Polimery w Medycynie

Polymers in Medicine

BIANNUAL ISSN: 0370-0747 e-ISSN: 2451-2699 polimery.umw.edu.pl

2023, Vol. 53, No. 2 (July–December)

Ministry of Science and Higher Education – 70 pts.
Index Copernicus (ICV) – 120.65 pts.

Wroclaw Medical University

Polimery w Medycynie

Polymers in Medicine

Polimery w Medycynie
Polymers in Medicine

ISSN 0370-0747 (PRINT) ISSN 2451-2699 (ONLINE) polimery.umw.edu.pl

BIANNUAL
2023, Vol. 53, No. 2
(July–December)

"Polymers in Medicine" is an independent, multidisciplinary forum to exchange scientific and clinical information, which publishes original papers (technical, analytical, experimental, clinical), preliminary reports and reviews regarding the use of polymers (natural and synthetic) and biomaterials in different specialties of medicine (biochemistry, clinical medicine, pharmacology, dentistry, implantology), biotechnology and veterinary science.

Address of Editorial Office
Marcinkowskiego 2–6
50-368 Wrocław, Poland
Tel.: +48 71 784 11 33
E-mail: redakcja@umw.edu.pl

Publisher
Wroclaw Medical University
Wybrzeże L. Pasteura 1
50-367 Wrocław, Poland

Online edition is the original version of the journal

Editorial Policy

During the review process, the Editorial Board conforms to the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication" approved by the International Committee of Medical Journal Editors (http://www.icmje.org/). Experimental studies must include a statement that the experimental protocol and informed consent procedure were in compliance with the Helsinki Convention and were approved by the ethics committee.

For more information visit the following page: https://polimery.umw.edu.pl

Polimery w Medycynie – Polymers in Medicine has received financial support from the resources of Ministry of Science and Higher Education within the "Social Responsibility of Science – Support for Academic Publishing" project based on agreement No. RCN/SP/0504/2021.

Ministry of Education and Science
Republic of Poland

Czasopismo Polimery w Medycynie – Polymers in Medicine korzysta ze wsparcia finansowego ze środków Ministerstwa Edukacji i Nauki w ramach programu „Społeczna Odpowiedzialność Nauki – Rozwój Czasopism Naukowych" na podstawie umowy nr RCN/SP/0504/2021.

Ministerstwo
Edukacji i Nauki

Indexed in: OCLC, WorldCat, PBL, EBSCO, MEDLINE, Index Copernicus

Typographic design: Monika Kolęda, Piotr Gil
Cover: Monika Kolęda
DTP: Wroclaw Medical University Press

Circulation: 11 copies

Polimery w Medycynie
Polymers in Medicine

BIANNUAL 2023, Vol. 53, No. 2 (July–December)

ISSN 0370-0747 (PRINT)
ISSN 2451-2699 (ONLINE)
polimery.umw.edu.pl

Contents

PREFACE

Dear Readers, Authors, Reviewers,
Members of the Scientific Committee and Section Editors,

The world of polymers applied in medicine and pharmacy may reveal a lot of its conundrums when it comes to detailed and diligent studies of the natural phenomena. We may present here only a segment of this fascinating field of research. We finish the year 2023 with an issue presenting a balanced collection of original and review papers.

As it is clearly seen from the contents of this issue, polymers in medical sciences have variable applications, and both biological and physico-chemical aspects are always important. Two original articles focus on polymers which interfere with or are synthesized in biological entities. The evaluation of rifaximin in biotic and abiotic models gives hope for better prophylaxis and combating *Pseudomona aeruginosa* infections. On the other hand, the analysis of amino acids derivatives, which are polymeric *par excellence,* and their influence on the course of amyotrophic lateral sclerosis (ALS) provides some information about further development of new medication methods for treating this disease. The known polymers may find new applications, also when studied using non-standard methods, as it is in the case of hyaluronic acid evaluated in terms of its surface tension, which is rather unusual compared to the standard viscosity measurements of this kind of specimens. The review articles cover two important areas: microbial exopolysaccharides as polymers for drug delivery applications, as well as scaffolds proposed as stem cells carriers for bone engineering.

The past year, like 2022, was filled with global events that we may want to ignore, but yet we have to face them. The civilized world, like it or not, is becoming a refuge for states and individuals who do not agree to disregard for basic values, for violence and injustice. Let us hope that natural and medical sciences also contribute through their voice to discovering real and fair principles of how societies function. I wish you good reading and fruitful conclusions. I would also like to convey to you the best wishes: Merry Christmas and a Happy New Year from the entire editorial team of *Polimery w Medycynie – Polymers in Medicine.*

Editor-in-Chief
Witold Musiał, Prof., PhD, DSc

Effect of subinhibitory doses of rifaximin on in vitro *Pseudomonas aeruginosa* adherence and biofilm formation to biotic and abiotic surface models

Lubna Ali Abd Al-Mutalib[A,B], Ayaid Khadem Zgair[A,C,F]

Department of Biology, College of Science, University of Baghdad, Iraq

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Polymers in Medicine, ISSN 0370-0747 (print), ISSN 2451-2699 (online) *Polim Med*. 2023;53(2):97–103

Address for correspondence
Ayaid Khadem Zgair
E-mail: ayaid.zgair@sc.uobaghdad.edu.iq

Funding sources
None declared

Conflict of interest
None declared

Received on April 8, 2023
Reviewed on May 29, 2023
Accepted on May 29, 2023

Published online on July 20, 2023

Abstract

Background. The adhesion of *Pseudomonas aeruginosa* to biotic and abiotic surfaces is responsible for the persistence and development of bacterial infection.

Objectives. To fill the gap in the knowledge regarding the relationship between rifaximin susceptibility and biofilm formation, and to investigate the effect of subinhibitory doses of rifaximin on the adhesion and biofilm formation.

Materials and methods. A total of 10 isolates of *P. aeruginosa* were obtained from 110 urine samples of urinary tract infection (UTI) patients. Biofilm formation on polystyrene microtiter plates, minimum inhibitory concentrations (MICs) of rifaximin against the 10 isolates of *P. aeruginosa* (Pa1–Pa10), the effect of sub-MICs of rifaximin (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC) on biofilm formation by the Pa4 isolate to polystyrene microtiter plates, and the adhesion to human epithelial cells (HECs) in vitro were evaluated.

