^{1+} + O_2$) [84,85]. The highly reactive oxygen intermediates are responsible for lipid peroxidation, oxidation of proteins, and cleavage of nucleic acids [85–87]. Additionally, free Cu-ions are able to oxidize sulfhydryl-groups, such as cysteine in proteins or the cellular redox-buffer glutathione [87,88]. Finally, Cu might cause inactivated proteins by replacing related metal cations such as those of zinc or iron from active sites.

Therefore, intracellular free Cu-concentrations are low and tightly regulated [89]. Thus, tight regulation of uptake, distribution and excretion of Cu is necessary for maintaining optimal cellular Cu levels. Defining the molecular mechanisms of Cu metabolism, including Cu uptake, intracellular trafficking, incorporation into Cu-requiring proteins, excretion, and regulation of these processes, is an important emerging research area. While high affinity Cu transporters play a critical role in Cu acquisition, excess Cu can enter cells by non-specific mechanisms. Once Cu is transported across the cell membrane, it must efficiently reach its appropriate destinations to be incorporated into Cu-requiring proteins without participating in harmful reactions. It has been demonstrated that target-specific cytosolic Cu carriers distribute Cu in eukaryotes ranging from yeast to humans [78,90,91].

Toxicity Mechanisms of Copper Oxide-NPs Compared to Copper Oxide-Microparticles and Bulk Forms

Since there is a published review paper available regarding the effects of DNA damage induced by CuO-NPs in *in vitro* studies [92], only a few studies are mentioned here. DNA damage, as a result of oxidative stress, identified by increased levels of 8-isoprostane and the ratio of glutathione disulphide to total glutathione (GSSG/GSH) in human airway epithelial (Hep-2) cells, has been reported [93]. Oxidative stress increased the expression of plasminogen activator inhibitor-1 (PAI-1), by mediating p38 phosphorylation in endothelial cells treated with CuO-NPs (42 and 200 nm) [93]. Elevated oxidative stress may lead to DNA damage, which, in turn, has the potential for carcinogenesis. In another study by Karlsson et al. (2008) on A549 cells (from adenocarcinomic human alveolar basal epithelial cells), CuO-NPs were the most potent, regarding cytotoxicity and DNA damage [94].

In a study conducted in 2009 by K. Kasemets et al. [95], toxic effects of ZnO-, CuO- and titanium oxide (TiO_2)-NPs on the single-cell eukaryotic organisms *Saccharomyces cerevisiae* (yeast), were evaluated. The effect of metal oxide NPs, as well as bulk oxide and ion formation, were compared. Both forms of ZnO showed the same toxicity, while CuO-NPs were60 times more toxic than bulk CuO. There was increased toxicity for both CuO-NPs and bulk CuO after 24-hour exposure when compared to an 8-hour exposure, due to an increased Cu ion dissolution. CuO-NPs were found about 60-fold more toxic than bulk CuO. In fact, at 8 h, the maximal effective concentrations needed to induce 50% response after exposure called EC50, for NPs and bulk, were, respectively, 20.7 and 1297 mg CuO/L and, 13.4 and 873 mg/L at 24 h. The increase in toxicity of both CuO forms after 24 h was due to the increased dissolution of Cu ions from CuO over time. A comparison of EC50 values for CuO-NPs, bulk CuO and Cu^{2+}, with bioavailable Cu concentrations in the growth medium, showed that the solubilized Cu ions explained only about 50% of the toxicity of both NPs and bulk CuO.

Three studies on CuO-NP-induced DNA damage in *in vitro* cellular models reported detection of significant single-strand break (SSB) lesions, using the alkaline comet assay [81,77,94], and two of the studies specifically applied the Fpg-modified comet assay (formamidopyrimidine-DNA glycosylase as a biomarker for the detection of oxidative DNA damage) to detect significant SSBs, due to the accumulation of oxidatively modified purine lesions [77,94]. Interestingly, all three studies noted significant oxidative damage to DNA at NPs concentrations of 80 μg/mL and, in each case, the DNA damaging effects of CuO-NPs were stronger than those of CuO-MPs or than added Cu^{2+} ions, or Cu^{2+} ions released from the CuO-NPs themselves. The mechanisms behind the induction of DNA damage are not clear, but it is apparent that released Cu^{2+} ions are not the causative factor for *in vitro* oxidative stress and the resulting DNA damage.

Cells treated with CuO-NPs presented reduced catalase and glutathione reductase enzyme activities and elevated glutathione peroxidase activities. The increasing ratio of glutathione disulphide (GSSG) to glutathione (GSH) indicated that CuO-NPs produce ROS and obstruct cellular antioxidant defences [96]. It is valuable information that CuO-NPs had higher toxicity than other metal oxides *in vitro* [97].

Moreover, it was shown that both CuO-NPs and bulk CuO exhibit higher toxicities on biofilms, compared to the low toxicity of 35% observed for Cu ions on planktonic cells. The concentration of Cu ions had to be 250 ppm to suppress bacteria growth completely; however, 90% of biofilm growth could be inhibited by 0.02 ppb [76]. Nonetheless, the detailed mechanism needs to be further investigated.

Cu/CuO-NPs caused a range of effects, including oxidative stress, cytotoxicity, neurotoxicity, DNA damage and DNA lesions, in a variety of cell lines. For Cu ions, the mechanism may involve oxidative stress [67]. The redox cycling of Cu ions results in the depletion of glutathione and affects the sulfhydryl groups of proteins, causing DNA damage and lipid oxidation [79]. Likewise, other *in vitro* studies using cultured lung epithelial cells have shown that exposure to Cu containing NPs led to increased intracellular ROS formation, oxidative DNA damage, and cell death [98,99]. Rushton et al. [100] showed that the native oxidant potential of metallic Cu-NP powders – as determined via oxidation of dichlorodihydrofluorescein and other methods – correlated well with oxidant stress related inflammatory mediator production in cultured cells and with their acute *in vivo* inflammatory potency.

Copper NPs Biocompatibility for Medical Applications

In 2008, Williams et al. [101] redefined biocompatibility as follows: ''biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy but generating the most appropriate beneficial cellular or tissue response in that specific situation and optimising the clinically relevant performance of that therapy''. In this context, NP toxicity refers to the ability of the particles to adversely affect the normal physiology, as well as to directly interrupt the normal structures of organs and tissues in humans and animals. It is widely accepted that toxicity depends on physicochemical parameters, such as particle size, shape, surface charge, chemistry, composition, and NP stability.

Cu is considered as an essential micronutrient, necessary for the proper growth, development and maintenance of bone, connective tissue, brain, heart and numerous other organs [102–104]. It is also involved in the stimulation of the immune system to fight infections, repair injured tissues and promote healing [105], and it aids neutralization of free radicals, which cause severe cell injury [106]. The average level of stored Cu in the body (mostly in the liver) is approximately 120-150 mg. Cu is an essential element for maintaining homeostasis in organisms [107]. Cu ions will become toxic once their concentration is not tolerated in the physiological range [108,109].

Toxicity assessment studies have primarily focused on investigating the effects of different exposure routes, such as the respiratory or gastrointestinal tracts. Yokohira et al. [110] analysed the carcinogen test from lung biopsy after intratracheal instillation of CuO-NPs. Histopathological assessment showed that they induced severe acute inflammatory changes in the rat lung at high doses, and chronically at low doses or with frequent instillations. Karlsson et al. [94] illustrated that CuO-NPs could lead to cell cytotoxicity and DNA damage in the human lung epithelial cell line A549. Oxidative lesions were verified by measuring the intracellular production of ROS with the oxidation-sensitive fluorescent probe, 2',7'-dichlorofluorescin diacetate. Associating the relationship between ROS generation and DNA damage, Chen et al. [111] reported that oxidative stress caused by CuO-NPs was the prior toxic effect. França et al. (2013) [112] investigated the effects of three chitosan differing only in their degrees of deacetylation and of carboxyethyl chitosan, as candidates for Fe_3O_4 NPs coatings. All the samples were found to be biocompatible and nontoxic before sterilization and remained so subsequently.

Cu ions may cause toxicity once they exceed the physiological tolerance range *in vivo* [113]. Therefore, the possible health effects and toxicology of CuO-NPs have caused great concern to both the public and scientific researchers.

Generally, metallic NPs show antibacterial and antifungal activity, even though there are environmental and human safety concerns regarding the release and consumption of metal NPs which are yet to be explored. Excessive release of silver, for example, causes environmental pollution which in turn makes silver harmful to humans and animals. And Cu is no exception, because an excess of Cu in the human body leads to generation of the most damaging radicals, such as the •OH [66]. However, there are Cu-transporting adenosine triphosphatases, including ATP7A and ATP7B, which play an important role in Cu homeostasis and export excess Cu through the intestine (ATP7A) as feces, the liver (ATP7B) as a bile product, and the mammary gland (ATP7B) as milk [66,114].

Copper NPs Nanotoxicity: Concerns and Challenges

Strain Differences: *E. coli* (Gr-) and *S. aureus* (Gr+)

It was previously hypothesized that a thick peptidoglycan layer of bacteria is a significant barrier to the NPs but in fact Gr+ bacteria often are more sensitive than Gr-. Data from previous studies suggest that NPs toxicity may vary significantly between Gr+ and Gr- bacteria, and in some cases, vary by organism [17]. For example, a range of studies showed higher antibacterial action of silver NPs against Gr- *rods* than against Gr+ *cocci*. The results indicated higher sensitivity of *E. coli* than *S. aureus*: the minimum inhibitory concentration against *E. coli* was in the range of 3.3-6.6 nM while against *S. aureus* – more than 33 nM. These differences in susceptibility were explained by the different structure of the cell wall, that is, by the higher concentration of peptidoglycan in the cell wall of *S. aureus*. Also, because peptidoglycan is a unique structure of bacteria absent in mammal cells Cu-NPs are promising agents with directed action on microorganisms by the facilitated influx of smaller-sized NPs into the cell wall of Gr- bacteria which consists of a unique outer membrane layer and a single peptidoglycan layer as compared to the cell wall of Gr+ bacteria with several peptidoglycan layers [115,116]. Thus, the cell wall is more exposed to NPs through the outer bacterial membrane. The unique high surface to volume ratio of Cu-NPs enables them to interact with the bacterial cell membrane through its surface [117], which leads to the death of the bacterium [118]. These findings underline that the size of the NPs plays an important role in their antimicrobial activity.

Bacterial Resistance to NPs

The scientists hope that the microbes are less likely to develop resistance to NPs, which means it could be used to combat the emerging problem of antibiotic resistance. Some evidence suggests that NPs accelerate horizontal gene transfer between bacteria, helping them to become resistant against multiple antibiotics. As Zhigang Qiu et al. [119] described in the Proceedings of the National Academy of Sciences, the transfer of genes can increase by up to 200 times, helping diseases to acquire resistance against antibiotics from harmless gut bacteria in wastewater. Nanoalumina in water increased the horizontal transfer of multidrug-resistance genes across genera with an increase in the concentration of nanoalumina, density of parent bacteria, conjugation time, and temperature. In contrast, aquatic factors had no or little effect on the transfer of antibiotic resistance genes. The mechanisms by which nanoalumina promotes the transfer of drug-resistance genes may involve the damage of bacterial membranes by oxidative stresses, an enhancement of the expression of conjugative genes, and the repression of global regulatory factor genes for RP4 plasmid conjugation. Qiu et al. (2012) [119] suggest that the application of nanoalumina in water and waste treatment should be evaluated carefully so as not to cause public health and environmental and ecological hazards. Further investigations are needed regarding other types of NP's contribution in bacterial resistance.

Bacterial Resistance to Metals

Several resistance mechanisms to metals have been described, the most common, which is enhanced efflux of metal ions from the cell, is a high-level, single-step and target-based mutation. This mutation enhances the efflux of metal ions from cell and makes metal resistance less probable owing to its multifaceted mode of action [120]. Bacteria have been shown to develop resistance to various heavy metals, including ionic Cu [76] and CuO [121]. Resistance mechanisms to heavy metals include sequestration of metals into complexes, reduction of a metal to a less toxic species and direct efflux of metal out of the cell [122]. However, these mechanisms are obviously ineffective for resistance to NPs, and may play a role in the removal of ions dissociating from the NPs surface only. Nonetheless, little is known about the effectiveness of CuO-NPs on biofilms and the changes in biological activity as a function of the size and shape of the NPs. Interactions between NPs and different bacterial structures are studied better for silver NPs; the mechanism of action of other NPs is still not well understood, especially the effect and mechanism of action of NPs combinations on bacterial cells.

Effect of NPs Size: Ions, NPs, MPs, and Bulk Material

A prevalent number of reports stated that smaller NPs demonstrated a higher toxicity rate in comparison to bigger NPs or bulk (micro scale) materials [123-125]. However, it was also observed that materials in NPs were more toxic than in MPs [94]. Other studies credited observed toxicity to soluble metal ions [126,127]. Moreover, it was shown that the toxicity of nanosized oxides (CuO-NPs) was much higher than their bulk counterparts [128]. The decline in the biotoxicity level of the Cu compounds in a series ions → NPs → MPs was observed in good agreement with the results of animal studies [111] estimating Cu-NPs toxicity (LD50 = 413 mg/kg) as being below the one of $CuCl_2$ but exceeding the one of MPs (LD50 = 110 mg/kg and LD50 > 5000 mg/kg, respectively). At the same time, the results indicate the similarity of this mechanism for various Cu compounds with NPs being less effective than Cu^{2+} ions but superior to MPs of this metal. Thus, nanosized particles are more toxic. This is also a possible reason to explain the toxicity of CuO-NPs, which exceeded that of bulk CuO by about 45- or 50-fold [64]. Midanderet al. [81] also showed that Cu-NPs were much more toxic than Cu-MPs in terms of DNA damage and cytotoxicity *in vitro*. Moreover, thisstudy [81] demonstrated that Cu can be released from CuO-NPs, and the soluble quantity was different in various media. Though NPs released more Cu ions than the MPs, the released fraction did not contribute significantly towards cytotoxicity when compared to particles themselves [92].

Effect of NPs Chemical Composition

Chemical composition of NPs is the base factor associated with antibacterial properties. Hence, the choice of an appropriate chemical element is extremely important and is linked to biocompatibility as well as toxicity properties [129]. For instance, Heinlaan et al. [130] pointed out that the toxicity of three oxides (both nano and bulk) to the Gr- bacteria *Vibrio fischeri* decreased as follows: TiO_2<CuO< ZnO.

Babushkina et al. (2010) [131] compared the antibacterial properties of Cu and iron NPs of 30-40 nm and 30-70 nm, respectively, on 10 clinical multidrug-resistant isolates of *S. aureus*. The authors revealed more noticeable antibacterial action of Cu-NPs on *S. aureus* compared with iron NPs: 30 min exposure to Cu-NPs even at the concentration of 0.001 mg/mL significantly reduced the number of living cells compared with a control without NPs, while iron NPs reduced bacterial growth only starting from 0.1 mg/mL concentration after 30 min exposure. Moreover, besides chemical composition, it was demonstrated in the study of Raffi et al. (2010) [132] that the antibacterial activity of Cu-NPs against *E. coli* behaved in a concentration-dependent way in liquid and solid media where 100 μg/mL of Cu-NPs of 12 nm totally inhibited bacterial growth, while concentrations of 20 and 40 μg/mL were less effective [64,95].

Oxidation of Copper based-NPs Results in Particle Aggregation and Reduces ROS Production Capabilities

Copper-based-NPs on the other hand face a major limitation which is rapid oxidation upon exposure to air, which can result in particle aggregation [133]. As the oxide layer is formed, the ROS generating capacity of the Cu-NPs is reduced. Since Cu oxidizes to CuO and cuprous oxide (Cu_2O), and it converts to Cu^{2+} during preparation and storage, it is difficult to synthesize pure Cu-NPs in an ambient environment. Therefore, alternative pathways exist to preserve metal NPs antibacterial properties. In fact, the idea is to form a coating on the surfaces of NPs, generally in the presence of polymers (e.g., polyvinyl pyrrolidone, polyethylene glycol, and chitosan) and surfactants (cetyl trimethyl ammonium bromide) as stabilizers. In fact, even though the Cu nanopowder can be completely reduced by a strong reducing agent, such as hydrazine or sodium borohydride, it can also be instantly oxidized by the dissolved oxygen (O_2(aq)) that exists in the aqueous reduction system [134,135]. Therefore, the development of an efficient approach for preventing the oxidation of Cu nanopowders during synthesis in an aqueous solution is required urgently.

Corona Phenomenon Effect

The organic rich environment present in nature is simulated by the presence of organic media ingredients in the nutrition broth. Kim et al. (2012) [136] demonstrated that the uncontaminated and surfactant-free NPs themselves possess moderate cytotoxicity to human cells in a cell-dependent manner. They used MTT assays using HeLa human cervical cancer cells, PC3 human prostate cancer cells, and MCF-7 human breast cancer cells, to address the inherent toxicity of the prepared ultra-pure NPs with nascent surfaces. Cells were maintained in tissue culture plates at 37°C in an atmosphere of 5% CO_2 in MEM/EBSS (Minimum Essential Medium with Earle's Balanced Salts) media for HeLa cells and in RPMI-1640 media for PC3 and MCF-7 cells, all supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. In order to keep cells in log phase, the cultures were re-fed with fresh media two or three times/week. However, the MEM/EBSS or RPMI-1640 media contains inorganic salts, amino acids, vitamins and other components which could «contaminate» these supposed «ultra-pure» NPs by the corona phenomenon. The corona phenomenon which depends on nanoparticle properties such as surface charge, hydrophobicity, presence of ligands, size, and morphology; medium composition such as protein source; medium condition such as pH; and exposure time; is defined as the phenomenon where NPs or any synthetic material will become quickly covered by resident proteins when they come in contact with any biological fluid. The corona layer composition depends on all previously mentioned NPs' properties and its architecture is made of two distinct layers: the hard corona, in direct contact and strongly interacting with the NP; and the soft corona, more external and unsteady.

Eco-Toxicity Depends on NPs Concentration Accumulation and Should Be Addressed Early

Since modern environment contains enhancing amounts of metal NPs, including NPs of Cu, in electronics, in biomedicine and in other technical processes, an evaluation of potential risks against human and animal health is of great importance. While the bactericidal effect for deliberate disinfection of pathogenic bacteria via application of antimicrobial coatings to various surfaces [137,138] is a desirable feature, the uncontrolled and unmanageable potential bactericidal effect from NPs released into the environment could damage the ecosystems of creeks, rivers, wetlands and other ecological systems that harbour bacterial communities. The accumulation of NPs in the environment might shift ecological niches and damage ecological systems.

Environmental research into CuO-NPs' toxicity has mostly focused on the effects on organisms, especially those in aqueous environments. The most common experiment models are algae and zebrafish, whose growth and toxicity are treated as environmental relevance indicators. Aruoja et al. [126] studied the toxicity of CuO-NPs on the algae *Pseduokirchneriella subcapitata* using bulk formulation of metal oxide as a control. At low concentrations, CuO-NPs (EC50 = 0.71 mg Cu/L) were more soluble and more toxic than the control (EC50 = 11.55 mg Cu/L). The results showed that the toxicities of bulk and nanosized CuO were largely influenced by soluble Cu ions. These findings were similar to the conclusions drawn by Grosell et al. [139] and Griffitt et al. [140]; whose publications both proved that the soluble Cu forms were highly toxic to fish. Some studies also reported that CuO-NPs' suspensions might damage gill lamellae and inhibit epithelial cell proliferation by altering plasma metal levels [140], as well as chloride cell number and diameter [141]. Therefore, Gomes et al. [142] considered that mussel digestive gland could aggregate CuO-NPs and result in toxicity. The results of Shi et al. [143] indicated that CuO-NPs decrease chlorophyll content of the duckweed, and that CuO-NPs toxicity is three to four times higher than that of ionic Cu, because of the larger uptake of NPs-released Cu. Griffitt et al. [140] compared the responses of fish exposed to Cu-NPs solution and soluble Cu and reported that the effects of gill morphology and transcription were not solely due to the dissolution of Cu-NPs.

Cu-NPs Synthesis Methods Reviewed

The synthesis procedures affect to a great extent the properties of Cu-NPs. Chemical, physical and biological techniques, considering bottom-up or chemical or biological methods and top-down or physical methods have been studied, FIG. 2 shows the different synthesis methods for Copper Oxides NPs [144].

The main techniques for NPs synthesis through the chemical approach are: Chemical reduction [145], microemulsion (colloidal) techniques [146], sonochemical reduction [144], electrochemical [147], microwave-assisted [148], and hydrothermal [149] synthesis. Biological or biosynthesis [150], techniques are also considered as bottom-up or chemical processes.

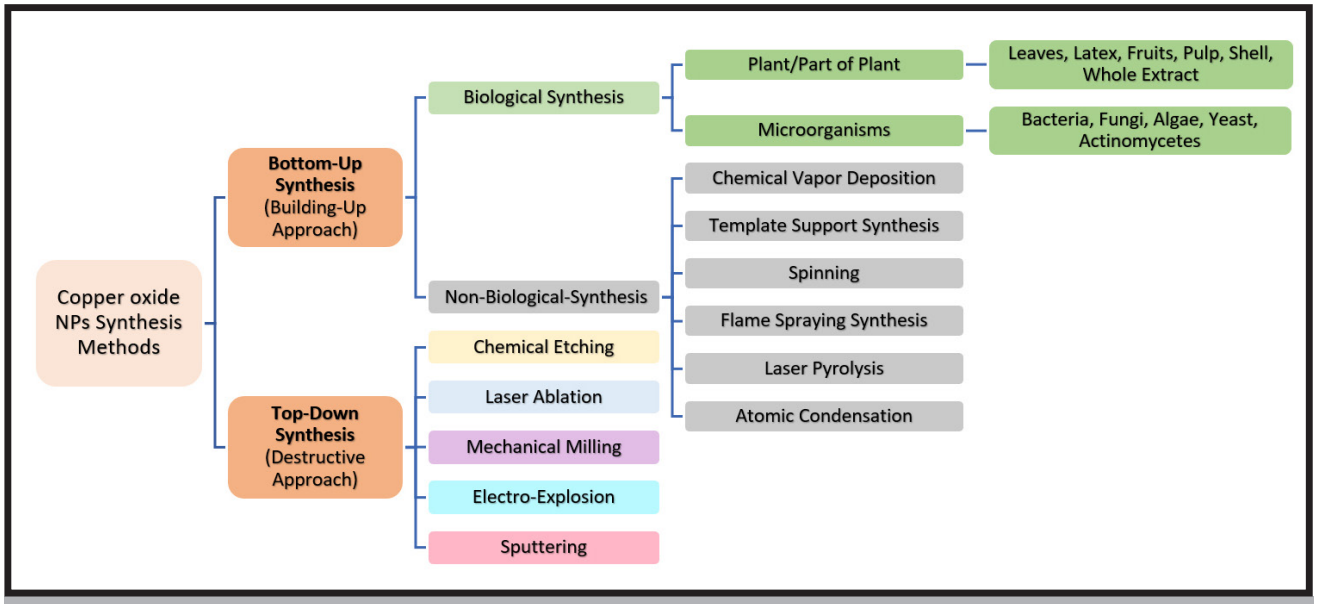

FIG. 2. Copper Oxide Nanoparticles synthesis methods [155].

Physical methods for nanoparticles synthesis are laser (pulse) ablation [151], vacuum vapour deposition [152], pulsed wire discharge [153], and mechanical milling [154]. The main disadvantages of these physical methods are the quality of the product, which is less as compared to nanoparticles produced by chemical methods. Additionally, these methods require costly vacuum systems or costly equipment to prepare nanoparticles such as plasmas.

Why Chemical Reduction is Often Preferred?

Among all methods, the chemical reduction is the most preferred because it has the advantages of being simple and economical, and it is also easy to control the particle size and morphology of the generated NPs. The following TABLE 1 summarizes the variety of NPs sizes produced and the concentration and incubation time used to assess a bactericidal effect against *E. coli* and *S. aureus*. Most reactions are carried out in water given the considerations of mass production, cost, and environmental consistency. However, using water as the solvent for the chemical reduction of Cu is a great challenge in contrast to its use for the synthesis of Au or Ag nanopowders because Cu is less chemically stable than Cu^{2+} and Cu^{1+} oxides; thus, a major problem is the usual occurrence of surface oxidation during its synthesis.

A detailed comparative study published by Umer et al. (2012) [156] revealed that chemical reduction methods are most suitable for the synthesis of Cu-NPs. Chemical reduction of Cu salts using ascorbic acid (Vitamin C) is a new and green approach in which Vitamin C is used both as the reduction and the capping agent. This approach is the most effective and is also economical.

Besides, another aqueous reduction method for the efficient synthesis of nonoxidative Cu nanopowders without using a protecting agent or introducing an inert gas is presented by Chang et al. (2013) [157]. In this approach, a capping layer composed of a hydrophobic solvent with a high boiling point is applied to and covered on the top of the reduction system. The proposed approach based on the use of a capping solvent is highly effective for preventing the oxidation of the produced Cu nanopowder. By this method, high purity Cu nanopowders can be produced that exhibit good electrical conductivity with an electrical resistivity on the order of 10^{-4} Ω·cm.

Physical Method Using Laser Ablation

A laser-assisted method called laser ablation has proven itself as an alternative physical nanofabrication method [158–163]. It consists in the ablation of a target (mostly solid) by intense laser radiation and leads to an ejection of its constituents and the formation of NPs. [158–163]. Laser radiation is used to ablate a solid target (Ag, Au, Co and Cu) placed on the bottom of a glass vial filled with a biocompatible aqueous solution (7 mM NaCl solution or neat water). In other words, size selection of CuO-NPs is obtainable using laser ablation. Laser ablation in liquid has several advantages over the traditional chemical reduction methods for NPs, such as technical simplicity and chemical pureness, because this method when performed in a clean and well-controlled environment enables us to avoid the use of toxic chemical reduction agents to control the growth of the colloidal NPs, resulting in production of ultrapure NPs with nascent surfaces [158–163]. Laser ablation provides a solution for the study on the biological effects of NPs since conventional NPs lead to cytotoxicity possibly caused by contamination with chemical precursors or additives during their synthesis and/or purification procedures. Thus, laser ablation is expected to offer novel opportunities to provide insight into the origin of NP toxicity.

Kim et al. (2012) [136] introduced this novel laser-assisted method called laser ablation to generate Ag-, Au-, Co-, and Cu-NPs in biocompatible aqueous solution and they obtained supposedly ultra-pure NPs. They evaluated the intrinsic/inherent toxicity of ultra-pure Ag-, Au-, Co-, and Cu-NPs generated by laser ablation in a liquid ambient, revealing that the chemical nature of metallic NPs strongly influences the toxicity and cellular uptake into the model cells. For instance, a research group investigated the dependence of the particle properties on the *in situ* annealing temperatures and selection sizes. CuO- and Cu_2O-NPs with narrow size distributions were well dispersed in the samples [164]. Nath et al. (2011) [165] revealed that high pressure (60 GPa) increases the material covalency at Cu-water interface. The growth of CuO phase was thermodynamically stable under tightly focused conditions. Reduction in size incited lattice distortion transformation into highly symmetric Cu_2O containing insufficiently oxidized Cu-NPs [165]. In another study, this synthesis procedure, which was used for the ROS generation by Cu-NPs, seems to be an appropriate procedure for the enhancement of bactericidal effect of these NPs [166].

TABLE 1. Copper and Copper Oxide Nanoparticles synthesis method, size and concentration used against bacteria in several references from the literature.

Nanoparticle Type	Bacteria Strain	Authors	NPs size	Concentration	Incubation time	Synthesis Method
Cu	*E. coli*	Raffi, 2010	12-15 nm	0.06 mg/mL	48 h	Thermal plasma
		Chatterjee, 2012	80 nm	12 µg/mL	18 h	
		Cubillo, 2006	2-5 nm	1 wt%	24 h	Reduction in gelatin medium
		Yoon, 2007	90 nm	33.49 µg/mL	24 h	
		Valodkar, 2011	32 nm	0.63 mg/L	24 h	
		Ruparelia, 2008	9.25 nm	160-300 µg/mL	24 h	
		Deryabin, 2013	24 nm			
		Giannousi, 2014	44 nm	71.31 µg/mL	24 h	Chemical synthesis
		Bagchi, 2014	10-20 nm	63.5 µg/mL		
	S. aureus	Betancourt-Galindo, 2014	<20 nm	1.6 mg/mL	16 h	Chemical synthesis, 99% inhibition
		Valodkar, 2011	2-5 nm	1 wt%	2 4	
		Ruparelia, 2008	32 nm	1.11 mg/L	24 h	
		Ruparelia, 2008	9.25 nm	160 µg/mL	24 h	NCIM 2079
		Ramyadevi, 2012	9.25 nm	160 µg/mL	24 h	NCIM 5022
		Bagchi, 2012	44 nm	>100 µg/mL		Hydrothermal method
CuO	*E. coli*	Das, 2013	15-30 nm	1 mg/mL	9 h	Reduction
		Ren, 2009	22.4-94.8 nm	0.25 mg/mL	24 h	
		Azam, 2012	29.11 nm	25 µg/mL	24 h	Gel combustion method
		Azam (2) 2012	20-28 nm	30-95 mg/mL	24 h	Hydrothermal method
		Baek, 2011	20-30 nm	28.6 mg/mL	24 h	Hydrothermal method
		Giannousi, 2014	16 nm	>100 µg/mL	24 h	
		Giannousi, 2014	12 nm	80.93 µg/mL	24 h	
		Ren, 2009	22.4-94.8 nm	250 µg/mL	24 h	Thermal plasma
	S. aureus	Das, 2013	15-30 nm	1 mg/mL	6 h	
		Ren, 2009	22.4-94.8 nm	2.5 mg/mL	24 h	
		Baek, 2011	28 nm	100 mg/mL	24 h	Hydrothermal method
		Giannousi, 2014	20-30 nm	65.9 mg/mL	24 h	Hydrothermal method
		Giannousi, 2014	16 nm	>100 µg/mL	24 h	
		Ren, 2009	12 nm	96.13 µg/mL	24 h	
		Ren, 2009	22.4-94.8 nm	250-2500 µg/mL	24 h	Thermal plasma

Physical Method Using Plasma Induction Process

Plasma induction method is generated through an inductive coupling mechanism. When an alternative current of radio frequency and a high voltage is imposed on a spiral coil, the conductor placed in the center of the coil will be heated up under the alternative electromagnetic field. Introducing a continuum gas flow into such a coil, the gas could be ionized and heated into plasma. The plasma so generated is called inductively coupled plasma or induction plasma for short.

The plasma induction method used for nanopowder synthesis has many advantages over alternative techniques such as high purity, high flexibility, ease of scale-up and ease of operation and control. For example, the plasma induction system developed by Tekna [167] has been successfully used in the synthesis and preparation of advanced materials such as new ceramics, nanometric metallic powders, biomaterials, and superconductors. The typical size range of the nanoparticles produced is from 20 nm to 100 nm, depending on the quench conditions employed. Materials at such a small-scale display unusual properties, as chemical, physical, electrical, optical, mechanical, magnetic, etc., different from their bulk state.

Recently, our team synthesized CuO powders from the reaction of copper with oxygen in plasma using HF-75 torch of Tekna [167]. Since copper has two states of oxidation, i.e., tenorite (CuO) and cuprite (Cu_2O), a preliminary effort has been made in the study to convert pure copper into tenorite. The extent of the conversion is mainly determined by two factors: the oxygen concentration in plasma and the temperature in the reactor. The antibacterial activities of these CuO powders are published in a separate paper [168].

Flow-Levitation (FL) Method

Flow-levitation (FL) method is based on the levitation melting technology [169] and has been used as a successful method for the preparation of some metals [170–172] and ZnO-NPs [173]. Moreover, this method has been used for the preparation of alloy NPs. Sivaprahasam et al. [174] used this method for the synthesis of FeCu nanopowders and other groups used it to produce intermetallic Ag_2Al^-, $FeAl^-$, and $FeNi_3$-NPs. [175,176].

In comparison to the conventional evaporation-condensation, the main advantages of the FL method are high purity of the product (due to the container less nature of the process) and high production rate (due to the rapid heating and continuous manner of the method) [177]. FL method is a novel method capable of producing high purity intermetallic NPs with a relatively high production rate. Cu- and CuO-NPs of various average sizes and chemical compositions were synthesized via FL condensation method [170].

However, main restrictions of FL method are as follows: relatively low productivity (1-100 g/hour), impossibility to work with refractory metals (with melting point over 2500°C) and relatively wide particle size distribution (that causes different chemical composition of particles with different sizes) [178].

Among main advantages one should emphasize an opportunity of fast and effective variation of particle production regimes to provide a wide range of particle sizes and chemical reaction conditions, and an opportunity to fulfill some steps of successive chemical transformations of the particles *in situ* within the one experimental apparatus in controlled atmosphere and temperature.

Addition of Stabilizing Agent to Counteract NPs Oxidation and Aggregation

We have established now that NPs' properties can be controlled depending on the synthesis method. One of the main effects, which are enhanced by controlling particle size, is their antimicrobial action [78,75]. Nonetheless, one main drawback in most of the synthesis technique is the rapid oxidation of copper-based-NPs upon exposure to the air.

For protecting Cu-NPs from oxidation during synthesis, the sufficient addition of surfactants such as hexadecyltrimethylammonium bromide [135,179], poly(N-vinyl-2-pyorrolidone) [134], oleic acid [180], gelatin [181], diethanolamine [182] and sodium dodecylbenzenesulfonate [137] is necessary for full coverage of the particle surface. However, the electrical properties of the Cu-NPs can be hindered because of the presence of the insulating surfactants. In fact, a mixture of metallic Cu and Cu_2O can be seen. It has been largely reported that Cu_2O or CuO generally accompanies the production of Cu nanopowders, even though the added amount of reductant is sufficient [134,179].

One approach is to add a capping layer composed of a hydrophobic solvent with a high boiling point and apply it to cover NPs. Without using this layer, O_2(aq) can be easily supplemented from air [134,179]. That is, when O_2(aq) is consumed by the reductant or the less-oxidized Cu^0 or Cu^I colloids, oxygen is immediately supplemented from air based on dynamic equilibrium. The additional O_2(aq) from air maintains the oxidation of the Cu^0 and Cu^I colloids. However, with the hydrophobic capping layer, the dissolution of oxygen is unfavourable, and thus the reduction system can be secluded from air and the system can remain deficient in O_2(aq). Without the addition of supplemental O_2(aq), oxidation of the produced Cu^0 colloids will not be easy. In addition to efficiently reducing the concentration of O_2(aq) in the reduction system, this method has other advantages. Unlike an inert gas, the capping solvent can be easily recycled and reused. Moreover, the use of surfactants as protecting agents is not a must, and thus good electrical conductivity of the as-synthesized Cu^0 can be anticipated.

Therefore, the Cu nanopowder is generally generated along with a mixture of its oxides (Cu_2O or CuO). Contrarily, when the capping solvent is applied, only the product of metallic Cu NPs is present, generally obtained in a face-centred cubic crystal structure. It becomes then obvious that the proposed approach based on the use of a capping solvent is highly effective for preventing the oxidation of the produced Cu nanopowder. This result may be attributed to the less favourable dissolution of oxygen in hydrophobic n-octane, which prevents the penetration and dissolution of oxygen in the aqueous solution [179,183].

Chemical Methods using Chitosan Stabilizer

It is known that Cu-NPs tend to agglomerate on synthesis due to the high tendency of Cu nuclei to bond. The aggregation may also be due to the high surface area of the Cu-NPs [133]. Therefore, the use of a polymer coating is essential to avoid NPs clusters.

Usman et al. (2013) [184] investigated the antimicrobial properties of metallic Cu-NPs synthesized in chitosan polymer medium through chemical means. The surfaces of chitosan-Cu-NPs are covered by fragments of chitosan (CS) which protect against aggregation and oxidation [185]. CS is a natural copolymer of D-glucosamine and N-acetyl-D-glucosamine and is produced by alkaline deacetylation of chitin. The nuclei of the individual nanocrystals were attracted to each other by weak van der Waals forces, and the stabilizer (CS) provided insulation between the particles by overcoming these forces, a phenomenon seen with both polymers and surfactants [154,185]. No other CuO or Cu_2O impurity peaks were observed in any of the spectra, suggesting that the synthesized particles were of high purity. The images obtained for the various concentrations of the stabilized crystals indicate that the Cu-NPs were embedded within the matrix of the polymer. These findings accentuate the important role of the polymer as a stabilizer. It is worth noting that the chitosan stabilized Cu-NPs exhibited both antibacterial and antifungal activity against Gr+ bacteria, Gr- bacteria, and yeast.

However, CS is deemed to also have antioxidant properties that may inhibit ROS generated by Cu-NPs. For instance, Xie et al. (2001) [186] studied the antioxidant activities of water-soluble chitosan derivatives which were considered to be hydroxyl radical scavengers. CS is also biodegradable, biocompatible, and non-toxic and, therefore, has been employed in biomedical applications such as drug delivery [187–190]. CS has many significant biological properties including bioactivity and biodegradability with a reactive chemical group including OH and NH_2. CS has shown good antioxidant activity, dependent on the molecular weight and the degree of deacetylation. The mechanism of action is still not exactly known, but it is postulated that it reacts with unstable free radicals to form more stable macromolecular radicals.

Other Examples of Polymeric Embedding of NPs

Esteban-Cubillo et al. [69] have shown that Cu/sepiolite NPs strongly inhibit the growth of *E. coli* and *S. aureus* by 99.9%. Ruparelia et al. [78] showed the specificity of Cu-NPs to selectively inhibit the growth of few strains of *E. coli*. The bactericidal and fungicidal properties of Cu-NPs are known [67,90,191]. Shameli et al. (2012) [192] reported that plant extracts could be used to stabilize NPs in green synthesis. Cu-NPs (about 6 nm) embedded in polyvinyl methyl ketone films exhibit a noticeable inhibitory effect on the growth of microorganisms [86]. Due to the stability of Cu-NPs supported on a matrix and their disinfecting properties, Cu-NPs can be used as a bactericide agent to coat hospital equipment. Christian et al. (2008) [12], revealed that Cu-NPs get more attention due to their excellent thermal properties after the introduction of nanofluids. Nanofluids of Cu-NPs are used in both heating and cooling applications [193].

Concluding remarks

It is noteworthy that in 2006 the US had about 720,000 hospital-acquired infections, causing $125 billion in excess hospital charges and more than 74,000 fatalities [194], (numbers for the US were extrapolated from data acquired for Pennsylvania). A hospital-acquired infection raised a patient's percent mortality from 2.1 to 12.3%, the length of hospitalization from 4.4 to 19.3 days and the cost per visit from $33,000 to $176,000. These numbers emphasize the need for new antimicrobial approaches that tackle this severe public health problem.

Despite the many effective antimicrobial strategies against planktonic bacteria, most antimicrobials are rarely tested or effective against biofilms [195]. Novel approaches to treat established biofilms are thus urgently needed. Clearly, new strategies for battling biofilms are warranted.

The potential usefulness of metallic Cu in hospitals and other public places to control and reduce hospital acquired infections is very promising. Nevertheless, research is also required that investigates the possible emergence and spread of Cu surface resistant bacteria. CuO- and Cu-NPs have shown to have adverse effects on bacteria, and Cu^{2+} dissolving from the NPs induced also toxic effects by triggering ROS production and DNA damage in bacteria. Therefore, copper-based NPs can be used as a novel antifungal agent in agriculture to control the plant pathogenic fungi as well as a potent disinfectant in poultry and animal husbandry.

However, in any organism, the molecular mechanisms responsible for killing and the cellular target(s) of Cu toxicity have not been delineated yet and remain elusive.