Results. The MICs of rifaximin against 10 isolates ranged from 62.5 µg/mL to 1000 µg/mL. The Pa4 isolate produced the highest level of biofilm formation, while the MIC of Pa4 was 125 µg/mL. There was no correlation between bacterial susceptibility to rifaximin and biofilm formation ($r: -0.016$; $p > 0.05$). Sub-MIC doses of rifaximin significantly reduced the biofilm formation on abiotic surfaces, while only 0.5 × MIC, 0.25 × MIC and 0.12 × MIC of rifaximin reduced the adhesion to HECs significantly ($p < 0.05$) in a dose-dependent manner.

Conclusions. This pioneering study demonstrated the negative effect of sub-MIC doses of rifaximin on biofilm formation and adhesion to abiotic and biotic surfaces in vitro.

Key words: adhesion, biofilm formation, *Pseudomonas aeruginosa*, rifaximin, subinhibitory doses

Cite as
Abd Al-Mutalib LA, Zgair AK. Effect of subinhibitory doses of rifaximin on in vitro *Pseudomonas aeruginosa* adherence and biofilm formation to biotic and abiotic surface models. *Polim Med*. 2023;53(2):97–103. doi:10.17219/pim/166584

DOI
10.17219/pim/166584

Background

Pseudomonas aeruginosa is a common bacterium that can cause a range of infections in humans, including pneumonia, urinary tract infections (UTIs) and bloodstream infections.[1] One of the unique characteristics of *P. aeruginosa* is its ability to adhere to and form biofilms on biotic and abiotic surfaces. A biofilm is a complex community of microorganisms that adhere to surfaces and are surrounded by a protective matrix of extracellular polymeric substances (EPS). Several factors may affect the ability of *P. aeruginosa* to form biofilms, such as quorum sensing and flagella.[2] Biofilms are difficult to eradicate and are associated with chronic infections that are often resistant to antibiotics. The biofilms cause complications in infections which lead to serious issues and difficulties treating *P. aeruginosa* infection.[3]

The first step in *P. aeruginosa* infection is the adherence of the bacterium to the host human epithelial cells (HECs).[4] Adherence is a critical step in *P. aeruginosa* infection, as it allows the bacterium to establish a foothold in the host and initiate the infection process. The mechanisms by which *P. aeruginosa* adheres to epithelial cells are complex and involve multiple bacterial factors and host cell receptors. Once *P. aeruginosa* has adhered to host epithelial cells, it can initiate the infection process by secreting virulence factors such as toxins and proteases.[5] These factors can cause tissue damage, impair host immune responses, and promote bacterial survival and growth.[6]

Treatment of *P. aeruginosa* biofilm infections can be challenging and may require a combination of antibiotic therapy, removal of infected devices, and surgical debridement.[7] Antibiotics are the most important strategy for treating bacterial infections, and increasing resistance to antibiotics represents the biggest challenge for treating bacterial infectious diseases nowadays.[7] The use of antibiotics is limited to not only killing bacteria, but many experiments have proven that the use of sublethal doses of antibiotics reduces the ability of different types of bacteria to adhere to surfaces, which contributes significantly to reducing the virulence of bacteria.[7,8] Subinhibitory antibiotic concentrations can also lead to decreased biofilm formation by *P. aeruginos*a. Several studies have reported that certain antibiotics can impair bacterial attachment to surfaces and biofilm formation,[9] but there is no previous study that highlighted the impact of a subinhibitory concentration of rifaximin on biofilm formation.

Rifaximin is a broad-spectrum antibiotic used to treat a variety of infections, including traveler's diarrhea, hepatic encephalopathy and other infectious cases.[10,11] It works by inhibiting bacterial RNA synthesis, which ultimately leads to bacterial cell death.[12] Previous studies did not evaluate the role of rifaximin in reducing the ability of *P. aeruginosa* to adhere to surfaces and form biofilm. Therefore, our current pioneering study highlighted the effect of subinhibitory doses of rifaximin on the ability of *P. aeruginosa* to adhere to HECs (biotic surface model) and form a biofilm on polystyrene (abiotic surface model).

Materials and methods

Ethical approval

The current study was conducted after obtaining the approval from the human ethical committee of the Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq (Reference No. 609, date: April 14, 2022).

Isolation and identification of bacteria

Urine samples were collected aseptically from 110 patients suffering from UTIs and treated at Baghdad Teaching Hospital (Baghdad, Iraq). The average age of the patients was 42.3 ±11.2 years (59 years for females and 51 years for males). All patients did not receive antibiotic treatment 72 h before the date of sample collection and gave consent to participate in the study. Briefly, 500 µL of urine sample was placed in 4.5 mL of Pseudomonas Asparagine Broth medium (HiMedia, Mumbai, India). The containers were incubated for 48 h at 37°C with vigorous shaking at 210 rpm. Then, 50 µL of bacterial suspension was streaked onto asparagine agar plates (1.5% agar; HiMedia) and incubated at 37°C until colonies developed.[13] A VITEK 2 DensiCheck instrument and fluorescence system (bioMérieux, Marcy-l'Étoile, France) (ID-GNB card) were used to identify the isolates of *P. aeruginosa*.[14]

Rifaximin minimum inhibitory concentrations (MICs)

The standard broth micro-dilution technique described by Wiegand et al. was followed to determine the MICs of rifaximin against 10 clinical isolates of *P. aeruginosa* (Pa1–Pa10).[15] Briefly, a 1 mg/mL stock concentration of rifaximin (Mylan, Potters Bar, UK) was prepared by dissolving the powder in sterile distilled water. Double-fold dilutions (100 µL) were prepared in the microtiter plate with sterile Mueller–Hinton broth (MHB; HiMedia). After overnight growth, the *P. aeruginosa* isolates were washed 3 times with sterile phosphate-buffered saline (PBS; 0.1 M, pH 7.2) using centrifugation at 10,000 rpm for 10 min (High-Speed Centrifuge, Avanti JXN-26; Beckman Coulter, Brea, USA), and the number of bacteria was adjusted to 10^6 CFU/mL using the spectrophotometric method (double-beam spectrophotometer model SP-MUV8000T; Bioevopeak, Jinan, China) at 600 nm; then, 5 µL of the bacterial solution was added to each well. The plates were gently mixed. Three technical controls were made: MHB inoculated with bacterial isolates, sterile MHB and different double dilutions of antibiotics. The MICs were

determined after overnight incubation at 37°C and were defined as the lowest antibiotic concentrations completely inhibiting growth.[15]