Applications for Cu NPs include antimicrobial, antibiotic, and antifungal(fungicide) agent when incorporated in coatings, plastics, textiles, in Cu diet supplements, in the interconnect for micro integrated circuits, for its ability to absorb radioactive cesium, in super strong metals, in alloys, in nanowire, in nanofiber, in certain alloy and catalyst applications. Further research is being done at various levels for their potential electrical, dielectric, magnetic, optical, imaging, catalytic, biomedical and bioscience properties. It is interesting to note that Cu-NPs are generally immediately available in most volumes.

Synthesis and use of NPs are influenced by a variety of factors which affect the activity of the NPs, such as chemical composition, concentration, contact time, size, shape, target microorganism and photo-activation. Further investigations should be devoted to the assessment of influence of biological fluids, pH, oxygen pressure and other chemical and biological factors on antibacterial properties of NPs, biocompatibility of NPs, and interactions between different physico-chemical properties of NPs in their combined use. Special attention should be paid to the study of activity of NPs and their combinations with other classes of antimicrobial agents against multidrug-resistant microorganisms.

Researchers have also recommended the use of silver and Cu ions as superior disinfectants for wastewater generated from hospitals containing infectious microorganisms. Cu-NPs, due to their excellent physical and chemical properties and low cost of preparation, have been of great interest. Cu-NPs have wide applications as heat transfer systems [84], antimicrobial materials [85,86], sensors [86], super strong materials [87,88], and catalysts [89,196]. However, Cu-NPs can easily oxidize to form CuO. To protect Cu-NPs from oxidation, they are usually encapsulated in organic and inorganic coating such as carbon and silica [11,85,197,198].

Considerable interest has been focused on noble metal NPs in recent years because of their potential applications in a wide range of fields such as catalysis, optics, biology, microelectronics, and electrical conductors. Among the popularly used noble metals, Cu is non-toxic; non-magnetic and has good thermal and electrical conductivity at the least cost compared to Au, Pd and Ag. **Therefore, Cu nanopowders are believed to be the most competitive material for the replacement of Ag nanopowders, which have been widely used to date in the above applications.**

Abbreviations

NPs	Nanoparticles
EPS	Extracellular polymeric substances
E. coli	Escherichia coli
NIs	Nosocomial infections
UTIs	Urinary tract infections
S. aureus	Staphylococcus aureus
P. aeruginosa	Pseudomonas aeruginosa
K. pneumoniae	Klebsiella pneumonia
A. baumannii	Acinetobacter baumannii
MRSA	Methicillin-resistant S. aureus
BSIs	Bloodstream infections
VRE	Vancomycin resistant Enterococci
VRSA	Vancomycin resistant S. aureus
NDM-1	New Delhi metallo beta-lactamase
MDR	Multidrug-resistant
PDR	Pan-drug-resistant
Gr+	Gram-positive
Gr-	Gram-negative
Cu	Copper
CuO	Cupric Oxide
Cu_2O	Cuprous oxide
Cu-NPs	Copper NPs
Cu-MPs	Copper microparticles
ROS	Reactive oxygen species
EC10	Maximal effective concentration to induce 10% of response after exposure
EC30	Maximal effective concentration to induce 30% of response after exposure
EC50	Maximal effective concentration to induce 50% of response after exposure
A549	Adenocarcinomic human alveolar basal epithelial cells
937	Human lymphoblast lung cells
HeLa cells	Human epitheloid cervix carcinoma cells
Hep-2	Human airway epithelial cells
PAI-1	Plasminogen activator inhibitor-1
p38	Mitogen-activated protein kinases
E. coli RFM443	Escherichia coli strain RFM443
ppm	Part per million
ppb	Part per billion
SSB	Single-strand break
Fpg	Formamidopyrimidine-DNA glycosylase
H_2O_2	Hydrogen peroxide
•OH	Hydroxyl radical
TiO_2	Titanium dioxide
ZnO	Zinc oxide
O_2•−	Superoxide
GC/MS	Gas chromatography-mass spectrometry
DCFH-DA	Fluorescent probe, 2',7'-dichlorofluorescin diacetate
GR	Glutathione reductase
GPx	Glutathione peroxidase
DCFH	Dichlorodihydrofluorescein
CFU	Colony-forming units
ZVCN	Zero valent Cu-NPs Cu0
Vitamin C	Ascorbic acid
CS	Chitosan
CMCH	Carboxymethyl chitosan
PC3	Human prostate cancer cells
MCF-7 cells	Human breast cancer cells
LD50	Lethal Dose inducing 50% mortality
MEM/EBSS	Minimum Essential Medium with Earle's Balanced Salts
RPMI-1640	Roswell Park Memorial Institute medium N1640
LD100	Lethal Dose inducing 100% mortality
S. albus	Staphylococcus albus
E.coli AB1157	Escherichia coli strain AB1157
E.coli K12	Escherichia coli strain K12
AAS	Atomic absorption spectroscopy
$CuCl_2$	Cupric chloride
O_2(aq)	Oxygen in aqueous medium GSH Glutathione

Acknowledgements

This work was supported by NSERC (Engage). We acknowledge GRSTB-FRSQ for supporting us in this review. Special thanks to Hind Khadraoui for her support and her work in formatting and proofreading this article.

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IN SITU-FORMED BACTERIAL EXOPOLYSACCHARIDE (EPS) AS A POTENTIAL CARRIER FOR ANCHORAGE-DEPENDENT CELL CULTURES

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Abstract

The study involved the use of a bacterial strain isolated from environmental samples which produce the biopolymer in the form of pellets in the submerged culture. This material (bacterial exopolysaccharide) is produced by bacteria of the Komogateibacter xylinus which are prevalent in the environment. The aim of this study was to characterize bacterial exopolysaccharides and commercial dextran-based "microcarriers" in terms of their roughness and cell culture effects, including the morphology and viability of the human hybridoma vascular endothelial cell line EA.hy926. The pellets were characterized using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The resulting structures were used for cell culture of adherent cells (anchorage-dependent cells). At the same time, the cultures with commercial, dextran-based "microcarriers" were carried out for comparative purposes. After completion of the cell culture (24 hours of culture), the cellulose and commercial "carriers" were analyzed using SEM and AFM. Finally, the obtained cell densities (fluorescence labelling) and their morphological characteristics (SEM) were compared. The obtained results strongly support the applicability of bacterial exopolysaccharide (EPS) in tissue engineering to build innovative 3D scaffolds for cell culture, the more so that it is technologically possible to produce EPS as spatially complex structures.

Keywords: *bacterial exopolysaccharides, dextran-based "microcarriers", scanning electron microscopy, atomic force microscopy, roughness parameters, three-dimensional cell culture*

[Engineering of Biomaterials 159 (2021) 18-23]

doi:10.34821/eng.biomat.159.2021.18-23

Introduction

Bacterial exopolysaccharides (EPSs) have various environmental functions and different physicochemical properties, depending on their structure. One of the first microbial exopolysaccharides to be characterized was microbial cellulose, which was discovered over 100 years ago [1]. This material is produced by *Komogateibacter xylinus* bacteria that are prevalent in the environment [2]. EPSs protect microbial cells in their natural environments against unfavorable conditions, such as drying, osmotic stress, antibiotics, toxic compounds, phagocytosis, etc. [3,4]. In addition to this, the EPS layer protects *Komogateibacter xylinus* bacteria environment against the competitive colonization by other microbial species. Furthermore, the presence of a gelled polysaccharide layer around a cell may have a predominant effect on its diffusion properties both into and out of the cell [5]. Among other functions, the polymer allows cells to remain on the surfaces of different liquids, where they have access to oxygen and nutrients. Despite many years of research, work on the potential applications of EPSs is still underway in many research centers around the world, most of which is focused on its use as a film on the surface of culture medium in static cultures [6]. However, only a small number of literature reports have discussed the possibilities offered by using this biopolymer formed as pellets. In such a form, EPSs could be used as blood flow improving agents in medicine, as microcarriers in tissue cultures and also as an immobilization matrix [7]. Many of the biological applications of EPSs have been previously discussed [8-12], mostly in relation to their structure, composition, and molecular weight. Additionally, bacterial EPSs have been explored as agents that could be useful in creating a more environmentally friendly, metal nanoparticle production process. This is due to the fact that they can reduce metal ions to form nanoparticles and by acting as capping agents can also stabilize them [13]. Of all known EPSs, bacterial cellulose (BC) has been studied most thoroughly as a material for dental implants, wound dressings, and as a temporary skin substitute. This research has resulted in the commercialization of several biomedical products [14-17]. Another very important aspect is the structural and spatial arrangement of EPSs, which could constitute a scaffold for eukaryotic cells and their potential use in bone regeneration [18].

In recent years, three-dimensional (3D) cultures of various cell types have become very popular [19-21]. The transition from 2D to 3D cell culture makes it possible to simulate conditions that are closer to the physiological processes observed in the human body [22]. The question is whether EPSs are suitable as matrices of micro scaffolds used for the cell culture and how their physicochemical properties will affect the cell culture. The aim of this study was to characterize bacterial exopolysaccharides and commercial dextran-based "microcarriers" in terms of their roughness and effects on the cell culture, regarding the morphology and viability of the human endothelial cell line EA.hy926. Endothelial cells line the inner surface of blood vessels and are one type of barrier cells responsible for the control of processes involved in transporting substances from barrier tissues to blood and from blood to deeper tissues [23,24]. The study was performed using atomic force microscopy and scanning electron microscopy working in an environmental mode, whereas the endothelial cell viability test was performed by labelling nuclei after the 24 h cell culture.

Materials and Methods

The EPS pellet production by *Komogateibacter xylinus* bacteria was developed in the framework of this work. Two commercial samples of dextran-based microcarrier beads, i.e. Cytodex™ 1 (17-0448-03) and Cytodex™ 3 (17-0485-01) were purchased from GE Healthcare Ltd, UK, and were used as reference samples. The particle size of Cytodex™ 1 and 3 averages 190 and 175 μm when swelled in a liquid solution, respectively. Cytodex™ 3 reference sample contains a layer of acid-denatured porcine collagen on the surface of the microporous matrix. This data refer to the specification sheets provided by the manufacturer. Reference samples were processed according to the manufacturer's manual.

Atomic Force Microscopy and Scanning Electron Microscopy

The substrate topography was measured using NTEGRA scanning probe microscope (NT-MDT, Russia) with NSG-01 cantilevers in the air (with resonant frequency from 87 to 230kHz, NT-MDT Probes, Russia) in a semi-contact mode. To obtain the precise visualization, at least 5 scans were performed of 5, 15, and 50 μm scan size. Additionally, the average roughness parameters (Sq – root mean square, Sa – average roughness, St – area peak-to-valley height, S10z – ten-point height, Sdr – surface area ratio, Sds – density of summits of the surface, Ssc – mean summit curvature) from 15 μm x 15 μm AFM images of the bacterial EPS surface, EPS surface etched with 0.1M NaOH and reference commercial samples were calculated. The statistically significant differences between the samples are marked as (*) for $p < 0.05$ and (**) for $p < 0.01$ (ANOVA).

The scanning electron microscopy (SEM) evaluation was performed using a Quanta FEG 250 microscope (FEI, The Netherlands) equipped with a large-field detector (LFD). The microscope was working in a "wet" environmental mode of scanning electron microscopy (ESEM) at an accelerating voltage of 5 kV, the sample temperature of -15°C, humidity 100%, and pressure 194 Pa. The SEM images were collected in five randomly selected areas.

Cell culture

The human hybridoma vascular endothelium cell line EA.hy926 (ATCC CRL-2922) was grown in culture medium Dulbecco's Modified Eagle Medium (DMEM, ATCC, USA) with 10% of Fetal Bovine Serum (Gibco, USA) and Penicillin/streptomycin cocktail (ATCC, USA) solution at 37°C in a humidified atmosphere of 5% CO_2. All the cell culture reagents were sterile. The cell culture protocol is consistent with our previous work [25]. The cells were passaged every 2-3 days when reached 90-100% confluence on the cell culture dishes. The cells were detached from the culture dishes with Trypsin-EDTA (ATCC, USA) applied for 3 min, and then neutralized in the 10-fold volume of complete DMEM medium. Next, the cells were centrifuged for 5 min at 800 rpm and seeded at the desired density on specified substrates (EPS-etched samples and cytodex microcarries). The sterile substrates were placed into a 96-well plate prior to seeding the cells. All types of the samples were processed in 5 replicates.

Cell morphology

A scanning electron microscope was used to observe differences in cell morphology on the surface of the tested samples. After 24 h of incubation with the suspension of cells, the culture medium was removed and the samples were rinsed twice with phosphate-buffered saline (PBS) and fixed for 1 h at 4°C with 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer.

Fluorescence labelling

The evaluation of the proliferation rate of EA.hy926 cells on the tested substrates was performed by measuring the blue fluorescence of the cells nuclei stained with Hoechst 33342 dye (Life Technologies, USA), which intercalates into DNA. The fluorescence was measured by Multifunctional microplate reader Victor X4 (Perkin-Elmer, USA). Each experiment was repeated in triplicate, and five independent procedures were performed for each experiment.

Results and Discussions

Characterization of EPS pellets by AFM

In FIG. 1, the surface topography of EPS pellets obtained for the selected growing time periods of 6 h (FIG. 1A, D), 10 h (FIG. 1B, E), and 24 h (FIG. 1C, F) is presented. Due to the larger scanning range of the surface (upper images), *Komogateibacter xylinus* bacteria can be observed, as well as the global pattern of EPS. To accurately compare the cellulose fibers themselves, AFM was performed for a smaller surface scan range of about 5 μm in the areas between the bacteria. The AFM images show a random microfiber network of cellulose chains, in which the length and arrangement of the fibers change with the growth time (FIG. 1D, E, F). FIG. 2 shows a comparison of the roughness parameters for bacterial cellulose in the form of pellets obtained for different growth times. The highest roughness values were obtained for 24 h, therefore this EPS growth time was used for further experimental purposes.

In the next step, etching with NaOH was carried out in order to remove bacteria. The surface topography of etched EPS pellets was compared with commercially available "microcarrier" beads (FIG. 3). Both reference samples present a microporous matrix, but with different packing densities and pore sizes. TABLE 1 shows the mean values of the roughness parameters for commercial samples and for the etched EPS. Based on the obtained results, one can conclude that the highest roughness values are observed for the etched EPS samples. Substrates with higher roughness values are most often characterized by better eukaryotic cell adhesion [26]. Nano/micro-topography stimulates behavioral changes in cells and plays a critical role in modifying their proliferation and vitality, as well as the strength of adhesion to substrates [27].

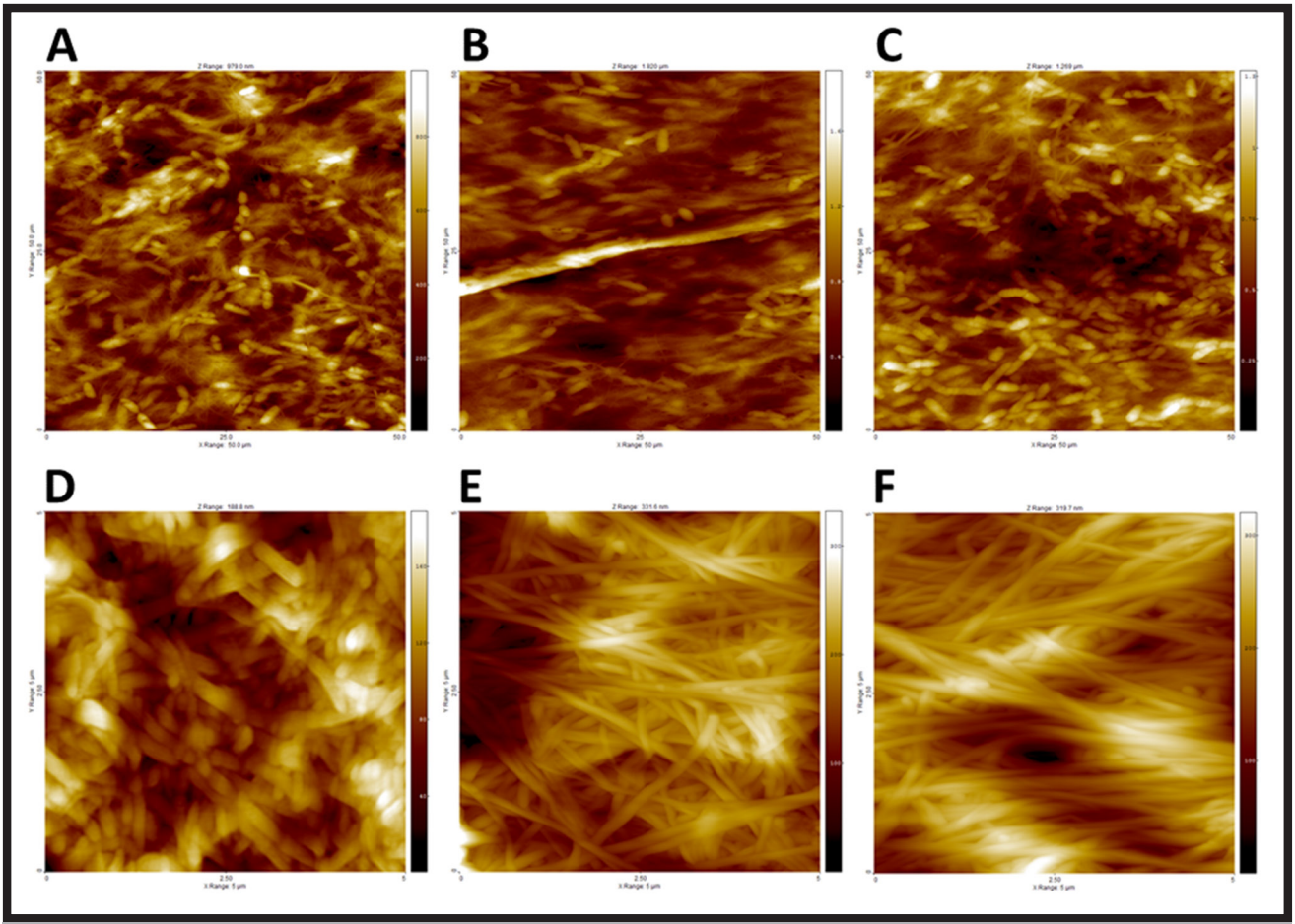

FIG. 1. AFM images of EPS surface for selected growing time periods, scan sizes 50 μm x 50 μm (A-C) and 5 μm x 5 μm (D-F).

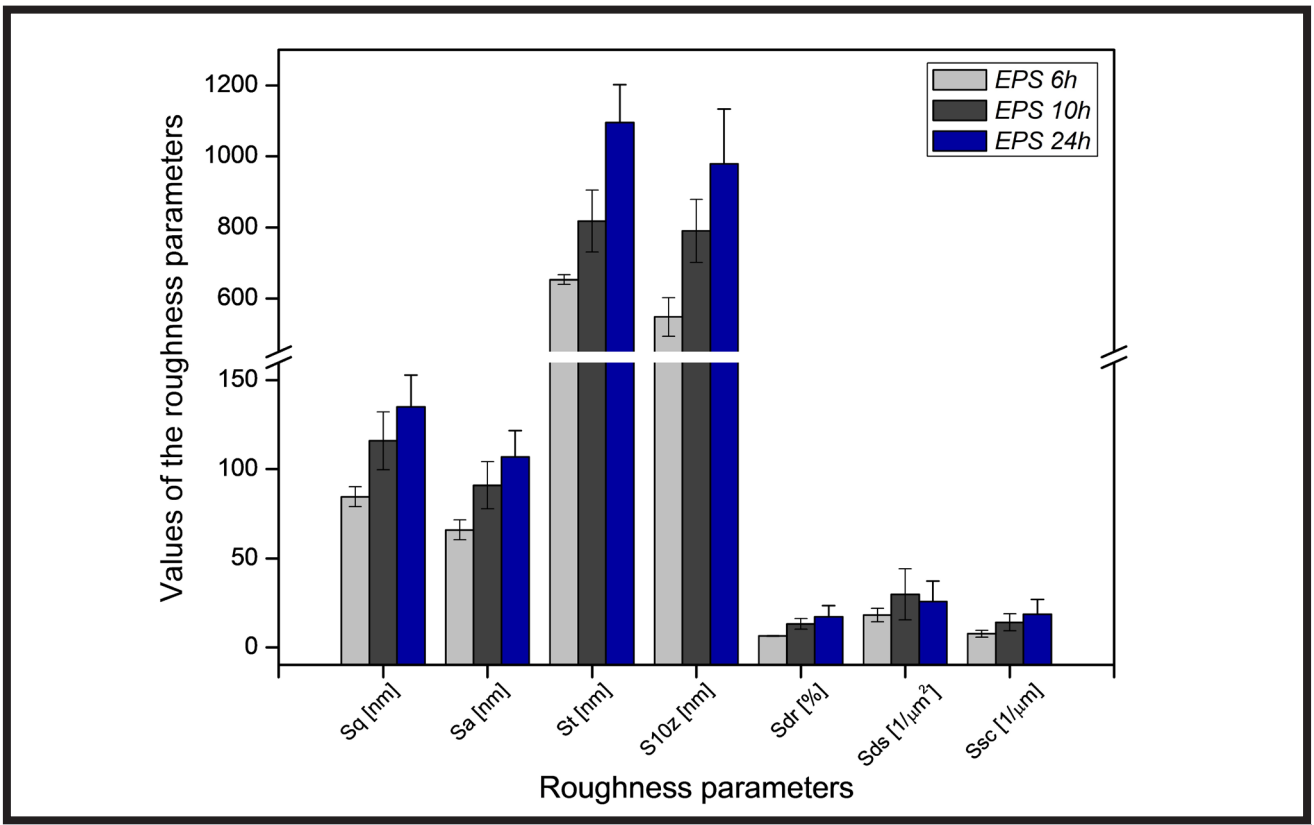

FIG. 2. Values of selected roughness parameters for EPS samples.

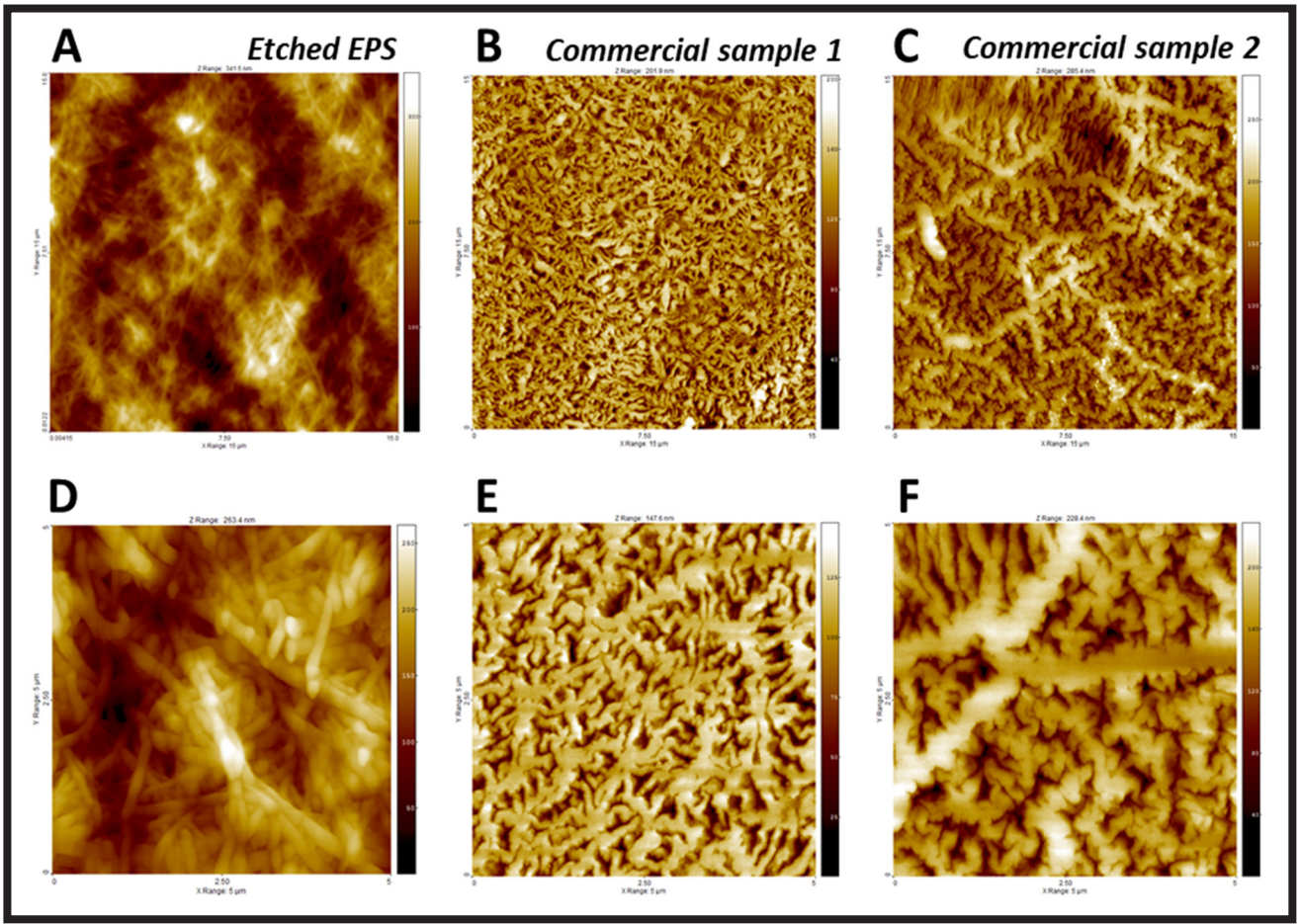

FIG. 3. AFM images of etched EPS surface and reference samples, scan sizes 15 µm x 15 µm (A-C) and 5 µm x 5 µm (D-F).

TABLE 1. Average values of roughness parameters for commercial samples and etched EPS.

Roughness parameters	Commercial sample 1	Commercial sample 2	Etched EPS (0.1M NaOH)
Sq [nm]	25.69 ± 1.21	37.77 ± 1.04	63.89 ± 15.03
Sa [nm]	19.96 ± 1.37	29.42 ± 0.31	51.65 ± 12.32
St [nm]	281.40 ± 123.17	359.63 ± 62.88	434.69 ± 76.28
S10z [nm]	247.74 ± 79.95	324.90 ± 71.80	394.56 ± 75.48
Sdr [%]	13.77 ± 5.31	9.47 ± 0.10	2.55 ± 1.29
Sds [1/um²]	68.64 ± 16.58	56.75 ± 3.29	18.49 ± 3.59
Ssc [1/um]	13.66 ± 1.64	13.88 ± 0.52	6.17 ± 1.58

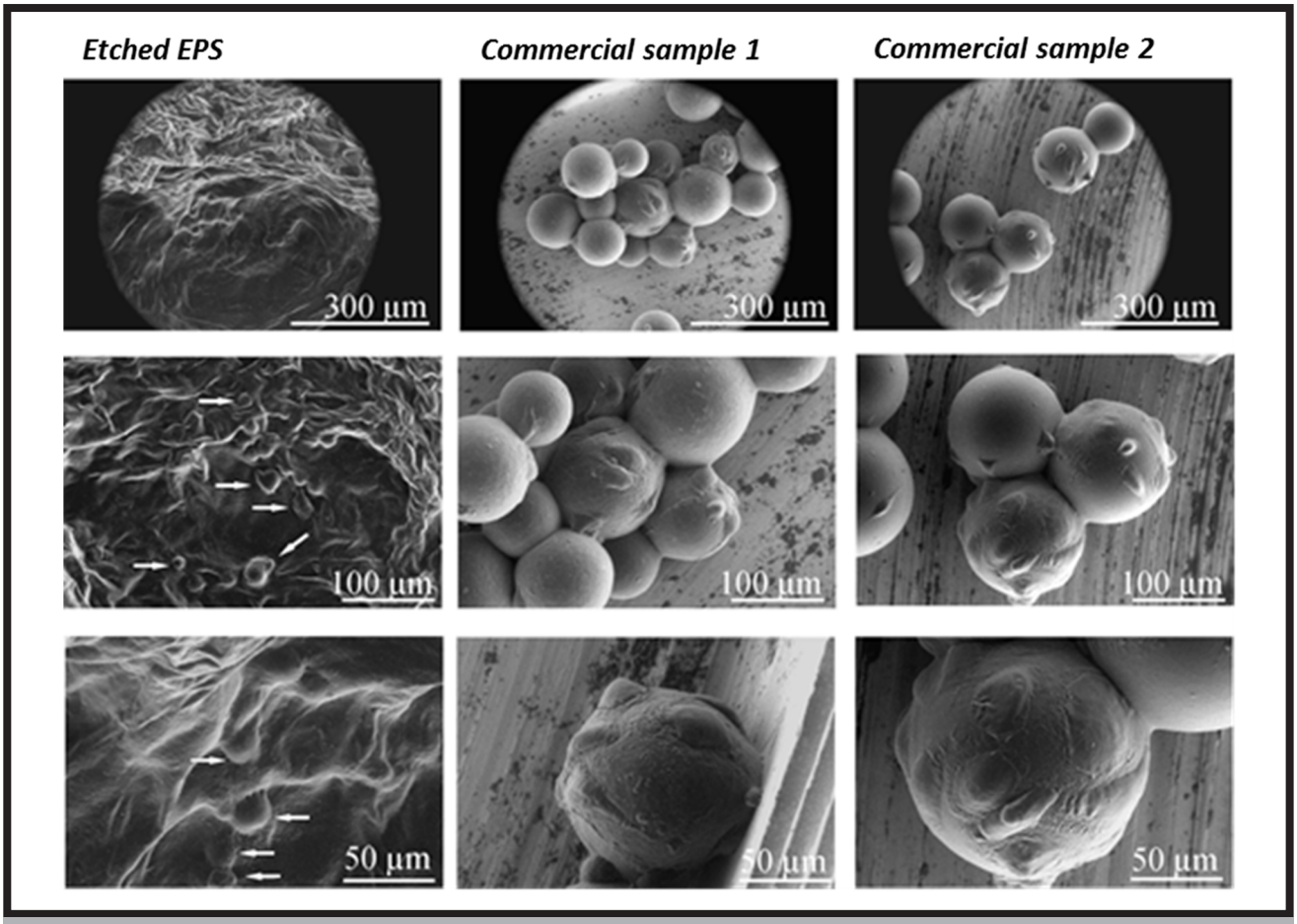

FIG. 4. SEM images of etched EPS surface and reference samples after 24 h of endothelial cell culture. White arrows indicate the EA.hy926 cells on the etched EPS surface. SEM images were collected for different magnifications, the scales are marked on the images.

Cells morphology visualized with SEM and fluorescence labelling

The well-flattened endothelial cells adherent to the selected substrates (etched EPS strains and commercial samples 1 and 2) can be observed in the SEM images (FIG. 4). On the etched-EPS pellets after the cell culture, the central parts of the endothelial cells can be distinguished (the areas with the cell nucleus marked with the white arrows). It can be concluded that EA.hy926 cells adhering to the etched-EPS pellets conform to their undulating surface. In the case of the reference samples, we found both single cells and whole microcarrier beads covered by endothelial cells. The highest cell proliferation values were obtained for the endothelial cells cultured on an etched EPS sample (statistically significant difference, $p < 0.05$). For commercial sample 1 and commercial sample 2 there were no differences comparing to the control sample, as shown in FIG. 5. This may indicate the good properties of the EPS substrate, produced by *Komogateibacter xylinus* bacteria, for the adhesion and proliferation of endothelial cells. The adhesion of cells to the surface of the biomaterial is very important for proper integration with tissues and should be taken into account when designing new biomaterials [28]. Many proteins from the extracellular matrix (including fibronectin, collagen, laminin, etc.) as well as cytoskeleton proteins (e.g. actin, vinculin) and membrane receptors (integrins) are involved in the cells adhesion processes to the 2 or 3D substrates. Mainly the interactions between these proteins and their specific receptors induce signal transduction, which in turn influences cell growth and differentiation [29].

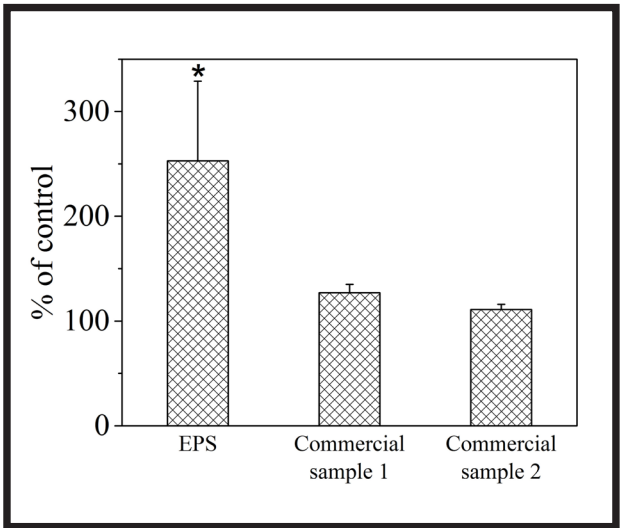

FIG. 5. Proliferation of endothelial cells (EA.hy926) on different surfaces after 24 h of cell culture.

Conclusions

In the present study, an innovative model based on bacterial exopolysaccharide was developed for potential three-dimensional cell cultures. It can be concluded that extending the time of bacterial cellulose synthesis results in higher parameters of its surface roughness. Moreover, the roughness parameters (Sq and Sa) for the etched EPS surface are much higher, as compared to the commercial reference samples, which may suggest better adhesion of the cells grown on the EPS surface. This paper also shows the importance of fully characterizing the substrate topography and measuring roughness parameters. Nano/micro-topography has been recognized as fundamental in the design of bio-inspired materials. The obtained results strongly support the idea of using bacterial EPS in tissue engineering to build innovative 3D scaffolds for cell culture, more so that it is technologically possible to produce EPS in the form of spatially complex structures.

Acknowledgments

This work is partially funded by The National Science Centre, project title: 'The influence of selected nanoparticles on the elastic properties of endothelial cells evaluated using atomic force microscopy', agreement no. 2017/26/D/ST4/00918.

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THE INFLUENCE OF AUTOLOGOUS AND HETEROLOGOUS EXTRACT OF ANTIMICROBIAL PEPTIDES ON LEUKOCYTES ISOLATED DURING TITANIUM IMPLANT INSERTION IN RABBIT AND OVINE MODEL

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Abstract

This study evaluated the in vitro leukocyte response to titanium implants in the presence of autologous or heterologous antimicrobial peptides extracts. Antimicrobial peptides (AMPs) appeared to be a new approach both against microorganisms and for regulation of inflammatory and repair processes. To evaluate their potential usefulness in regenerative medicine, we prepared different extracts of neutrophil-derived AMPs from rabbit, ovine or porcine blood which contained AMPs of different compositions, mainly defensins, cathelicidins and fragments thereof. Then, we assessed in vitro the influence of different AMPs extracts on the neutrophils and monocyte-derived macrophages (MDM) activity. For this purpose, these cells were obtained from experimental animals, rabbits, or sheep submitted to insertion of a titanium implant into the tibial defect. The cultured cells stimulation was autologous or heterologous, dependently on the AMPs extract origin and the experimental animal species. The neutrophil activity was assessed on the basis of the enzymes release from azurophilic and secondary granules and the free radicals generation. The MDM functional assessment was based on the NO and superoxide generation and arginase activity. Additionally, morphological changes were evaluated in the cell cultures. Our results indicated that the origin of AMPs extract is crucial for its activity. The autologous extracts stimulated anti-inflammatory responses, whereas the heterologous extracts displayed pro-inflammatory effect on neutrophils and macrophages. These results might be considered during the introduction of new preparations in regenerative medicine.

Keywords: *titanium implants, neutrophil extract, implantation of biomaterial, neutrophils, monocyte-derived macrophages, natural antimicrobial peptides*

[Engineering of Biomaterials 159 (2021) 24-30]

doi:10.34821/eng.biomat.159.2021.24-30

Introduction

Titanium (Ti) is considered to be a relatively inert biomaterial which evokes minimal adverse effects. However, it has been described in the literature that the titanium particles released to circulation may be harmful under some circumstances. The adverse effect is noted especially during frictional wear of prostheses or screws used for the implant to bone attachment, the bone to bone fixation or the soft tissue fixation. Side effects in soft tissues with the presence of foreign body giant cells have been described in reaction to rough surface titanium alloy medical prostheses together with increased plasma concentration of Ti ions. The presence of Ti particles in the regional lymph nodes and in the tissue around the implant can lead to long-term metabolic, neoplastic and immunologic effects [1].

Despite these limitations, Ti is favourable because it is endowed with advantageous biomaterial characteristics, such as mechanical strength, corrosion resistance, chemical stability, and biocompatibility. For instance, due to the thin TiO_2 layer, the surface becomes highly resistant to corrosion. However, in the last decade, an increasing number of reports have been published about excessive inflammatory reactions and bone loss around implants, so-called peri-implantitis involved in implant side effects [2].

Within the white blood cells (WBC) population both neutrophils and blood monocytes migrate to the site of injury and participate in tissue repair [3]. These cells are essential for the regeneration, repair and remodelling in many tissues and can act as secondary pro-inflammatory factors capable of releasing cytokines and other stimuli. It should be emphasized that, apart from other effects, titanium ions form particles that induce inflammatory cells activation, especially monocytes and macrophages [2,4-7]. On the other hand, neutrophils are increasingly considered as a heterologous population with high regulatory potential. However, chronic inflammation and persistent neutrophils and macrophages activity are often involved in tissue destruction and fibrosis [3,8]. For these reasons, current studies are focus on interactions between components of the host defence and medical implants in order to improve the implanted biomaterials properties. Therefore, we decided to conduct the research that goes beyond basic biocompatibility studies and extends their scope.

Antimicrobial peptides are widely recognized for their multifunctional antimicrobial and immunomodulatory activities. These functions refer to modulation of pro- and anti-inflammatory responses, including macrophage differentiation and modulation of wound healing. AMPs can suppress pro-inflammatory responses, as in the case of human cathelicidin LL-37, thanks to limiting the release of pro-inflammatory mediators and the lipopolysaccharide (LPS) neutralization [9]. A similar effect was observed for porcine cathelicidin PR-39 [10]. PR-39 is a proline-arginine rich peptide from porcine neutrophils with a broad antibacterial effect. This peptide, first identified in the porcine small intestine, displays a specific antibacterial activity against multiple gram-negative bacteria. It inhibits the protein synthesis and exerts the membrane-disruptive effect on microbes.

Moreover, PR-39 improves wound healing by upregulating the surface expression of syndecan-1 and syndecan-4 on mesenchymal cells. In the mouse model of sepsis, this peptide showed a protective effect as it increased the nitric oxide (NO) production in the liver and limited the reactive oxygen species (ROS) generation [11]. Furthermore, AMPs influence the functions of neutrophils, i.e. the major innate immune effector cells of the early-phase response to injury. Neutrophils are both producers and receivers of AMPs. They are a source of both defensins and cathelicidins which are stored and released from granules during the neutrophil degranulation. AMPs can enhance the neutrophils influx both by a direct chemotactic function and indirectly by promoting the secretion of these neutrophil products [12].

It was estimated that, dependently on their properties, AMPs can induce the macrophage differentiation towards M1, M2 or the intermediate phenotype between pro-inflammatory M1 and anti-inflammatory M2 macrophages. PR-39 influences the porcine macrophages polarisation from the M2 to a M1 phenotype, and promotes the bactericidal functions of these phagocytes [11].

In our experiment, we evaluated the influence of various AMPs extracts on the activity of neutrophils and monocyte-derived macrophages (MDM) obtained from experimental animals, rabbits or sheep, having inserted the Ti implants into their bones.