Biofilm formation

The protocol of Zgair and Chhibber was followed.[16] Briefly, 200 µL of sterile tryptic soy broth (TSB) was added to the wells of flat-bottom polystyrene tissue culture plates. Then, 5 µL of overnight growth of *P. aeruginosa* (Pa1–Pa10) was washed 3 times with sterile normal saline using centrifugation at 10,000 rpm for 10 min (High-Speed Centrifuge, Avanti JXN-26; Beckman Coulter). The number of bacteria was adjusted to 10^6 CFU/mL using the spectrophotometric method (double-beam spectrophotometer model SP-MUV8000T; Bioevopeak) at 600 nm. Then, 5 µL of adjusted bacterial suspension was added to each well, and the plates were incubated at 37°C for 18 h. The media were discarded, and non-adherent bacterial cells were removed by washing them 5 times with normal, sterile saline. The quantity of biofilm formation was measured with the spectrophotometric method. The resultant biofilms of different *P. aeruginosa* isolates were dried and fixed by incubating at 65°C for 35 min. Then, 200 µL of Hucker crystal violet (0.4%) was added to each well and incubated for 6 min at 21°C. The plates were washed 4 times with distilled water and dried for 30 min at 37°C. Then, 200 µL of acetone:ethanol (30:70) was added to each well. The absorbency of each well was measured at a wavelength of 570 nm using a microplate reader (BioTek 800 TS; BioTek, Winooski, USA).[16] The experiment was repeated 3 times.

Effect of sub-MICs of rifaximin on biofilm formation

To determine the effect of sub-MICs of antibiotics on the biofilm formation of the *P. aeruginosa* isolate that produces the highest level of biofilm, a similar method of biofilm formation was followed. However, instead of TSB, double-fold dilutions of sub-MICs of rifaximin were used (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC). Tests were performed in triplicate.

Adherence of *P. aeruginosa* to HECs

The standard method of Ali and Zgair was followed to prepare HECs in vitro.[17] The volunteers from whom the HECs were collected did not take any antibiotics 3 days before the HEC sample collection. The method of Zgair and Chhibber[16] was followed to evaluate the adhesion of clinical isolates of *P. aeruginosa* that produced the highest level of biofilm to HECs in vitro. Briefly, in each well of a 24-well tissue culture microtiter plate (Thermo Fisher Scientific, Waltham, USA), 1×10^5 HECs were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 10 mM of L–glutamine. Then, 100 µL of *P. aeruginosa* (5×10^7 CFU/mL) was added to each well. The plates were incubated for 1 h at 37°C and then washed 3 times with PBS (0.1 M, pH 7.2). The HECs were washed 3 times with PBS (0.1 M, pH 7) by centrifugation (1000 rpm, 10 min, 4°C). The HEC pellets were lysed with PBS containing 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, USA), diluted 10-fold, and plated on nutrient agar to estimate the number of bacteria adhering to HECs.

Effect of sub-MICs of rifaximin on adhesion of *P. aeruginosa* to HECs

To study the effect of different concentrations of rifaximin (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC) on the ability of *P. aeruginosa* (the isolate that produced the highest level of biofilm) to adhere to HECs, the colonies of bacteria that grew on Mueller–Hinton agar (MHA) were suspended in TSB (HiMedia). The bacterial number was adjusted to 1×10^7 with MHB (HiMedia) that was prepared in different concentrations of rifaximin (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC). The broths were incubated for 18 h at 37°C. The bacterial cells were washed 3 times with normal, sterile saline (10,000 g for 10 min). The final number of bacteria was adjusted to 1×10^7 CFU/mL with TSB. A similar procedure to investigate the adherence of *P. aeruginosa* to HECs was followed in order to determine the ability of bacteria treated with different concentrations of rifaximin to adhere to HECs in vitro. The results were compared with the ability of bacteria (untreated with rifaximin) to adhere to HECs (control). The experiments were performed in triplicate.

Leishman's stain

Smears of HECs were prepared and dried at 37°C. The standard method of Sareen et al.[18] was followed to stain 3 groups of HECs: HECs that were untreated with bacteria and rifaximin, HECs that were treated with *P. aeruginosa* only, and HECs that were treated with *P. aeruginosa* and different concentrations of rifaximin (0.5 × MIC, 0.25 × MIC, 0.12 × MIX, and 0.06 × MIC). The slides were examined under a light microscope (Lomo Mikned 2; Lomo, Sankt Petersburg, Russia), and the images were captured using a smartphone camera over the microscope eyepiece.

Statistical analyses

The statistical analysis was performed and the graphs were created using Origin v. 8 software (OriginLab, Nothampton, USA). The data were expressed as means ± standard error (M ±SE). The differences were evaluated using a Student's t-test and one-way analysis of variance (ANOVA). The relationship was assesed using Pearson's correlation coefficient. A value of $p < 0.05$ was considered statistically significant.

Results

Isolation and identification

Ten isolates of *P. aeruginosa* were obtained from 110 urine samples collected from patients suffering from UTIs. Gram stain and biochemical tests were used to identify the bacterial species. The VITIK 2 Technology (bioMérieux) proved that the isolates were *P. aeruginosa* (Pa1–Pa10).

Rifaximin MICs and biofilm formation

In the current study, the MIC of rifaximin against 10 clinical isolates of *P. aeruginosa* (Pa1–Pa10) was measured. The highest MIC of rifaximin was observed against Pa10 (1000 µg/mL), while the lowest MIC of rifaximin was seen against Pa8 (62.5 µg/mL) (Fig. 1A).

Figure 1B shows the biofilm formation of the 10 clinical isolates of *P. aeruginosa* that were obtained from urine samples. The highest level of biofilm was produced by Pa4 (optical density (OD): 0.49 ±0.029), followed by Pa10 (OD: 0.34 ±0.021), while the lowest biofilm mass was formed by Pa9 (OD: 0.11 ±0.019). The present study showed that all isolates can produce biofilm, but Pa4 produced the highest level of biofilm mass. Thus, this isolate was used for further experiments aimed at evaluating the role of a subinhibitory dose of rifaximin in reducing biofilm formation and bacterial adherence in vitro.

Figure 2 shows that there is no relationship between the values of MICs of rifaximin against 10 clinical isolates of *P. aeruginosa* and biofilm formation of the same isolates. The present study proved that there is no relationship between the ability of clinical isolates of *P. aeruginosa* to form biofilm and the susceptibility of these bacterial isolates to rifaximin.

Effect of sub-MICs of rifaximin on biofilm formation

The effect of sub-MICs of rifaximin on biofilm formation by *P. aeruginosa* (Pa4) was also evaluated. It was found that all the concentrations used (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC) reduced the ability of Pa4 to form a biofilm on polystyrene microtiter plates, and the decrease in biofilm formation was shown in a concentration-dependent manner. Whereas the highest decrease in biofilm formation was observed when treating bacteria with 0.5 × MIC of rifaximin (0.207 ±0.032), the lowest decrease in biofilm formation was found when treating bacteria with 0.06 × MIC of rifaximin (0.403 ±0.035) (Fig. 3).

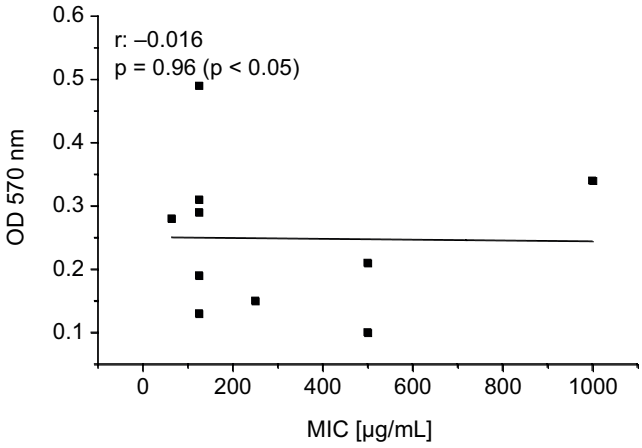

Fig. 2. Relationship between minimum inhibitory concentrations (MICs) of rifaximin against 10 clinical isolates of *Psedomonas aeruginosa* (Pa1–Pa10) and biofilm formation of the same isolates of *P. aeruginosa* (p > 0.05)

OD – optical density.

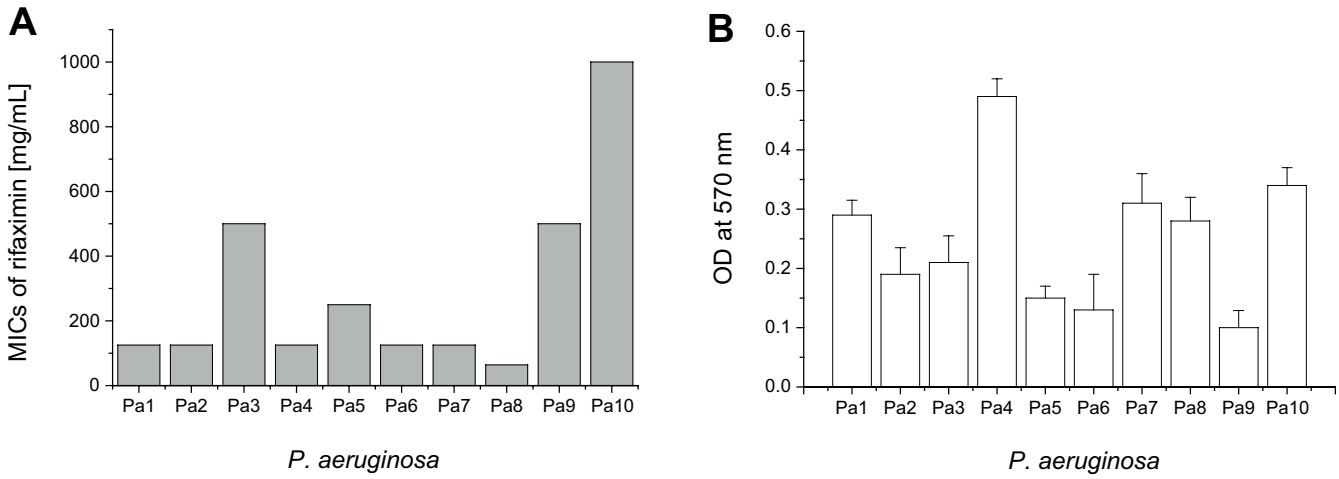

Fig. 1. A. Minimum inhibitory concentration (MIC) of rifaximin against 10 clinical isolates of *Psedomonas aeruginosa* (Pa1–Pa10) obtained from the urine samples collected from patients suffering from urinary tract infections (UTIs); B. Biofilm formation of the same 10 clinical isolates of *P. aeruginosa* to polystyrene microtiter plates

OD – optical density.

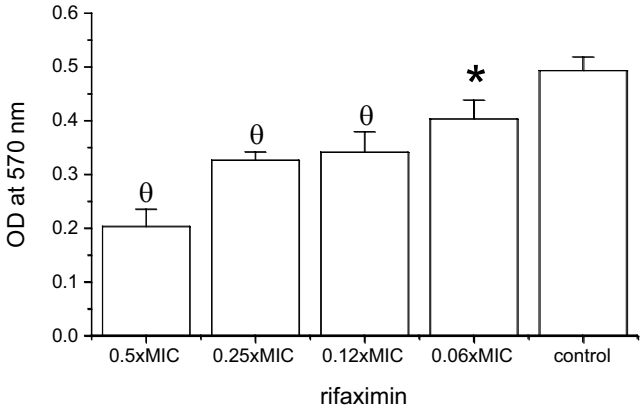

Fig. 3. Effect of subinhibitory doses of rifaximin (0.5 × minimum inhibitory concentration (MIC), 0.25 × MIC, 0.12 × MIC, and 0.06 × MIC) on biofilm formation by *Psedomonas aeruginosa* (Pa4). All concentrations of rifaximin significantly reduced the biofilm formation by Pa4

* $p < 0.05$; θ – $p < 0.001$; OD – optical density.

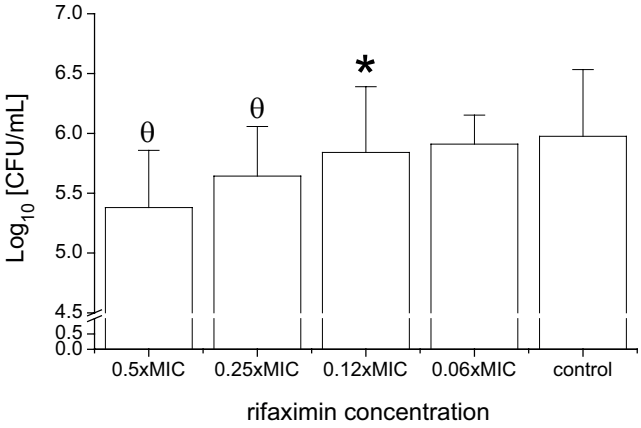

Fig. 4. Effect of subinhibitory doses of rifaximin (0.5 × minimum inhibitory concentration (MIC), 0.25 × MIC, 0.12 × MIC, and 0.06 × MIC) on *Psedomonas aeruginosa* (Pa4) adhesion to human epithelial cells (HECs) in vitro. All concentrations of rifaximin significantly reduced the number of adhered bacteria (CFU/mL) to HECs

* $p < 0.05$; θ – $p < 0.001$; OD – optical density.