Materials and Methods

The study was conducted on a rabbit and ovine model for biomaterial implantation. The response of neutrophils and MDM was evaluated *in vitro* after the titanium implant insertion. The neutrophil activity was assessed on the basis of the enzymes release, reactive oxygen species-ROS and reactive nitrogen intermediates-RNI generation. The MDM response was assessed based on the nitric oxide (NO) and superoxide generation, arginase activity and morphological changes of these cells. Before the experiment, the AMPs extracts of autologous or heterologous origin were prepared. Additionally, some components of the AMPs extracts (namely PR-39 and protegrin mixture) were separated via the gel filtration chromatography.

Titanium implants

Titanium Grade 2 discs were prepared for this experiment, having pre-treated the implants surface with a triple surface etching formula (TSE). The Tollens method was used to incorporate silver on the titanium surface so as to obtain the titanium implants modified with silver nanoparticles [13].

Rabbits and surgical procedure

Six healthy male New Zealand White (NZW) rabbits, aged between 7 and 9 months, with the body weight of approx. 4000 g were used for the experiment. The rabbits were housed and treated according to the laboratory animal treatment and care guidelines. The study protocol was approved by the Local Ethics Committee of the University of Life Sciences in Lublin and the experiment was performed in compliance with the animal protection regulations.

Prior to the surgical procedure, the rabbits were carefully examined. After premedication with xylazine (Sedazin; Biowet, Pulawy, Poland), 5 mg/kg and ketamine (VetaKetam; Vetagro, Lublin, Poland) 30 mg/kg intramuscularly, the animals received ketamine (0.35 mg/kg/min) intravenously. The surgical procedures were conducted under standard sterile conditions. After the hair removal, shaving, disinfection and draping, a straight 3-cm skin incision was made over the medial proximal tibia. Then, after the surgical exposure, the 4 mm defect was made using an electric surgical drill. The titanium implant was inserted into the tibial defect and fixed to the cortical bone. Then, the muscle tissue and skin were sutured. After the surgery, the rabbits were examined daily for clinical signs of complications or adverse reactions.

Preparation of autologous (rabbit) AMPs and heterologous (porcine) AMPs extract and its isolated components (PR-39, protegrin mixture), neutrophils isolation and stimulation

Porcine neutrophils for the AMPs extract were isolated from the blood collected at an abattoir. The red blood cells were lysed by the addition of 0.83% ammonium chloride to the blood sample and then centrifuged. The remaining pellet was washed twice with phosphate-buffered saline (PBS). The final cells were homogenized to release the neutrophil granules. The granules were collected (25 000 x g, 40 min, 4°C), suspended in the 10% acetic acid and stirred overnight at 4°C to extract the antimicrobial peptides. The solution containing the peptides was separated from the granules (25 000 x g, 20 min, 4°C) and the obtained extract was considered as the AMPs neutrophil extract. Then, the peptides were isolated according to their molecular mass via the gel filtration chromatography. The obtained products, namely the crude AMP extract, PR-39 and protegrins were used to stimulate the neutrophils or macrophages cultures. Rabbit AMPs were prepared similarly from rabbit blood.

In order to prepare the rabbit AMPs extract and cell culture, rabbit neutrophils were isolated from the blood collected from the ear vein. The blood was obtained 7 days before and 24 hrs after the surgery. The cell suspensions were supplemented as follows: the control group with PBS (marked as unstimulated), the other groups were stimulated with crude porcine antimicrobial extract (EXT), PR-39, protegrins or the crude rabbit antimicrobial extract (AMPs). Then, the cultures were incubated for 30 min and for 22 hrs at 37°C in the presence of 5% CO_2. Next, the neutrophils activity was assessed. The enzyme release from azurophilic granules was assayed based on the elastase or myeloperoxidase (MPO) release and compared to the maximal enzyme content obtained after the cells treatment with 0.5% Triton X-100. The elastase activity assay was based on the cleavage of azocasein as a substrate at 25°C for 10 min; thereafter, the absorbance value was assessed at 490 nm. The MPO release was measured as absorbance at 490 nm after cleavage of the substrate- o-phenylendiamine (Sigma-Aldrich, Poznan, Poland). The alkaline phosphatase (ALP) release, constituting a marker of specific granule response, was estimated after the 10 min incubation at 25°C with an equal volume of 4-nitrophenyl phosphate disodium salt hexahydrate, after which absorbance was measured at 405 nm. The nitric oxide level was determined by means of the Griess reaction [14]. Briefly, the equal volumes of the culture supernatant and Griess reagent (0.1% N-[1-naphtyl] ethylendiamine dihydrochloride 1% sulphanilamide and 2.5% H_3PO_4) were mixed and incubated at room temperature for 10 min and absorbance was measured. The obtained values were expressed as the nitrite concentration. The superoxide anion generation was measured by incubating neutrophils with a 0.1% nitroblue tetrazolium solution at room temperature for 10 min and reading absorbance at 545 nm. The superoxide generation was assessed using the extinction coefficient 21.1 nM [14].

Evaluation of the rabbit AMPs extract influence on the rabbit MDM

The influence of the rabbit AMPs extract on the rabbit MDM was assessed basing on morphological and functional changes. The blood for leukocytes isolation was obtained 7 days before and 24 hrs after the Ti implant insertion into the tibial defect. Mononuclear cells were isolated from the whole blood by gradient centrifugation over Histopaque-1077 and immediately cultured at a concentration of 1.0×10^6 cells/mL into 96-well flat-bottomed tissue culture plates at 37°C and 5% CO_2 for 72 hrs in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum (BCS) to obtain MDM [14]. The cultures described as BCS did not undergo any additional stimulation. The other cultures were stimulated with 40 µg/mL of AMPs and marked as the rANE group. All these cultures were incubated for 3 days at 37°C and 5% CO_2, then the functional analysis was performed on the basis of the superoxide and NO generation and arginase activity of cultured MDM [15]. The morphology microscopic analysis was conducted using a reversed phase microscope (Olympus).

The study on the ovine model

The study was conducted on eight female sheep, BCP local breed, 4 months old, approximately 20 kg body weight, from the Bezek Experimental Farm, University of Life Sciences in Lublin. The sheep were clinically examined for possible infections and were found healthy with normal blood hematology and biochemistry parameters. The animal management and surgical protocol were approved by the local Ethics Committee number II in Lublin. The animals were anaesthetized with intramuscular xylazine (0.1 mg/kg) and butorphanol (0.1 mg/kg). For the local anesthesia, 2% Lignocaine was applied. The standard surgical approach for proximal tibia was used and a commercially available Ti plate was inserted. The postoperative care was Melovem (Meloxicam 5%, Dopharma Research B.V. 1.2 mL SC) as analgesic and Combi-ject (200 000 IU/mL Penicillin and 200 mg/mL Streptomycin) to prevent infections. The animals were monitored postoperatively for ten days to assess their breathing, heart rate, and body temperature. The postoperative wound and the skeletal system were examined to exclude motor disorders of the operated limb.

7 days before the experimental procedure the blood for hematological assays was collected from the jugular vein into tubes containing EDTA as an anticoagulant. A complete blood count was performed using an Abacus Junior Vet analyzer (Diatron, Budapest, Hungary) and hematological parameters in all animals were within the reference ranges. At the same time, blood aliquots of approximately 25 mL with 3.8% sodium citrate as anticoagulant were obtained for the AMP extract.

Evaluation of the ovine macrophages response to the AMPs extract

The blood for the MDM culture was obtained from each sheep before the Ti plate implantation into the proximal tibia and 5 months after the surgery. The AMPs extracts were prepared as previously described and stored. The mononuclear cells fraction (MNC) was isolated by gradient centrifugation over Histopaque-1077 to obtain the MDM, as in the case of the rabbit MNC. Then, the cultured MDM was stimulated with the ovine or porcine AMPs extract or left without additional stimulation as an unstimulated group. The MDM morphology and function was then assessed as described in the case of the rabbits.

Statistical analysis

Statistica 13.1 Software (Statsoft Poland) was used for statistical data analyses, with a two-tailed Student t test to compare two datasets and ANOVA for multiple comparisons, with the post hoc comparisons using Tukey's test. The values of $p < 0.05$ were considered as significant, the results were expressed as mean ± SD.

Results and Discussion

The rabbit neutrophil response to the autologous AMPs and the heterologous (porcine) AMPs extract and its isolated components (PR-39, protegrin mixture)

Having stimulated rabbit neutrophils with the autologous AMPs extract, their response diminished in comparison with the unstimulated cells (described as PBS). We observed the decreased MPO and ALP activity, as well as the diminished nitric oxide and superoxide generation in the measurements both before and after the implantation. On the contrary, the heterologous crude AMPs extract (described as EXT) had a pro-inflammatory effect on the neutrophils obtained before and after the implantation. We noted the increased elastase activity from 51 ± 0.8% before to 54.83 ± 0.98% after the implantation. On the other hand, we observed that some components of the porcine extract, namely PR-39 and protegrins, had a different influence on the neutrophil activity. PR-39 in most cases acted as a factor decreasing the neutrophil secretory activity both before and after the implantation, as compared to the unstimulated groups. In all the measurements, the activity of neutrophils treated with PR-39 was significantly lower ($p < 0.05$) than after the crude AMP extract stimulation. Similar results were noted after treating the neutrophils with PG (FIG. 1).

These findings confirmed the previous studies on the anti-inflammatory influence of PR-39 [11] and simultaneously indicated the AMPs potential to regulate the neutrophil inflammatory response. We also proved that the autologous AMP extract has an anti-inflammatory effect and could be a mean to decrease the excessive neutrophil response during surgical procedures.

The influence of the rabbit AMPs extract on morphology and function of the rabbit MDM obtained before and after the biomaterial implantation

We discovered that the rabbit macrophages treated with the autologous AMPs extract showed partially anti-inflammatory features with the decreased nitric oxide and superoxide generation and the unaltered arginase activity. The concentration of nitrite and superoxide was lower after stimulation with rANE, in comparison with the BCS groups in the two time points. After the implant insertion, the NO generation decreased from 3.63 ± 0.2 µM in the unstimulated groups (marked as BCS) to 3.24 ± 0.13 µM ($p < 0.05$) in the group treated with rANE. Similar results were obtained before the biomaterial implantation. The superoxide generation decreased from 5.6 ± 0.4 nM before to 5.4 ± 0.34 nM after the rANE addition in the group after the surgery (FIG. 2).

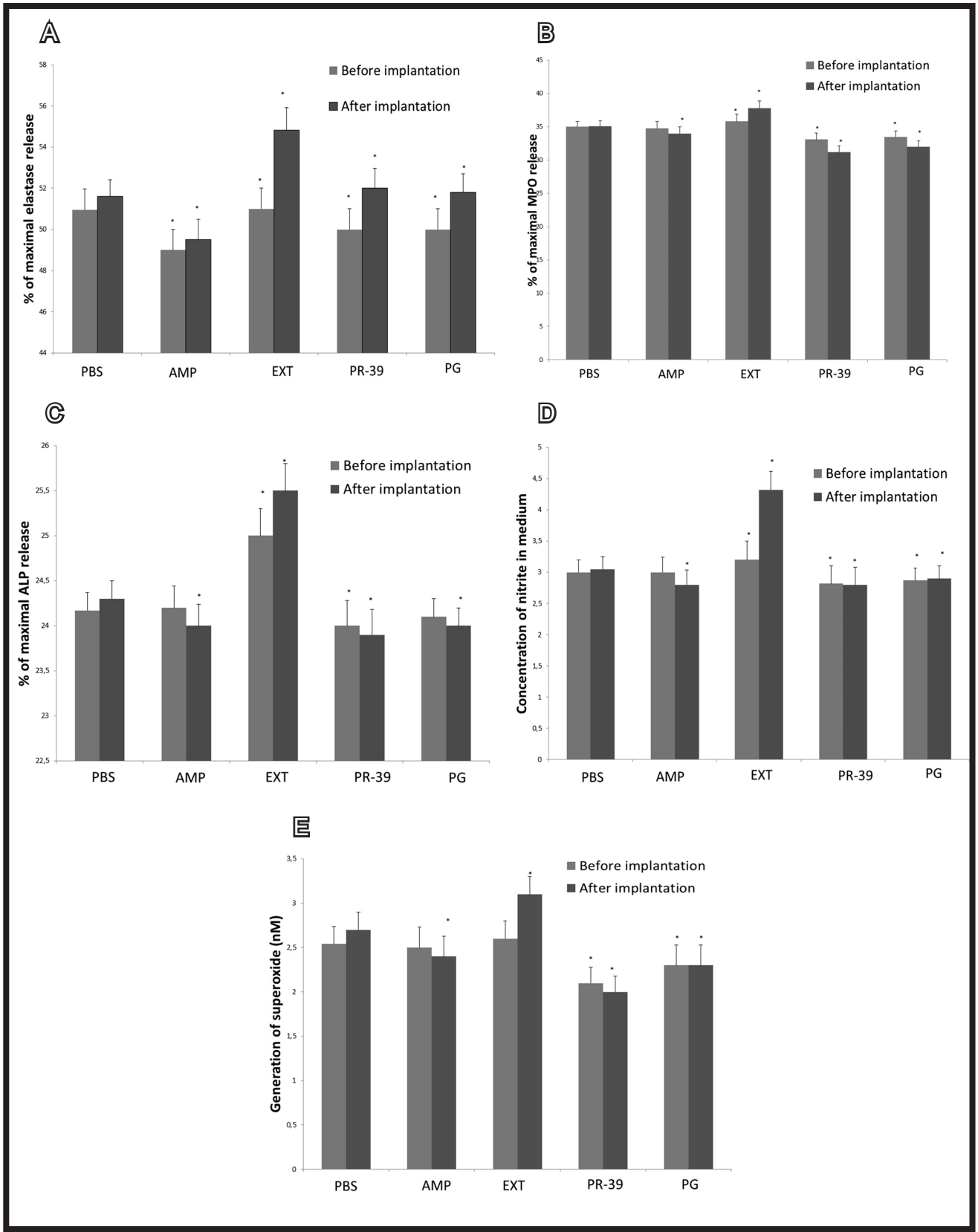

FIG. 1. The response of rabbit neutrophils to the autologous rabbit AMPs extract (AMP), the heterologous porcine extract (EXT) and isolated products of the porcine extract (PR-39, protegrin-PG) before and after the implantation. (A) The elastase release, (B) myeloperoxidase release, (C) alkaline phosphatase release, (D) nitric oxide generation, (E) generation of superoxide. All of the data expressed as the mean ± SD. *p < 0.05.

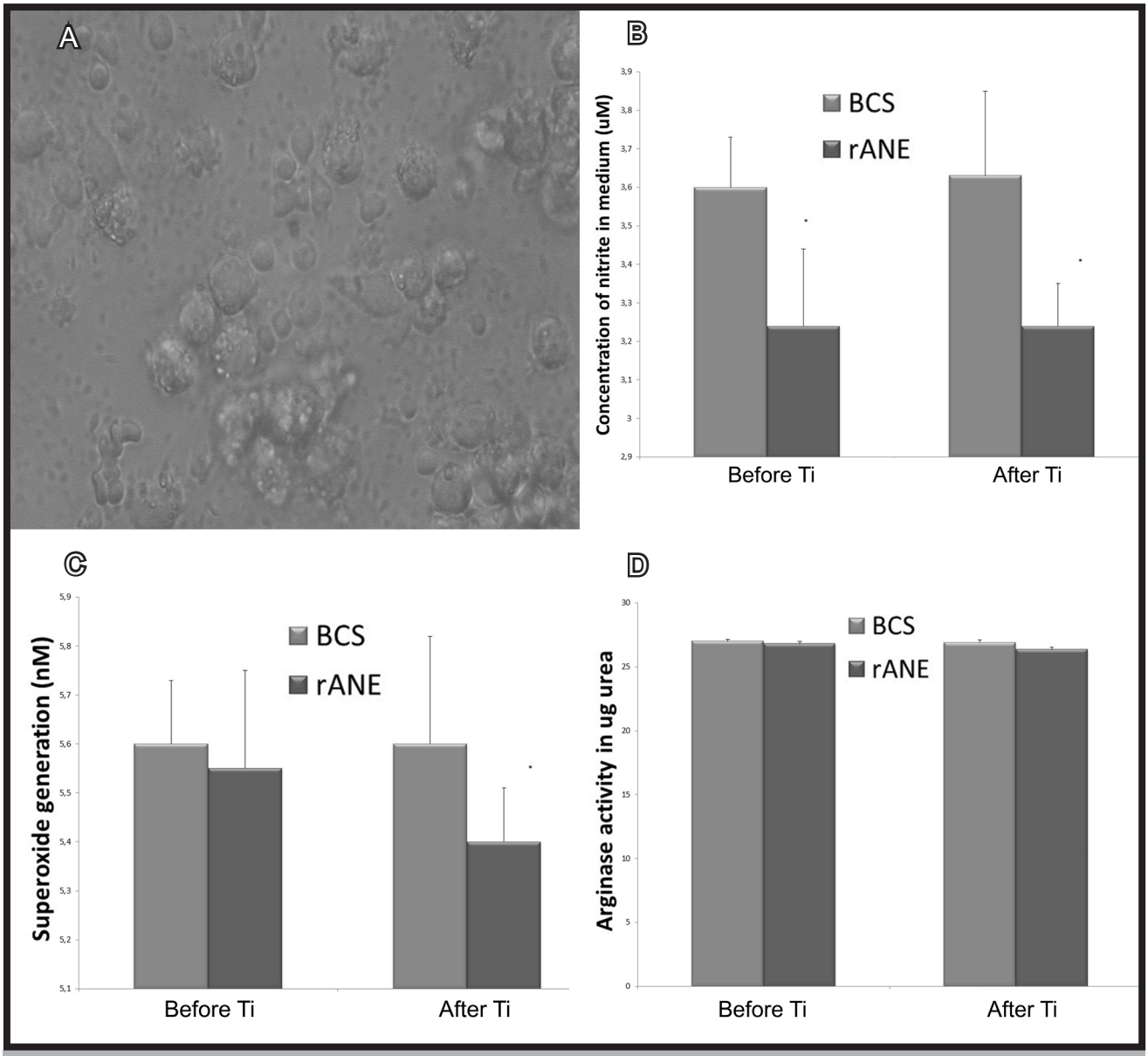

FIG. 2. The influence of the rabbit AMPs extract (rANE) on morphological and functional changes of MDM before and after the Ti implant insertion. (A) The MDM morphology after the rANE treatment , (B) nitric oxide generation, (C) superoxide generation, (D) arginase activity. All of the data expressed as the mean ± SD. *p < 0.05 compared with the cultures before the implantation.

Apart from their antimicrobial activity, AMPs are regulatory molecules that limit inflammation. Therefore, they promote immune homeostasis. The AMPs anti-inflammatory function was previously confirmed by several studies on animal models. It was demonstrated that the deficiency of these peptides resulted in the overexpressed inflammatory responses; i.e. cathelicidin- deficient mice exhibited a more severe inflammatory phenotype, as compared with wild-type mice. Similarly, the reduced expression of β-defensin in human enterocytes has been noted in the Crohn's disease. Moreover, the critical role of defensins in maintaining the integrity of intestinal mucosa and immune homeostasis is well established. The exogenous application of AMPs, such as human cathelicidin LL-37, CATH-2, BMAP-28 or HBD2, and synthetic peptides (e.g. IDR-1 and IDR-1002) has proved to control inflammation in various animal models of infection and sepsis. Similarly, the LL-37-derived peptide controlled the disease process in a mouse model of inflammatory arthritis and the IDR-1002 effectively suppressed the airway inflammation *in vivo* [12].