Effect of sub-MICs of rifaximin on adhesion to HECs

In the current study, epithelial cells isolated from the human mouth were used as a model for biotic surfaces that were utilized to evaluate the adhesion of *P. aeruginosa* (Pa4) to biotic surfaces (HECs) and to estimate the effect of sub-MICs of rifaximin on the adhesion of *P. aeruginosa* (Pa4) to HECs in vitro. The 0.5 × MIC, 0.25 × MIC and 0.125 × MIC concentrations of rifaximin significantly reduced the adhesion of Pa4 to HECs ($p < 0.05$), while no significant decrease in the adhesion of bacteria to HECs was observed when the bacteria were treated with the 0.06 × MIC of rifaximin ($p > 0.05$). The decrease in adhesion was detected in a rifaximin concentration-dependent manner. While the highest significant decrease in bacterial adhesion was observed when treating bacteria with 0.5 × MIC of rifaximin ($p < 0.001$), the lowest significant decrease in bacterial adhesion was found when treating bacteria with 0.12 × MIC of rifaximin ($p < 0.05$) (Fig. 4).

The light microscopy images of the Pa4 adhesion to HECs with and without exposure to rifaximin (0.5 × MIC, 0.25 × MIC, 0.12 × MIC, and 0.06 × MIC) are shown in Fig. 5. Figure 5A shows a low number of bacteria (pretreated with 0.5 × MIC of rifaximin) attached to HECs that prove the 0.5 × MIC of rifaximin has a pronounced effect on the adhesion of Pa4 to HECs. However, pretreating Pa4 with 0.25 × MIC and 0.12 × MIC resulted in a moderate effect on the Pa4 attachment to the HECs (Fig. 5B,C). Figure 5D shows that the high number of bacteria pretreated Pa4 with 0.06 × MIC that attached to HECs, and this finding is similar to the attachment of Pa4 (untreated with rifaximin) to HECs (Fig. 5E) that proved there was no significant effect of 0.06 × MIC of rifaximin on the ability of Pa4 to attach to HECs. Figure 5F shows the HECs that were not exposed to the Pa4 (control). The present results showed the negative effect of rifaximin on the ability of *P. aeruginosa* to attach to HECs in vitro (biotic surface model).

Discussion

Biofilm formation is a process by which microorganisms, such as bacteria, adhere to surfaces and form a protective community of cells encased in a matrix of EPS. This matrix provides a protective barrier for the microorganisms, making it difficult for antibiotics and the immune system to reach and eliminate the bacterial cells.[19] The adhesion to HECs is an important step in the establishment of many infections, since HECs are the first line of defense against invading microorganisms and serve as a physical barrier to infection. Many bacterial pathogens have developed mechanisms to adhere to and invade HECs, allowing them to establish an infection and evade the immune system. The process of adhesion to the epithelial cells represents the first step for the bacteria to activate several mechanisms (secretion of some lytic enzymes such as protease) that enable them to penetrate this barrier.[20]

The ability of *P. aeruginosa* to form biofilms and adhere to HECs is a major factor in its capacity to cause chronic infections.[21] The present study has shown that all clinical isolates of *P. aeruginosa* can produce biofilm in vitro, and that was not related to rifaximin susceptibility. Moreover, it was highlighted for the first time that the subinhibitory concentrations (sub-MICs) of rifaximin reduced the ability of *P. aeruginosa* (Pa4) to produce the biofilm (in vitro) to polystyrene microtiter plates (abiotic surfaces) and adhesion to biotic surfaces (HECs) significantly (except 0.06 × MIC). The absence of a relationship between the ability of *P. aeruginosa* to form a biofilm and its response to the rifaximin indicates that *P. aeruginosa*