The influence of the ovine and porcine AMPs extract on morphology and function of the rabbit MDM obtained before and after the biomaterial implantation

In the experiment on the MDM stimulation with the autologous (ovine) and heterologous (porcine) AMPs neutrophil extracts, we found out that the MDM response was different and related to the extract origin. The autologous extract causes the NO generation decrease, particularly after the implantation, from 3.03 ± 0.23 μM (unstimulated) to 2.65 ± 0.22 μM (stimulated with oANE). Moreover, the superoxide generation was also lower in comparison to the cultures described as BCS, whereas the arginase activity remained unchanged. It was confirmed that the local arginase activity is required for wound healing [16]. Thus, this effect could be considered as beneficial in the repair process. Conversely, the heterologous extract stimulation showed a significant ($p < 0.05$) increase in the free radical generation. We detected that the nitrite concentration in the cultures after implantation reached 4.48 ± 0.22 μM in comparison with 3.03 ± 0.23 μM in the unstimulated group. Additionally, the arginase activity was higher before and after the treatment (FIG. 3).

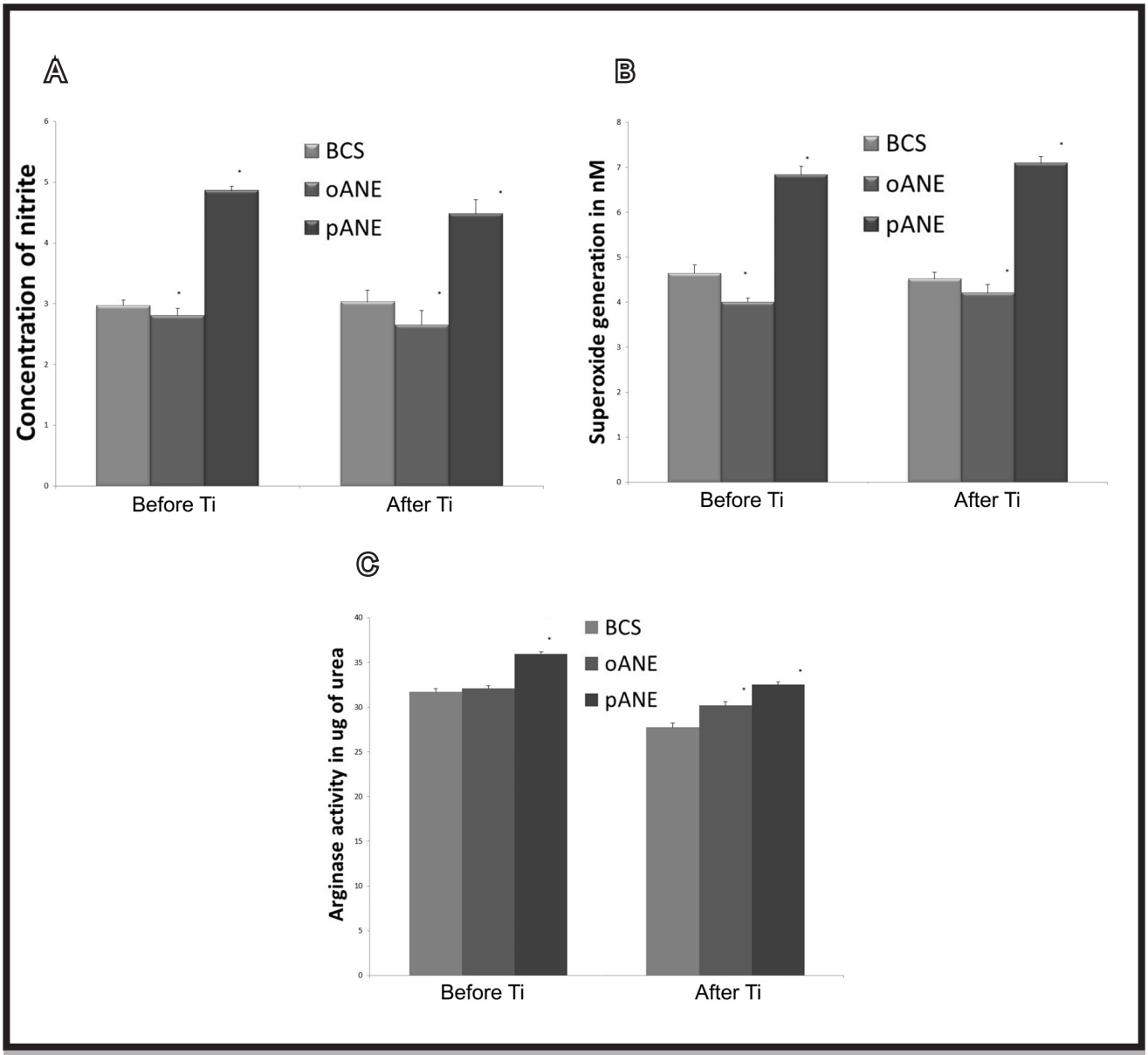

FIG. 3. The influence of the autologous (oANE) and heterologous (pANE) extract on the MDM function before and after the Ti implant insertion in the ovine model (A) nitric oxide generation, (B) superoxide generation, (C) arginase activity. The data expressed as the mean ± SD. *p < 0.05.

With regard to these results, we studied the influence of autologous and heterologous AMPs extracts on some components of the white blood cells system (WBC). Our study revealed that the response depended on the extract origin, the animal species, the cell type and the animal status (before or after the implantation). The study of neutrophils revealed that the autologous AMPs decreased their activity in respect of the enzymes release and the free radicals generation. Contrary to this, the heterologous AMPs extract increased the secretory activity of these cells.

After the implant insertion, MDMs are among the first cells at the implant site and they are considered as key regulators of both the initiation and the resolution of inflammation [17]. Therefore, in our experiment, we also evaluated the MDM response to the AMPs extracts. The MDM after stimulation with the autologous extract showed the decreased ROS and NO generation with the intact arginase activity in comparison with the cultures stimulated only with BCS. After the heterologus AMPs extract stimulation, in turn, these cells generated higher amounts of superoxide and NO and the higher arginase activity. These results indicated a mixed subpopulation of both pro- and anti-inflammatory features in which the unchanged arginase activity ensures an undisturbed healing process. A similar intermediate state was described in response to the synthetic IDR-1018 peptide when macrophages developed a unique capability to maintain particular proinflammatory activities while producing anti-inflammatory and regulatory mediators [9,18].

It should be emphasized that differences between the cellular response before and after the implantation resulted both from the biomaterial itself and the overall host reaction to the implantation procedure. The implant surface characteristics also influenced the host immune response. Different modifications of the implant surface, e.g. the pectin coatings, were described previously [19]. The authors evaluated the *in vitro* interactions of the implant and human bone marrow stromal cells regarding f the cell adhesion and proliferation. In our experiment, we used commercially available Ti discs with a silver coating and the response of circulating neutrophils and MDM was studied before and after the implantation.

Conclusions

The inflammatory response may be modulated using blood-derived products which not only display antimicrobial activity, but also regulate the inflammation processes, dependently on the organism needs. Therefore, different AMPs extracts can be used to enhance or suppress the inflammatory response. This may be applicable in surgical procedures where antimicrobial peptides will act both as an antimicrobial agent and a factor inhibiting the excessive reaction to the biomaterial implantation. The obtained results could be used to develop novel autologous blood-derived products for bone healing.

Acknowledgements

This work was financed from the statutory funds of the Sub- Department of Pathophysiology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin.

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CHEMICAL AND PHYSICAL MODIFICATIONS OF ELECTROSPUN FIBERS AS A METHOD TO STIMULATE TISSUE REGENERATION – MINIREVIEW

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Abstract

Fibrous scaffolds based on (bio)polymers are observed as mimicking the microstructure of the extracellular matrix. Thus, they are considered as an example of a utilitarian scaffold, useful for the regeneration of various types of tissues. The techniques described in the literature are well known to obtain submicrometric and nanometric fibers that, when randomly arranged, mimic the ECM.

The biomimetic scaffold criterion might be even better reflected if the cell adhesion sites are present on the surface of such fibers. They promote the formation of the focal adhesion contact or facilitate the formation of a protein film on the fiber surface. Such a process is enhanced by an appropriate physical or chemical modification that activates the protein adsorption and the subsequent cell adhesion. The aim of this paper is to present different methods of physical and/or chemical modifications of fibrous materials: which can serve as scaffolds to support the regeneration processes of various tissues. In terms of physical methods, only weak interactions between the surface and the modifier were observed. This technique is simple but not durable. Chemisorption used as a second method of fiber modification is possible if a covalent or ionic bond is formed between the fiber and the modifier. Therefore, the chemical adsorption may not be fully reversible and requires a sequence of chemical actions to form a chemical bond. The most commonly used methods are the combined methods where the first step is the physical activation of the fiber surface, which facilitates the chemical modification step.

Keywords: *electrospun fibers, fibrous scaffold, surface modification, extracellular matrix*

[Engineering of Biomaterials 159 (2021) 31-41]

doi:10.34821/eng.biomat.159.2021.31-41

Introduction

The main purpose of the scaffold-based approach is not just to replace the missing tissue but also to create a microenvironment for its regeneration and, eventually, restoring its function. It could be achieved by a geometry which guarantees appropriate mechanical support and advantageous surface properties triggering cellular responses. The structure within the body responsible for these processes is the extracellular matrix (ECM) which is a three-dimensional mesh of macromolecules, such as collagen, glycoproteins, and enzymes [1]. The characteristic ECM structure provides the structural support for growing tissue, it induces the cell adhesion, migration and proliferation and affects its antibacterial properties. Therefore, the focus of tissue engineering is on mimicking the microstructure of the native extracellular matrix as well as its chemical activity.

Since the ECM is a permanent and unique part of every tissue in the human body, fibrous morphology is the most desired form of a scaffold for regenerative engineering. Research shows that the synergistic effect of the matrix composition, its architecture and stiffness play a big role in maintaining the tissue functions. The appropriate adjustment of its characteristic features at microstructural and structural level increases the chances of inducing regenerative processes [2]. Another study highlighted the importance of the surface topography in gene expression regulation. It revealed that fibrous microstructure has a greater potential for altering the human mesenchymal stem cells (hMSC) gene expression than a flat one [3]. It is reported that factors, such as the fiber diameter, porosity and the way they are organized, regulate the cells behaviour, especially their proliferation and differentiation. Finer PCL fibers ($\varnothing$ = 0.35 μm) promote the better differentiation of pre-osteoblastic cells (i.e. MC3T3-E1) while a larger diameter ($\varnothing$ = 6.5 μm) fibers exhibit the better proliferation [4]. Another study conducted by Lekshmi Krishna et al. also highlighted the impact of a fiber diameter on corneal epithelial cells (HCE-T) and the retinal pigment (ARPE-19). The research revealed that HCE-T seeded on PCL with a diameter of 500 nm exhibited the greater differentiation while those seeded on PCL with a diameter of 1300 nm showed the better proliferation. In the case of ARPE-19 cells seeded on larger fibers, the greater phagocytic activity and the lower apoptosis level were observed while on finer fibers the pluripotential behaviour and secretion of vascular endothelial growth factor A (VEGF-A) was higher [5].

There are lots of methods for ECM fibrillar structure restoration which include the phase separation technique, the molecular self-assembly and the electrospinning. Among them, the solution electrospinning is out of big interest because of its simplicity, the possibility of using various materials and generating nanoscale fibers with a high surface-to-volume ratio [6,7]. On the other hand, the use of a solvent increases the risk of the residual solvent remaining in the scaffold. Therefore, it may contribute to its toxicity towards the surrounding tissues and increase the production costs [8]. A method that does not involve a polymer solution is the melt electrospinning. In this technique, the fibers are formed directly by heating the polymer, therefore the disadvantages associated with the solvent application are eliminated, which, makes the melt electrospinning safe and green [8]. To reduce the fibers diameter, the process can be also aided by hot air (melt-blown electrospinning). By increasing the airflow velocity not only the fiber diameter is reduced but also its alignment and anisotropy are increased. It improves the Young Modulus and yield stress so the fibers are suitable for e.g. tendon regeneration [9,10]. The melt electrospinning writing is an additional manufacturing method that allows achieving aligned sub-micro- and microscale fibers (≈0.8 μm to ≈140 μm) [11]. The greater control over the fibers alignment ensures their arrangement into different shapes and obtaining the desired mechanical properties of a scaffold as well as the cellular alignment [11,12]. It is also proven that different geometric structures exhibit diverse drug release kinetics, which makes the melt electrospinning an attractive method for the wound dressing production [13].

The traditional electrospinning results in a two-dimensional microstructure and low porosity, which limits the cell migration within a scaffold. In the wet electrospinning, a metallic collector is replaced with a liquid solution, which enables achieving a three-dimensional, sponge-like microstructure that exhibits the improved porosity, hydrophilicity and cell adhesion and proliferation [14]. Another interesting technique for producing nanofibers is the rotary jet spinning (RJS). In this method, a polymer jet is ejected from the reservoir holes by a centrifugal force caused by the shaft rotation. Simultaneously, the solvent evaporates, therefore its low boiling point is necessary. Since the RJS uses the high-speed rotation instead of an electric field, a conductive solution is not required. This technique leads to aligned, submicrometric fibers with a rougher surface, in comparison to the electrospinning. Such a morphology limits the implant bacterial colonization without affecting the cell viability [15].

All the above-mentioned methods lead to obtaining scaffolds that differ in fibers dimensions, their arrangement, topography and porosity, including the diameters in the range of nanometers (<100 nm), sub-micrometers (<1 μm) and micrometers. The research shows that a scaffold consisting of both nano- and submicrometric fibers exhibits the most sufficient mechanical properties and cells penetration [16]. The diameter of the electrospun fibers differs from the collagen fibrils of the native ECM. However, the full functionality of the tissue is maintained also by a hierarchical microstructure of an extracellular matrix. Regardless of the type of tissue, this inhomogeneity plays a key role in maintaining homeostasis and inducing a cellular response and thus, it determines the proper regeneration and functionalization of the tissue. Therefore, there is a great demand for the substrates with a complex microstructure which allows for differentiation of the cells toward an appropriate phenotype, their infiltration into the scaffold and eventually the formation of the proper tissue. By changing the parameters of electrospinning it is possible to obtain fibers with different diameters, arrangements (aligned or randomly oriented) and morphology (smooth, porous, hollow, etc.). This diversity of nanofiber microstructure enables the control over the formation of a particular type of tissue. The fibers arrangement has also a big impact on the mechanical properties of the scaffold. Aligned fibers contribute to more anisotropic mechanical properties which are used in fibrous mats for bone and nerves regeneration [17,18]. The ability to mimic the morphology and composition of the ECM fibers is a reason behind the widespread use of electrospinning in biomedical applications. Unfortunately, the majority of synthetic electrospun fibers do not facilitate the cellular attachment due to the high hydrophobicity and inappropriate surface characteristics [19].

There are two ways of promoting the cells-scaffold interaction. The first one is a modification of the bulk material by the copolymers production, the piezoelectric materials application, and the plasma treatment of the pre-electrospinning solution, etc. [6,20]. The second way is the surface functionalization [20]. Both approaches facilitate the cell adhesion and antimicrobial behaviour of the scaffold by altering its surface morphology, roughness, hydrophilicity and free energy. However, the drawback of the first method is that any changes in the material's chemical composition may decrease its mechanical properties and thus its cell-supporting ability. Therefore, since the interactions between cells and scaffold occur at the interface, the promising strategy is to alter the surface properties, and not to modify the bulk material structure. Depending on the application, the surface characteristics requirements may vary but still there are a few that every scaffold must fulfil. These are: high biocompatibility, selective permeability, appropriate mechanical properties, antibacterial properties, lack of immunological response or cytotoxic behaviour [21,22]. Numerous technologies intended for fibers surface modification result in its various properties. All the methods derive from two long-known surface biofunctionalization strategies, i.e. physical and chemical modification (FIG. 1). The physical modification methods are simple, safe as well as cost and time efficient. However, the bond between the substrate and the coating material is not durable. On the other hand, chemical methods more closely reflect the conditions in the human body. They are also characterized by the higher durability, due to the new chemical bonds that appear between the fiber surface and the modifier. However, these methods are limited by the range of materials susceptible to chemical modifications. Since the aim of the ECM regeneration is to achieve the microstructural resemblance via the electrospinning, the challenge grows.

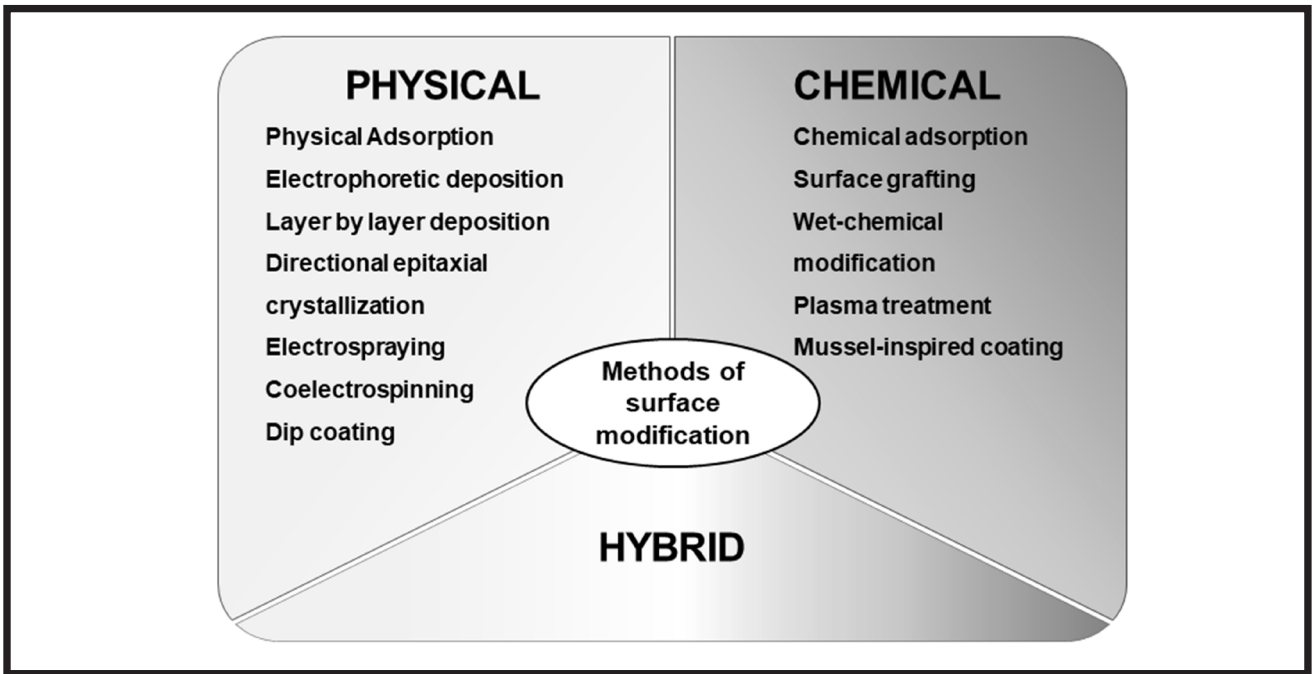

FIG. 1. Methods of fibers surface modification.

Physical Surface Modification

Physical methods of modification belong to the simplest ways of immobilization. They are based on the physisorption characterized by weaker interactions between the substrate and an attached biomolecule, such as van der Waals forces, hydrogen bonding or hydrophobic interactions [23]. The following is a brief description of physical methods used in the literature to modify fibers. These include: the physical adsorption (immobilization), the electrophoretic deposition, the dip coating, the electrospraying, the co-electrospinning, the directional epitaxial crystallization, the layer by layer deposition.

Physical Adsorption

The physical adsorption is the most commonly used technique because of its simplicity and the non-destructive character. It is based mainly on electrostatic interactions, such as hydrogen bonding or van der Waals forces between the substrate and the coating, without forming covalent bonds [24]. The main drawback of the physical adsorption is the random orientation of the molecules adsorbed on the surface, which may result in their functionality loss [25]. Chen et al. investigated the difference between the physical adsorption and the chemical treatment of silica/PVP composite fibers with laminin molecules. The outcomes indicated the covalent attachment as a better solution because the neural cells proliferation was significantly greater than that of the physically modified samples [26]. Jianhua Ye et al. modified the thermoplastic polyurethane surface by incorporating cellulose nanofibers (CNF) via the ultrasound-assisted method. The hydroxyl group of the adsorbed CNF improved the hydrophilicity and water retention ratio. It resulted in the better cell adhesion and proliferation, in comparison to the untreated polyurethane [4].