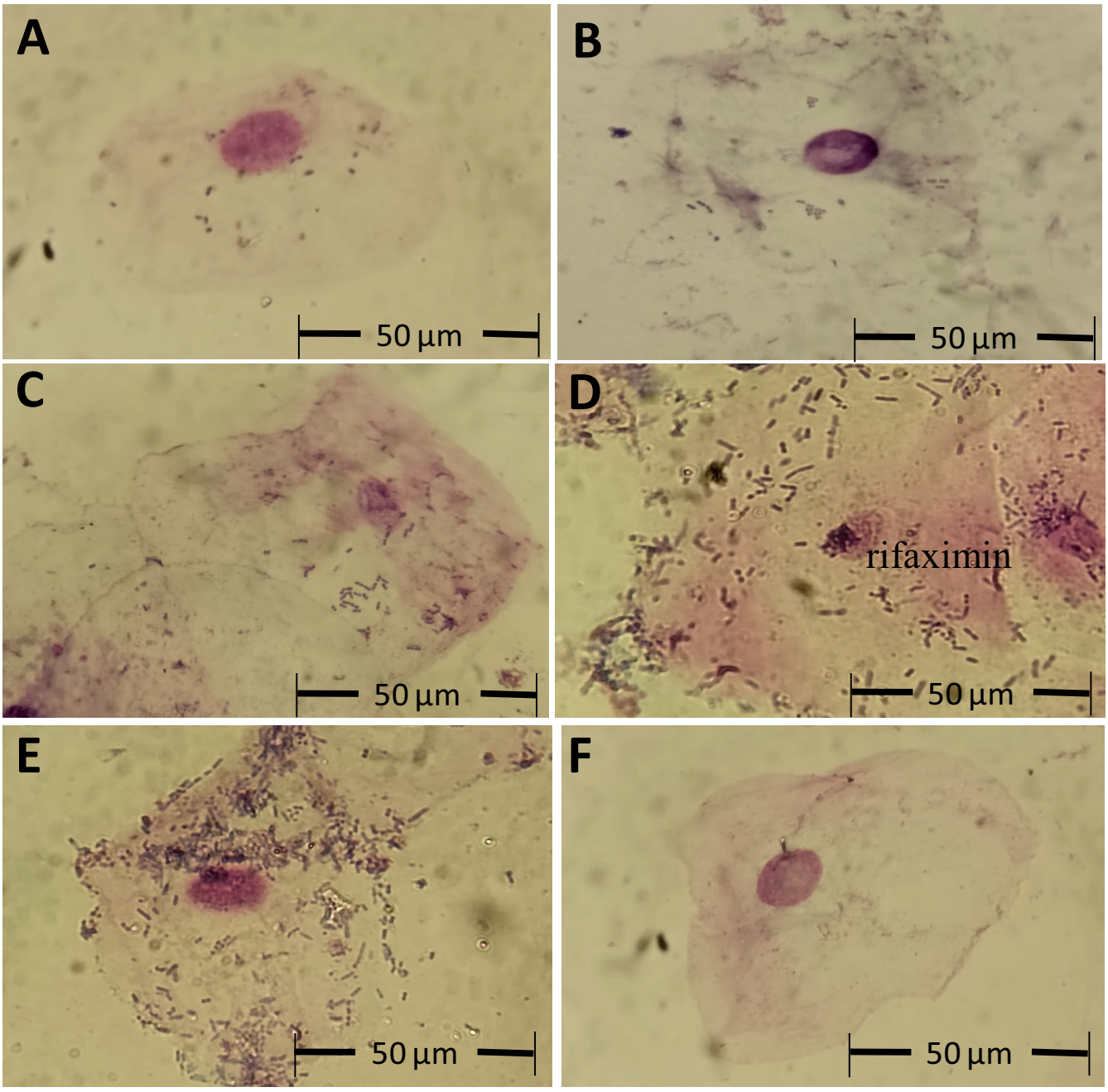

Fig. 5. Light microscopy images of Leishman-stained slides depicting adhesion of *Psedomonas aeruginosa* (Pa4) (pretreated with rifaximin) to human epithelial cells (HECs) after 2 h of incubation. A. Adhesion of Pa4 pre-treated with 0.5 × minimum inhibitory concentration (MIC) of rifaximin to HEC; B. Adhesion of Pa4 pretreated with 0.25 × MIC of rifaximin to HEC; C. Adhesion of Pa4 pretreated with 0.12 × MIC of rifaximin to HEC; D. Adhesion of Pa4 pretreated with 0.06 × MIC of rifaximin to HEC; E. Adhesion of Pa4 without rifaximin treatment to HEC; F. HECs alone

depends on other mechanisms rather than biofilm formation to resist this antibiotic.

The production of biofilm makes the bacteria more resistant to antibiotics by blocking the penetration of antimicrobial agents, thereby preventing them from reaching the bacterial cells. The EPS can sequester and deactivate antimicrobial agents. Bacteria within the biofilm can enter a dormant state in which they become less metabolically active and, therefore, less susceptible to antibiotics.[22] Furthermore, biofilms can provide a physical barrier that protects bacteria from host immune defenses, making it more difficult for the immune system to clear the infection, which poses a serious challenge to public health.[23] The reduction of the development of biofilm to the mature stage will reduce the chance of the bacteria causing infectious diseases by *P. aeruginosa*. Similarly, reducing bacterial adhesion to HECs by using the sub-MIC concentrations of rifaximin can be an effective strategy for preventing or reducing the severity of bacterial infections.

Overprescribing of treatments with antibiotics contributes to the creation of new strains of bacteria that are resistant to antibiotics,[24] so the use of effective low doses will have a positive economic and health impact if the doses can reduce the predominance of bacteria on adhesion and

biofilm formation. Moreover, this will help build a new treatment strategy to decrease bacterial infectious diseases by reducing the ability of pathogenic bacteria to adhere and form a biofilm on biotic and abiotic surfaces. However, such a strategy would require further study and development. Therefore, the strategy proposed in this study, which includes the use of low doses of antibiotics to reduce the ability of bacteria to adhere to biotic and abiotic surfaces and form a biofilm, is considered a promising strategy to prevent *P. aeruginosa* from causing infectious diseases.

Conclusions

The current study showed for the first time that there is no relationship between the susceptibility of *P. aeruginosa* to rifaximin and the ability of the bacteria to form a biofilm. Moreover, this is the first study that showed the role of subinhibitory doses of rifaximin in reducing the ability of *P. aeruginosa* to form the biofilm on abiotic surfaces (polystyrene) and to adhere to biotic surfaces (HECs) in vitro. This study contributes to developing a new strategy for the treatment of bacterial infections.

ORCID iDs

Lubna Ali Abd Al-Mutalib https://orcid.org/0009-0001-1849-784X
Ayaid Khadem Zgair https://orcid.org/0000-0002-2356-3338