Dip coating

Another physical surface modification method is the dip coating. It is simple, cost-effective and can be used even for complex geometries without wasting a lot of material. These advantages have contributed to its widespread use not only to produce scaffolds but also to modify the wettability and reduce the pore size of fibrous membranes [27]. In the dip-coating method, a fibrous sample is immersed in the coating solution for a certain time and then removed. Eventually, the adsorbed film is dried by the solvent evaporation. The coating thickness depends on the dipping time, the drying atmosphere, the solution viscosity, the withdrawal speed and the number of cycles [28].

The dip coating is also used to fabricate substrates for cell cultures. Immersing the cellulose mesh in a silk fibroin solution retains the three-dimensional microstructure of the cellulose substrate and contributes to its core-shell morphology (FIG. 2). The random secondary structure of silk fibroin present on the surface results in the better cell aggregation and formation of tumor spheroid [29].

Moreau et al. prepared a ligament implant of PVA fibers coated with PVA hydrogel and hydroxyapatite (HAp) via the combined dip-coating and physical cross-linking methods. The results indicated that the PVA/Hap layer did not significantly improve osseointegration as HAp was partially trapped within the PVA matrix, causing the inappropriate kinetics of its dissolution. However, the coating contributed to the reduction of fibrous tissue formation [30].

In our study of PCL fibrous scaffolds modified by the dip coating in the hyaluronic acid (HA) suspension, we obtained the non-uniform morphology of single fibers. The achieved layer was not homogeneous, but it did not lead to fiber sticking and the scaffolds did not lose their porosity. As a result, we observed a drop in the wettability of the fibrous scaffold (decrease of the wetting angle from 92° to 56°).

Electrophoretic deposition

The EPD is a two-step process consisting of electrophoresis followed by deposition. In the first step, an external electric field causes the migration of colloidal charged molecules/particles towards the oppositely charged electrode. During the deposition, the stage particles accumulate and coagulate at the electrode surface. The deposition occurs either on the anode or cathode, depending on the surface charge of the molecule [31].

Thanks to the electrophoretic deposition, a surface with antifouling properties can be obtained. Thinakaran et al. deposited the chitosan and polyethylene glycol coating containing silver nanoparticles onto the surface of the fibrous PCL mat. As a result, they achieved a uniformly distributed layer containing an antimicrobial agent, which made the samples completely resistant to biofilm formation [32].

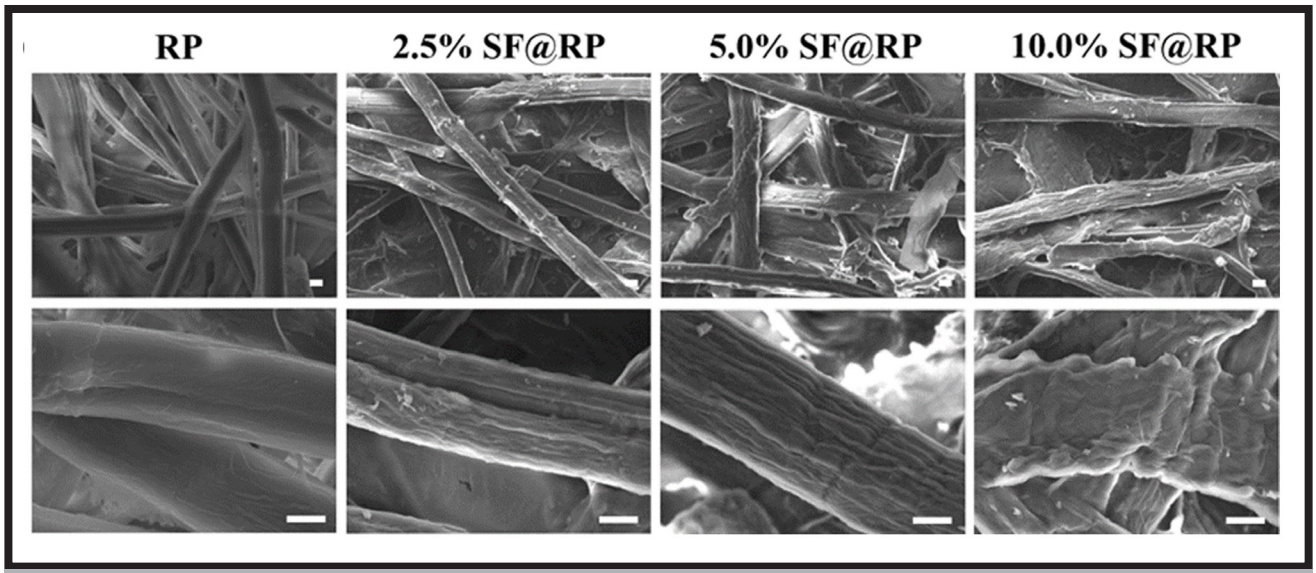

FIG. 2. FESEM pictures of rice paper (RP) and SF@RP (silk fibroin@rice paper) at different concentrations of SF [29].

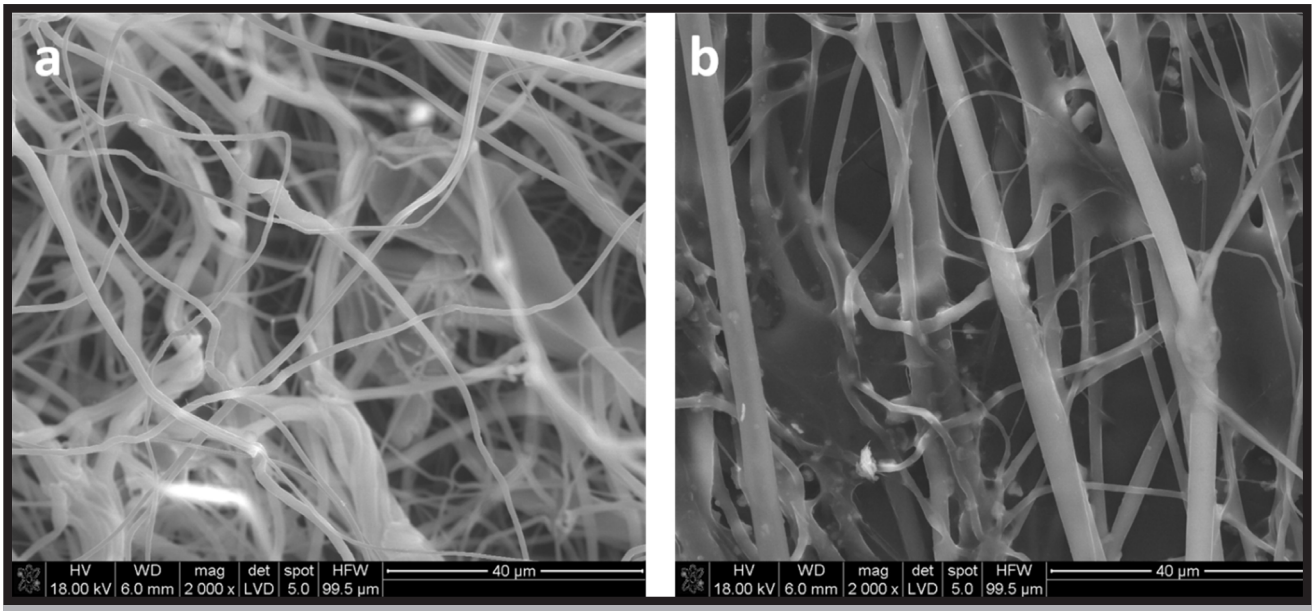

FIG. 3. Unmodified cellulose acetate (CA) fibers (a) and CA fibers surface modified by EPD method in SiO_2 suspension (b) [35].

In the case of porous materials, just as fibrous scaffolds, the main drawback of the EPD is the possibility of the pores blockage. Therefore, Taale et al. coated graphite fibers with hydroxyapatite nanoparticles using the periodic electrophoretic deposition. Their modification resulted in more uniformly covered fibers within the whole volume of the scaffold and the better promotion of osteoblasts activity [33].

This method could be also combined with other techniques of deposition. Yao et al. coated the graphene foam by a two-step process involving the dip coating in a poly(lactic-co-glycolic acid) and the electrodeposition of a chitosan and bone morphogenic proteins (BMP2) hybrid layer. The performed modifications improved mechanical properties and differentiated the human mesenchymal stem cells towards osteocytes caused by the BMP2 sustained release [34].

In the electrophoretic deposition method, the suspension composition must be carefully studied. Better results are observed if the suspension contains particles with the character (Dzeta potential) opposite to the chemical nature of the fiber material. If this condition is not fulfilled, agglomerates or solidified areas are observed on the fiber surface (FIG. 3).

Electrospraying

The electrospraying is a process in which a liquid is dispersed by a high electric field. As a result, nano- and submicron particles are formed [36]. The principles of electrospraying and electrospinning are similar. However, changing the solution properties (solvent, polymer concentration, viscosity) or process parameters (voltage, flow rate, distance between nozzle and collector) may lower the degree of the molecular interaction in the solution. Therefore, a droplet may form instead of a fiber [37,38]. Both methods could be also combined.

Jiajia Tang et al. produced a nanocomposite scaffold for nerve regeneration consisting of electrospun aligned PCL fibers. They modified its surface by electrospraying collagen and conductive polypyrrole nanoparticles (PPy NPs). The synergistic effect of the fiber orientation and conductive properties of electrosprayed PPy NPs provided appropriate mechanical properties and induced the neurite/axon elongation along the fibers. In combination with the external electrical stimulation, it also resulted in the enhanced neurogenesis [39].

In another study, Yuzhu He et al. prepared the nanocomposite chitosan (CS) and gelatin (Gln) guided bone regeneration membrane enriched in hydroxyapatite nanoparticles (nHAp) and antimicrobial peptides (AMP) (FIG. 4). Via the layer-by-layer electrospinning and electrospraying the osteogenic layer (CS/Gln/nHAp), the barrier layer (CS/Gln) and AMP-loaded PLGA microspheres embedded in between were formed. The results showed the excellent biocompatibility, osteogenic behaviour and long-term release of an antimicrobial agent, i.e. one week of bactericidal activity and antibacterial activity over one month (research conducted toward *E. coli* and *S. aureus*) [40]. Due to the particles morphology which ensures the adjustable release kinetics, the electrospraying is also widely used in the drug encapsulation [41].

Co-electrospinning

The first physical method used at the phase of the fiber mesh production is coelectrospinning. This surface modification has the same benefits as the traditional electrospinning, i.e. simplicity, possibility of using various materials and cost-effectiveness. Moreover, the addition of the second material with the different properties diminishes the drawbacks of the first one and thus improves the integration with the surrounding tissue [2,3].

Taskin et al. created a 3D scaffold consisting of PCL and polydopamine (pDA) by the single-step wet electrospinning. The hydrophilic pDA addition boosted the biocompatibility and the human mesenchymal stem cells adhesion as well as their penetration within the scaffold and differentiation towards fibroblasts [42]. Other biomolecules that could be incorporated into the scaffold are extracellular matrix components. Bhowmick et al. prepared co-electrospun nanofibers made of gelatin, chondroitin sulfate and sulfated hyaluronan which was modified with glycosaminoglycans. They observed a significant increase in the adhesion and proliferation of the mesenchymal stem cells, keratinocytes and fibroblasts [43].

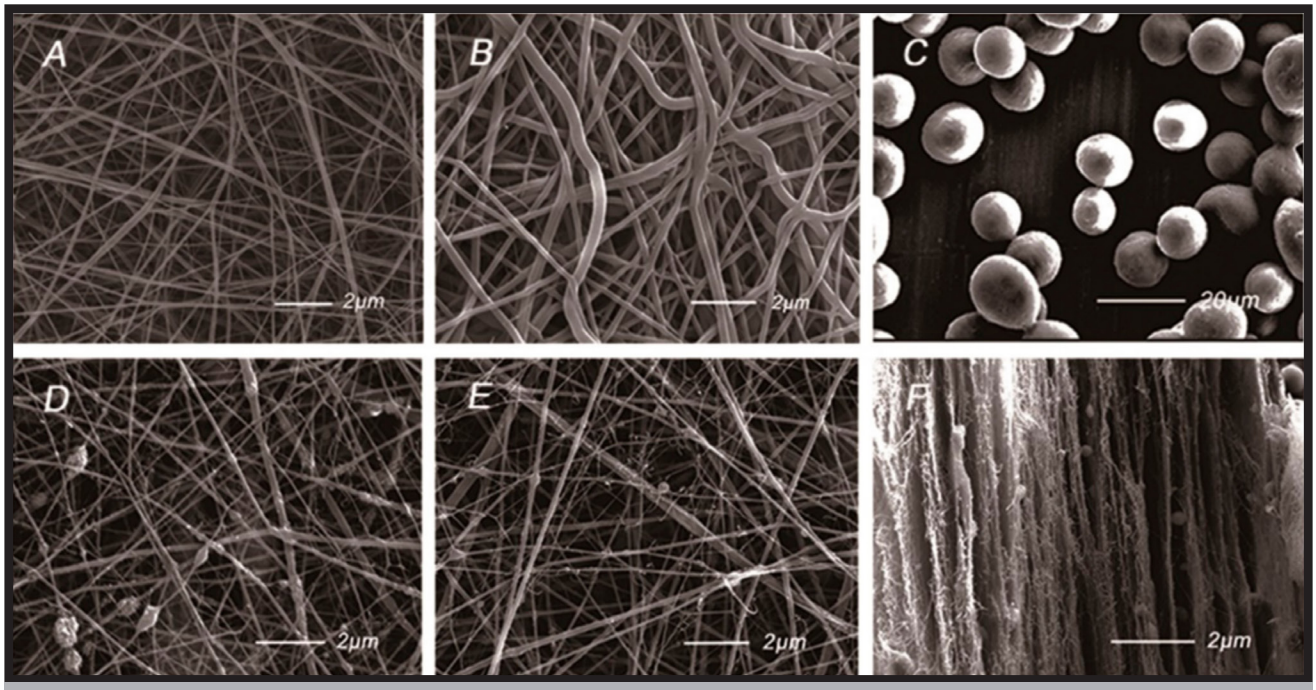

FIG. 4. SEM images of the microstructure of the Gln/CS composite membrane before (A) and after (B) crosslinking; (C) Electrosprayed AMP@PLGA; The layer of Gln/CS/nHAp by magnetic stirring (D) and ultrasonic dispersion (E); (F) Cross-sectional image of the membrane [40].

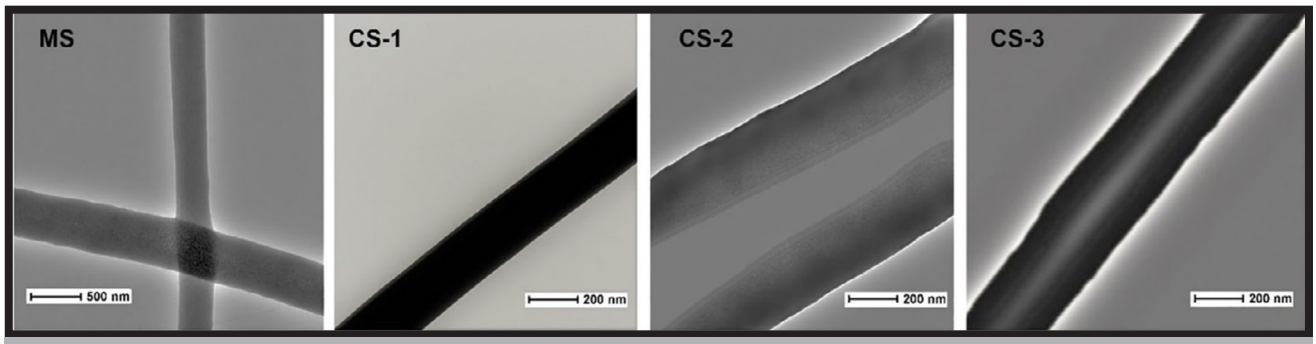

FIG. 5. TEM images of monostructural and core-shell fibers [45].

To improve the scaffold performance, the co-electrospinning can be combined with the surface grafting and used e.g. in bone tissue engineering. In the study conducted by Qingxia Zhu et al., the fibrous mesh consisting electrospun poly(ester urethane)urea (PEUU) was reinforced with $nTiO_2$. Moreover, poly(ester urethane) (PEU) was grafted onto the $nTiO_2$ to improve its interaction with the substrate. This approach resulted in the improved tensile strength (the increase of Young modulus) and better biomineralization and mesenchymal stem cells proliferation [44]. Another way of modification is the coaxial electrospinning. This approach has found a high interest in tissue engineering and wound healing applications (drug release). Eskitoros-Togay et al. used the coaxial electrospinning to produce fibers for the controlled release of doxycycline (DOXH). They prepared core/shell fibers of poly(ε-caprolactone)/poly(ethylene oxidase) loaded with DOXH in the core part (FIG. 5). The outcomes indicated that blending hydrophobic (PCL) and hydrophilic (PEO) polymers resulted in the more controlled drug release kinetics in the first 120 min, when compared to monolithic fibers [45].

Layer by layer deposition

The main advantages of this method are the possibility of producing multilayer coatings and the nanoscale control over deposited layers. In this technique, oppositely charged polymers are alternately deposited on the surface where they form an ultrathin and uniform film (FIG. 6). Moreover, the LBL uses natural forces, such as hydrogen bonding, electrostatic interactions and molecular interactions, which makes it cost-efficient [46]. The method has numerous benefits, such as: precise control over thickness and properties of the coating, homogeneity of the layers, versatility of the biomolecules and their controllable release [47]. Qian et al. used the layer by layer deposition to functionalize PCL nanofibers by silk fibroin. Additionally, heparin disaccharide (HD) was attached to the scaffold by the click chemistry to inhibit foreign body reaction and fibrosis development around it. The studies revealed that the HD incorporation resulted in the interleukin-4 (IL-4) adsorption which is responsible for the macrophages polarization toward M2 macrophages and, therefore, it improves anti-inflammatory properties of the PCL fibers [48].

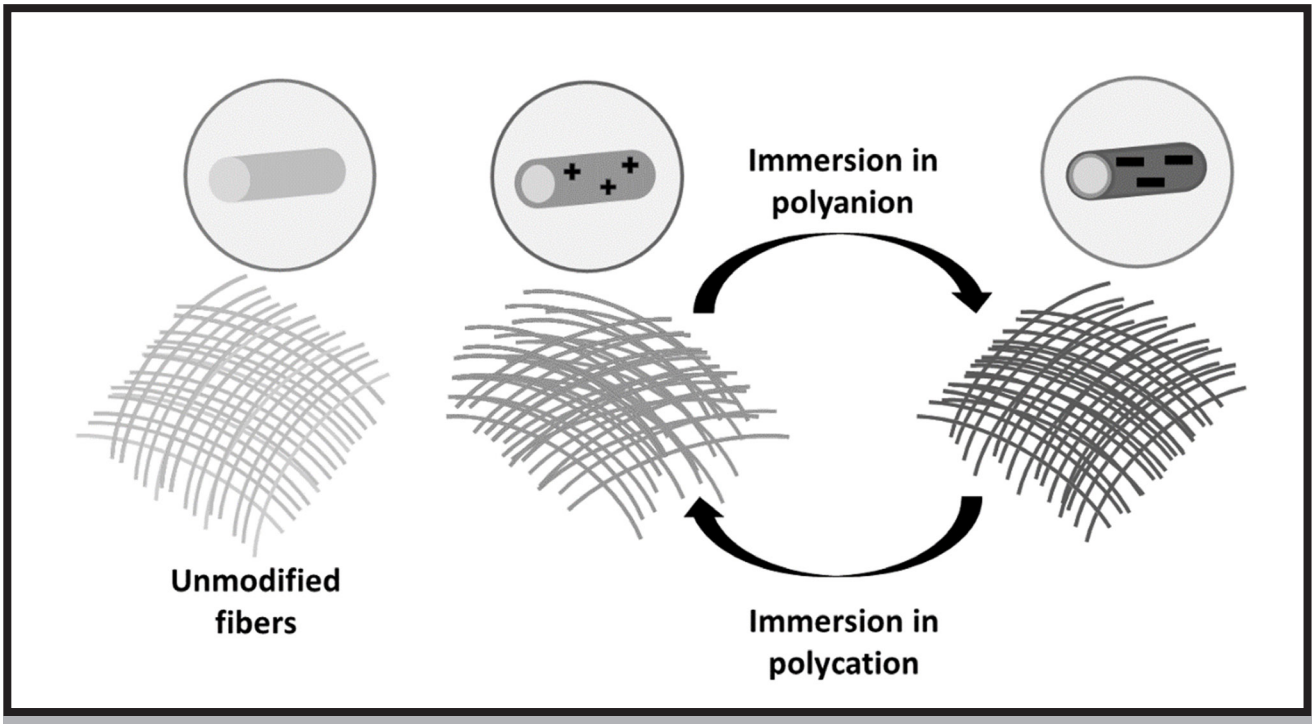

FIG. 6. Schema of the LBL process.