References

1. De Sousa T, Hébraud M, Dapkevicius MLNE, et al. Genomic and metabolic characteristics of the pathogenicity in *Pseudomonas aeruginosa*. *Int J Mol Sci*. 2021;22(23):12892. doi:10.3390/ijms222312892
2. Mohammed HA, Zgair AK. Detection of quorum sensing genes of *Pseudomonas aeruginosa* isolated from different areas in Iraq. *Iraqi J Sci*. 2022;63(1):4665–4673. doi:10.24996/ijs.2022.63.11.5
3. Lee K, Yoon SS. *Pseudomonas aeruginosa* biofilm, a programmed bacterial life for fitness. *J Microbiol Biotechnol*. 2017;27(6):1053–1064. doi:10.4014/jmb.1611.11056
4. Doig P, Paranchych W, Sastry PA, Irvin RT. Human buccal epithelial cell receptors of *Pseudomonas aeruginosa*: Identification of glycoproteins with pilus binding activity. *Can J Microbiol*. 1989;35(12):1141–1145. doi:10.1139/m89-189
5. Capasso D, Pepe MV, Rossello J, et al. Elimination of *Pseudomonas aeruginosa* through efferocytosis upon binding to apoptotic cells. *PLoS Pathog*. 2016;12(12):e1006068. doi:10.1371/journal.ppat.1006068
6. Mues N, Chu HW. Outsmarting the host: Bacteria maneuvering the immune response to favor their survival. *Front Immunol*. 2020;11:819. doi:10.3389/fimmu.2020.00819
7. Burnham JP, Rojek RP, Kollef MH. Catheter removal and outcomes of multidrug-resistant central-line-associated bloodstream infection. *Medicine (Baltimore)*. 2018;97(42):e12782. doi:10.1097/MD.0000000000012782
8. Haney EF, Mansour SC, Hancock REW. Antimicrobial peptides: An introduction. In: Hansen PR, ed. *Antimicrobial Peptides*. Vol 1548. Methods in Molecular Biology. New York, USA: Springer New York; 2017:3–22. doi:10.1007/978-1-4939-6737-7_1
9. Nolan C, Behrends V. Sub-inhibitory antibiotic exposure and virulence in *Pseudomonas aeruginosa*. *Antibiotics (Basel)*. 2021;10(11):1393. doi:10.3390/antibiotics10111393
10. Koo HL, DuPont HL. Rifaximin: A unique gastrointestinal-selective antibiotic for enteric diseases. *Curr Opin Gastroenterol*. 2010;26(1):17–25. doi:10.1097/MOG.0b013e328333dc8d
11. Axtell S, Shokoya A, Yocum C. Probable fidaxomicin-induced pancytopenia. *Am J Health Syst Pharm*. 2018;75(8):532–535. doi:10.2146/ajhp170239
12. Schey R, Iorio N, Malik Z. Profile of rifaximin and its potential in the treatment of irritable bowel syndrome. *Clin Exp Gastroenterol*. 2015;8:159–167. doi:10.2147/CEG.S67231
13. Ghafil JA, Zgair AK. Bacterial secretions in growth medium stimulate the mouse respiratory innate immune response. *J Med Microbiol*. 2022;71. doi:10.1099/jmm.0.001588
14. Funke G, Monnet D, deBernardis C, Von Graevenitz A, Freney J. Evaluation of the VITEK 2 system for rapid identification of medically relevant Gram-negative rods. *J Clin Microbiol*. 1998;36(7):1948–1952. doi:10.1128/JCM.36.7.1948-1952.1998
15. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc*. 2008;3(2):163–175. doi:10.1038/nprot.2007.521
16. Zgair AK, Chhibber S. Adhering ability of *Stenotrophomonas maltophilia* is dependent on growth conditions. *Mikrobiologiia*. 2011;80(4):459–464. PMID:22073545.
17. Ali M, Zgair A. Extracellular product of *Pseudomonas aeruginosa* in growth medium is involved in the pro-inflammatory cytokine response of human oral epithelial cells in vitro. *Polim Med*. 2022;52(2):77–82. doi:10.17219/pim/155849
18. Sareen R, Kapil M, Gupta G. Incubation and its effect on Leishman stain. *J Lab Physicians*. 2018;10(03):357–361. doi:10.4103/JLP.JLP_154_17
19. Lin Y, Zhou X, Li Y. Strategies for *Streptococcus mutans* biofilm dispersal through extracellular polymeric substances disruption. *Mol Oral Microbiol*. 2022;37(1):1–8. doi:10.1111/omi.12355
20. Posselt G, Backert S, Wessler S. The functional interplay of *Helicobacter pylori* factors with gastric epithelial cells induces a multi-step process in pathogenesis. *Cell Commun Signal*. 2013;11(1):77. doi:10.1186/1478-811X-11-77
21. Thi MTT, Wibowo D, Rehm BHA. *Pseudomonas aeruginosa* biofilms. *Int J Mol Sci*. 2020;21(22):8671. doi:10.3390/ijms21228671
22. Su Y, McCarthy A, Wong SL, Hollins RR, Wang G, Xie J. Simultaneous delivery of multiple antimicrobial agents by biphasic scaffolds for effective treatment of wound biofilms. *Adv Healthcare Mater*. 2021;10(12):2100135. doi:10.1002/adhm.202100135
23. François P, Schrenzel J, Götz F. Biology and regulation of staphylococcal biofilm. *Int J Mol Sci*. 2023;24(6):5218. doi:10.3390/ijms24065218
24. Chen Z, Wang Z, Ren J, Qu X. Enzyme mimicry for combating bacteria and biofilms. *Acc Chem Res*. 2018;51(3):789–799. doi:10.1021/acs.accounts.8b00011

Plasma levels of soluble RAGE, AGEs and AOPPs at the early stage of amyotrophic lateral sclerosis: A preliminary study

Stężenie rozpuszczalnego RAGE, AGE i AOPP w osoczu we wczesnym stadium stwardnienia zanikowego bocznego – badania wstępne

Judyta Juranek[1,2,A,C–F], Adam Osowski[2,B–D,F], Joanna Wojtkiewicz[2,A,E,F], Marta Banach[3,A–C,E,F]

[1] Department of Medicine, New York University Grossman School of Medicine, USA
[2] Department of Human Physiology and Pathophysiology, School of Medicine, University of Warmia and Mazury, Olsztyn, Poland
[3] Department of Neurology, Jagiellonian University Medical College, Cracow, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Polymers in Medicine, ISSN 0370-0747 (print), ISSN 2451-2699 (online) *Polim Med*. 2023;53(2):105–110

Address for correspondence
Marta Banach
E-mail: martabanach@yahoo.com

Funding sources
Statutory project awarded to Marta Banach (2004–2007) No. 501/NKL/32/L.

Conflict of interest
None declared

Acknowledgements
We would like to thank Dr. Michael Maclean, a PhD candidate at Sackler Biomedical Institute, New York University Langine Medical Center (NYULMC), at the time of manuscript preparation for his insightful remarks on statistical analysis.

Received on August 25, 2023
Reviewed on October 9, 2023
Accepted on November 20, 2023

Published online on December 19, 2023

Cite as
Juranek J, Osowski A, Wojtkiewicz J, Banach M. Plasma levels of soluble RAGE, AGEs and AOPPs at the early stage of amyotrophic lateral sclerosis: A preliminary study. *Polim Med*. 2023;53(2):105–110. doi:10.17219/pim/175544

DOI
10.17219/pim/175544

Abstract

Background. Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder with largely unknown pathogenesis and no effective cure. It is believed that several, not mutually exclusive mechanisms contribute to the pathogenesis and progression of this disease, including, among others, elevated oxidative stress, excitotoxicity, increased neuroinflammation, and protein aggregation. Receptor for advanced glycation end products (RAGE) is a part of immunoglobulin superfamily; it is believed to participate in ALS pathogenesis.

Objectives. Our previous studies on ALS demonstrated that RAGE is likely one of the key players in ALS, acting on its own and in tandem with its oxidative stress and pro-inflammatory ligands, such as advanced glycation end products (AGEs) or advanced oxidation protein products (AOPPs). In this study, based on our previous results, we aimed to establish blood levels of soluble RAGE, AGE and AOPP in ALS patients.