Directional epitaxial crystallization

Yet another way to modify the nanofiber surface is the directional epitaxial crystallization. Via this method the characteristic morphology called shish-kebab is formed (FIG. 7). It consists of the electrospun fiber which acts as a "shish" and of periodically arranged crystals which act as a "kebab". This type of morphology is achieved by immersing the scaffold in a crystal-forming polymer and the solution free chains crystallizing onto the surface of the electrospun fibers [49]. This method could be used with various materials, such as PLA [50], PCL [51] or PEO [52]. Guo et al. attempted to mimic ECM collagen fibrils by the self-induced crystallization on the PCL nanofibers. This modification enabled achieving the shish-kebab morphology which promoted further cell adhesion, migration and proliferation [51]. Such a method could be also used for bone regeneration. Liu et al. prepared a scaffold which consisted of co-electrospun PCL and β-tricalcium phosphate (β-TCP) which they modified by the self-induced crystallization. Both the presence of β-TCP and the shish-kebab morphology decreased the contact angle, protein adsorption and cellular adhesion. Moreover, the samples exhibited enhanced mechanical properties and biomineralization [53]. This method could be also combined with the coaxial electrospinning. Huang et al. prepared hierarchical core-shell nanofibers consisting of PCL as a shell and PVA as a core modified by the self-induced crystallization. Additionally, they enriched one group of the fibers with bone morphogenetic protein 2 (BMP2) in the core part. The results revealed that the shish-kebab morphology and the growth factor incorporation (BMP2) promoted the osteogenic cells differentiation. Moreover, a hierarchical structure of the scaffold allowed the sustained BMP2 release [54].

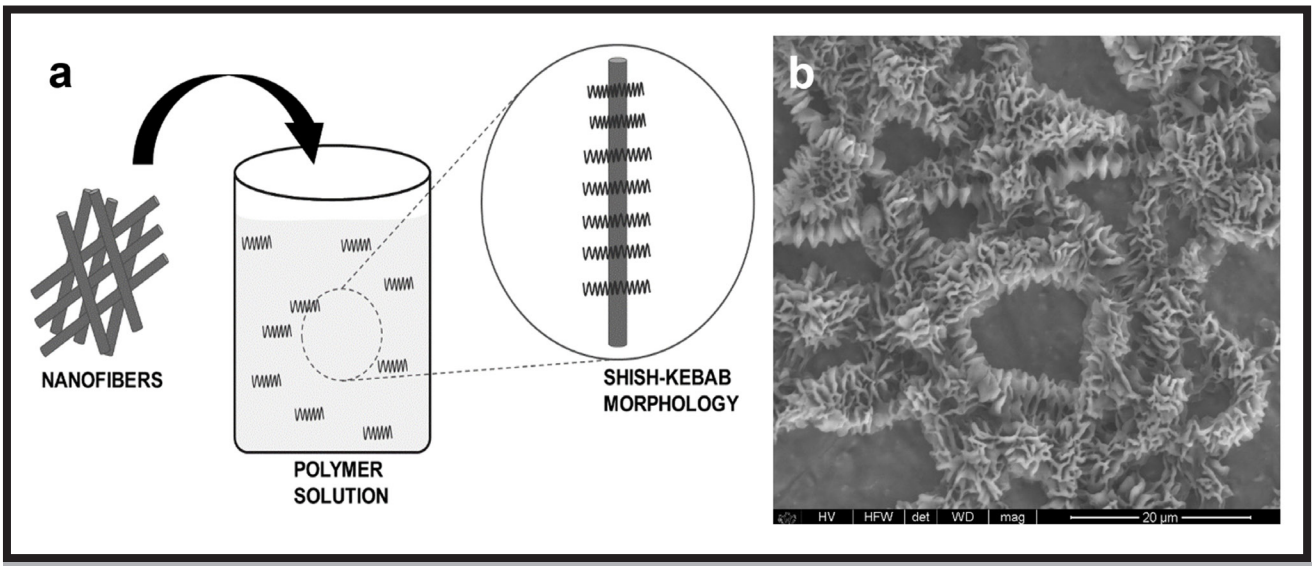

FIG. 7. Schema of the directional epitaxial crystallization process (a) and SEM image of shish-kebab morphology (b) [55].

Chemical Surface Modification

Chemical modification techniques consist in forming covalent bonding between the surface and the immobilized agent. In comparison to the physical methods, the molecules attached to the surface via chemical modifications are less prone to being leached out. There are numerous approaches, such as the wet chemical modification, the plasma treatment, the surface grafting with peptides or copolymers [56].

Chemical adsorption

In the chemical adsorption method (also called chemisorption) the molecule on the substrate is immobilized when the electrons of the adsorbate and the adsorbent form a covalent or ionic bond. Therefore, the chemisorption requires a surface with a significantly higher free energy than in the physical adsorption [57]. If active functional groups are not present on the surface, its chemistry has to be modified [58]. One of the strategies is to activate the surface via the covalent immobilization by means of an intermediary linker, e.g. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS). In their research, Guler et al. used EDC/NHS to activate the COOH- groups of P3ANA immobilizing RGD peptide onto the surface of the poly(ε-caprolactone)/poly(m-anthranilic acid) (PCL/P3ANA) fibers. As a result, the better cell adhesion and osteogenic activity were observed [55]. Another approach involves adapter molecules. Biotin-avidin is stable, unaffected by the pH change or the subsequent washing complex coupled by a strong non-covalent bond [59]. Such a set of properties determines its use in biomedical applications, e.g. to immobilize growth factors on the surface of the fibrous scaffold for bone tissue regeneration. The activation of the gelatin nanofibers coated with HAp with avidin enabled the attachment of biotinylated growth factors (bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2)) to its surface. As a result, the release profile of the BMP-2 and FGF-2 was more efficient for the biotin-avidin complex than for the physical adsorption [60]. The chemisorption can also be used for capturing microspheres on the polymer fibers surface. Ahrens et al. activated the surface of fibrous poly(ethylene terephthalate) (PET) with carboxyl groups to immobilize biotin. Then, they examined the efficiency of capturing of the avidin-functionalized cells-containing microspheres under different flow variants. The biotin high affinity toward avidin led to the sufficient capturing of the microspheres under all the tested conditions (i.e. vigorous mixing and perfusion flow) [59].

Surface grafting

One of the most recent approaches in the surface modification is the peptide grafting. The obtained high bioactivity, stability and low immunogenicity of the material makes the peptide grafting a good alternative. The commonly used biomolecules include fibronectin, collagen, laminin and peptides originated from them as an attractor for endothelial cells, fibroblasts and keratinocytes [61-64]. Moreover, antibacterial properties could be obtained by the surface functionalization with antimicrobial peptides (AMP). The main advantage of the AMP over antibiotics is limiting the antibiotic resistance development [65]. The peptide grafting method also provides a signal to the host cells, which results in their better integration with the surrounding tissue. To improve the cell interaction this method can be also combined with growth factors. Studies by Emre Yüksel et al. proved that Magainin II and the epidermal growth factor attached to the PLGA fibers surface could result in both the increased cell proliferation and the reduced bacteria activity (S. aureus) [66]. One of the most commonly used peptides is RGD (arginine-glycine-aspartic acid) which originates from fibronectin. It regulates the endothelial cells adhesion, migration and proliferation. As RGD is recognized by different integrins, it acts as a nonspecific peptide. In their study, Ge Peng et al. used different types of peptides: fibronectin originated (RGD, REDV) and laminin originated (YIGSR) on the silk fibroin scaffolds to achieve the complex cellular response to improve the vascular graft endothelization. The modification increased the surface hydrophilicity and the cells adhesion. However, the platelet activation and the cell proliferation depended on the combination of peptides grafted on the surface, which means that the specific cell behaviour could be obtained by the peptides proper selection [64]. Another example is the immobilization of the RGDC signal peptide on the

γ-PGA fibers by the click chemistry. The scaffold can also be loaded with GS-Rg3 to heal wounds and inhibit scar formation. The results showed high biocompatibility of the samples as well as the better kinetics of the drug release [67].

Wet-chemical modification

In this method, reactive functional groups at the surface are generated by immersing the fiber mesh in liquid reagents. The process is usually carried out in an acidic or alkaline environment, which contributes to breaking the chain at the site of specific groups. Due to the wet chemical modification, functional groups including -OH, -COOH and $-NH_2$ appear at the surface [68].

One of the most common techniques is the aminolysis (FIG. 8) which introduces amino groups at the biomaterial surface and thus it serves as an intermediate step in the surface functionalization. S. Asadpour et al. used the aminolysis for further gelatin or collagen immobilization on the surface of vascular grafts made of poly(ether ester urethane) urea. The authors observed the improved cell adhesion and the endothelial cells layer formed on the implant [69]. Hoseinpour et al. prepared polyethersulfone (PES) membranes and modified them with carboxymethylcellulose (CMC) or sulphated carboxymethylcellulose (SCMC) which are extracellular matrix derived peptides. The membranes were immersed in the 10 wt% diethylenetriamine solution to introduce the $-NH_2$ groups on the surface. Then the samples were rinsed with ethanol for the aminolysis solution removal. The next step was the membranes incubation in a CMC or SCMC solution. The employed modifications lowered the contact angle, protein adsorption and platelet adhesion values, thus increasing the hemocompatibility. Moreover, the antifouling properties of the PES-CMC or PES-SCMC membranes were improved [70]. Another biomolecule which was grafted on the PLA fibrous scaffold by the aminolysis functionalization is an epidermal growth factor. The results showed the enhanced cell viability and proliferation [71].

The hydrolysis (FIG. 8) is another wet technique which incorporates -COOH group at the surface via the base or acid treatment. Thanks to the hydrolysis, the surface hydrophilicity and roughness could be increased [68]. Brown et al. used this method to improve the viability of human hepatocytes. In their study, a PLGA scaffold was prepared by the wet electrospinning. The mesh was immersed in the NaOH solution and then incubated in a solution of collagen I and fibronectin. Thanks to this modification, the microenvironment conducive to the hepatocytes survival was obtained. It ensured the higher albumin secretion and activity of the hepatocyte-specific gene in comparison to the reference samples [72].

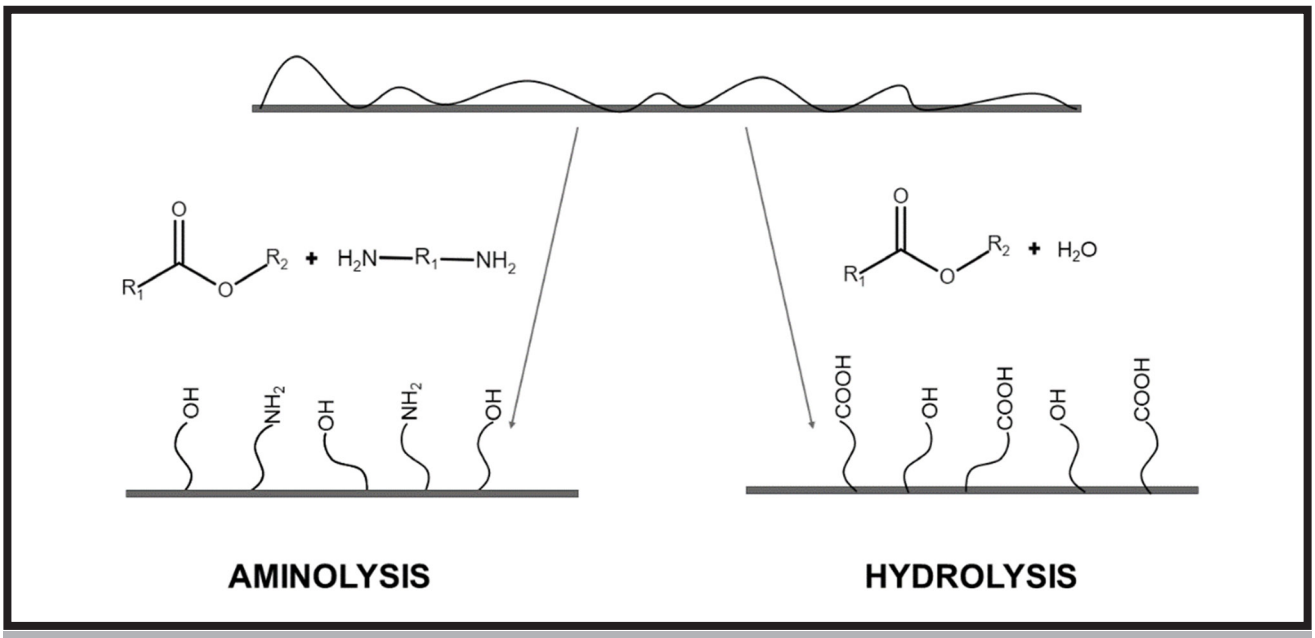

FIG. 8. Surface chemistry after hydrolysis and aminolysis.

It is also reported that combining the wet chemistry methods with adhesive molecules leads to the better cell-scaffold interaction. Pilipchuk et al. examined the impact of the different modifications on the PCL films biocompatibility. The applied methods were the amination, the hydrolysis, the fibronectin immobilization and the combination of hydrolysis with fibronectin incorporation. The results showed that, among all the options, the last approach had the best cellular response [73].

Plasma treatment

Another promising technique is the non-thermal plasma treatment of the fibers which enables the introduction of the various functional groups. This technique changes the surface properties without affecting its morphology. However, the outcomes are strongly dependent on two factors: the gas used for plasma creation and the time of the process. The samples treated with the argon plasma for a maximum time of 15s showed the unaltered fiber morphology, while those treated with the air plasma displayed the locally melted spots. Nevertheless, both methods increased the surface hydrophilicity by incorporating oxygen-containing groups [7].

Not only the oxygen-containing groups could be incorporated into the surface. In the research conducted by Mahtab Asadian et al., chitosan/polyethylene oxide nanofibrous mats were modified by the dielectric barrier discharge in the argon, nitrogen and ammonia/helium medium. Via the applied modification, polar functional groups (oxygen-containing and nitrogen-containing) were incorporated at the surface. The associated increase in free energy facilitated the cell adhesion. Another advantage was the increased tensile strength of the fibers [1]. In the next research, Mahtab Asadian et al. examined the plasma treatment effect before and after the electrospinning. The results indicated that the fibers morphology and surface chemistry were different when the plasma was applied before or after the process. In the first case, they achieved the beadless mesh with the unaltered surface properties. In the latter case, the surface wettability, as well as the cell adhesion and proliferation, increased without any changes in the fibers morphology [74]. There are many other studies on the plasma treatment used for the surface modification proving t its positive impact on the cells adhesion and mechanical properties [75-77]. The plasma pretreatment is also used to prepare surfaces for the physical deposition to enhance the coating adhesion. Akhavana et al. used the ion-assisted plasma pretreatment on the titanium surface. Then the material was immersed in the solution containing the antimicrobial peptides. Due to such a procedure, a covalent bond between the surface and molecules was formed [78].

Mussel-inspired coating

This biomimetic strategy is inspired by the adhesion mechanism governed by the mussel foot protein consisting of lysine and dihydroxyphenylalanine (DOPA). The catechol group in the lysine DOPA and amino groups interacts with the surface through electrostatic interactions, hydrogen bonds and covalent reactions. Dopamine is another molecule with both catechol and amino groups which can also self-polymerize on various substrates [79]. Carmagnola et al. used DOPA for the gelatin grafting on the surface of the PLGA electrospun membranes. This surface functionalization did not deteriorate the bulk material properties. The increased hydrophilicity improved the cellular adhesion and viability [80]. Chen et al. immobilized bromelain on the PCL electrospun membrane used for wound healing. Polydopamine (PDA) was applied as a linking agent between bromelain and the substrate. The BrPDA-PCL fibers exhibited good mechanical stability, very high hydrophilicity, biocompatibility and antibacterial behaviour against *E. coli* and *S. aureus*. The increased wound healing rate was observed during the *in vivo* examination [81].

Norepinephrine is another catecholamine molecule which could be used in tissue engineering. Liu et al. prepared a PCL fibrous scaffold coated with poly norepinephrine (pNE) via self-polymerization. The *in vitro* research revealed that the pNE addition facilitated the hydrophilicity and thus improved the skeletal muscle cell adhesion and proliferation. On the other hand, no toxic behaviour was observed during the 40-day *in vivo* examination [82]. The main drawback of this method is that it requires the alkaline environment for the dopamine polymerization and thus it cannot be used for materials that are unstable in these conditions. Another disadvantage is that this is a time-consuming process (up to several hours) [79].

Conclusions

In this mini-review, the most recent methods of modifying electrospun fibers for tissue engineering were discussed. The main limitations of pristine electrospun scaffolds can be overcome thanks to the properly selected modification technique. The specific biomolecules immobilization leads to obtaining the desired and customized implant functionality, which results in its better integration with the surrounding tissues, mechanical support and bioactivity.

The fibers physical modifications have numerous advantages, such as simplicity, short time, and low cost. Unfortunately, the physical modifications are not durable and are only suitable where the material can be applied immediately after the process.

The chemical methods are more demanding and consist of many stages. Very often they require additional processing, e.g. preparing the fiber surface or selecting fiber materials that enable the formation of primary bonds. Moreover, the chemical modification is not always sufficiently effective due to the substrate form i.e. the fiber submicron and micrometer diameters. Instead of obtaining the homogeneous fiber surface, most often its domain character occurs. Chemical modifications are not durable either due to the low process efficiency, but often the post-treatment is accompanied by the second-order interactions which are more numerous and contribute to the increased durability of the new layer on the fiber. Such hybrid substrates seem to be the future of ECM scaffolds. At the moment, most works focus on material modifications via the peptide grafting because of the obtained high bioactivity and possibility to control the cellular response. Nevertheless, the long-term impact of this approach on the *in vivo* effectiveness and biocompatibility is still unknown. Since fibrous scaffolds are intended to be used inside the human body, the selection of an appropriate sterilization process should be taken under consideration. Electrospun fibers could be sterilized by any of the commonly used sterilization methods such as autoclave, dry heat, gamma radiation, ethylene oxide, plasma or ozone. However, the selection of an appropriate method is dependent upon material and its properties (e.g. structure, melting temperature, glass transition temperature), microstructure (fibers diameter, porosity) or presence of active molecules on the surface. It is also important to choose a sterilization method that does not change not only the morphology of the fibers but also their physicochemical properties and biological activity. In the case of gamma radiation, the possibility of changes in molecular structure due to chain scission should be also taken under consideration [83]. On the other hand UV sterilization could attenuate the release kinetics of the growth factors [84]. Therefore, there is still a big urge to know the impact of different methods of sterilization on the bioactivity of the modified fibrous scaffolds. Therefore, the full understanding of the implant-tissue interface and its influence on the human body are crucial to yet develop the most beneficial scaffolds for tissue engineering.

Acknowledgements

We thank Barbara Trybalska and Agnieszka Wojteczko for taking a number of SEM photos. This work was supported from the subsidy of the Ministry of Education and Science for the AGH University of Science and Technology in Kraków (Project No 16.16.160.557).

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