Materials and methods. Forty-six coded and anonymized surplus plasma samples from ALS patients and non-neurological control were used in the study. The plasma levels of RAGE, AGE and AOPP were measured using enzyme-linked immunosorbent assay (ELISA) commercially available kits. Statistical evaluation of data was performed using one-way non-parametric analysis of variance (ANOVA) with Kruskal–Wallis post hoc test.

Results. Our results revealed a decline in soluble RAGE level, concurrent with an increase in the levels of AGEs and AOPPs in blood samples from ALS patients, signifying a loss of neuroprotective form of RAGE and a simultaneous increase in AGE and AOPP production and uptake at the early stage of the disease.

Conclusions. The results obtained from our study indicate that further longitudinal study of RAGE, AGE and AOPP levels would be beneficial, outlining the dynamics between RAGE and its ligand levels as the disease progresses, and making them valuable diagnostic tools and potential therapeutic targets.

Key words: receptor for advanced glycation end-products, advanced glycation end-products, advanced oxidation protein products, amyotrophic lateral sclerosis, plasma

Streszczenie

Wprowadzenie. Stwardnienie zanikowe boczne (amyotrophic lateral sclerosis (ALS)) jest wyniszczającą chorobą neurodegeneracyjną o nieznanej patogenezie i bez skutecznego leczenia. Uważa się, że w patogenezie i postępie tej choroby bierze udział kilka niewykluczających się wzajemnie mechanizmów. Należą do nich m.in. podwyższony stres oksydacyjny, ekscytotoksyczność, okołonerwowe odczyny zapalne i agregacja białek. Receptor końcowych produktów zaawansowanej glikacji (RAGE) należy do nadrodziny immunoglobulin i uważa się, że bierze on udział w patogenezie ALS.

Cel pracy. Nasze wcześniejsze badania ALS wykazały, że RAGE jest prawdopodobnie jednym z kluczowych graczy w tej chorobie, działając samodzielnie bądź w połączeniu z ligandami stresu oksydacyjnego i zapalenia, takimi jak zaawansowane produkty końcowe glikacji (AGE) lub zaawansowane produkty utleniania białek (AOPP). W niniejszej pracy, w oparciu o nasze poprzednie wyniki, mieliśmy na celu ustalenie stężenia rozpuszczalnego RAGE, AGE i AOPP w próbkach krwi pochodzących od pacjentów z ALS.

Materiał i metody. W badaniu wykorzystano 46 zakodowanych i anonimowych próbek krwi pochodzących od pacjentów z ALS i grupy kontrolnej. Badanie zostało zaprojektowane zgodnie z założeniami Deklaracji Helsińskiej i zatwierdzone przez Instytucjonalną Komisję Etyki. Poziomy RAGE, AGE i AOPP w osoczu mierzono za pomocą dostępnych komercyjnie zestawów ELISA. Statystyczną ocenę danych przeprowadzono za pomocą jednokierunkowej nieparametrycznej ANOVA z post-testem Kruskala–Wallisa.

Wyniki. Uzyskane wyniki wykazały spadek stężenia rozpuszczalnego RAGE przy jednoczesnym wzroście stężenie AGE i AOPP w próbkach krwi od pacjentów z ALS, wskazując na utratę neuroprotekcyjnej formy RAGE i jednoczesne nasilenie produkcji oraz wychwytu AGE i AOPP we wczesnym stadium choroby.

Wnioski. Wyniki uzyskane w naszym doświadczeniu wskazują, że dalsze długotrwałe badania stężenia RAGE, AGE i AOPP byłyby korzystne, umożliwiając określenie dynamiki zmian między stężeniem RAGE i jego ligandów w miarę postępu choroby, i tym samym dostarczając cennych narzędzi diagnostycznych i potencjalnych celów terapeutycznych.

Słowa kluczowe: receptor końcowych produktów zaawansowanej glikacji, końcowe produkty zaawansowanej glikacji, zaawansowane produkty utleniania białek, stwardnienie zanikowe boczne, osocze

Background

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal motor neuron disease first described in 1869. Since its discovery, it has been associated with progressive motor neuron death, muscle weakness, loss of motor functions, disability, and death within few years after the initial diagnosis.[1] It is an adult-onset disease with an incidence of 1–5/100,000 in most population, with a predicted 69% increase in its cases over the next 25 years. Clinically, depending on the onset of first clinically observed symptoms, ALS is divided into 2 forms: spinal (affecting limb muscles) and bulbar (impacting head and neck muscles). Of those, spinal form is far more prevalent, diagnosed in about 70% of ALS cases.[2]

It is believed that several, not mutually exclusive, mechanisms contribute to pathogenesis and progression of this disease. These include elevated oxidative stress, excitotoxicity, increased neuroinflammation, mitochondrial dysfunction, disruption of the neurofilament network, protein aggregation, cytoskeletal dysfunction, and involvement of non-neuronal cells within motor neuron surroundings.[3–7]

The receptor for advanced glycation end products (RAGE) is a part of immunoglobulin superfamily and belongs to the group of pattern recognition receptors involved in pathogen- or damage-associate molecular pathways (PAMP and DAMP, respectively).

Physiologically, RAGE is largely present on the surface of endotheliocytes, neural and immunocompetent cells, e.g., neurons, glia, lymphocytes, and macrophages, respectively.[8] Receptor for advanced glycation end products, aside from binding to advanced glycation end products (AGEs), interacts with over 25 different molecules, including advanced oxidation protein products (AOPPs), S100/calgranulin, HMGB1/amphoterin, amyloid-beta peptide (Aβ), transforming protein RhoA, protein diaphanous homolog 1 (Diaph1), and many others.[8] The engagement of RAGE by AGEs or its other ligands in a variety of settings initiates prompt generation of reactive oxygen species (ROS), upregulation of inflammatory pathways, and other RAGE signaling-dependent mechanisms. Receptor for advanced glycation end products is well known to be involved in the progression and pathogenesis of several neurodegenerative diseases and conditions.[9,10] Although the detailed mechanisms of RAGE involvement to the neurodegeneration remains unclear, studies indicate that it exerts its detrimental actions via its binding to the pro-inflammatory ligands such as AGEs, S100/calgranulin and amphoterin, and subsequent activation of downstream regulatory pathways such as NF-κB, STAT and JNK signaling. Soluble RAGE is a truncated form of RAGE, acting as a decoy for a full-length RAGE. Studies have implicated that soluble RAGE counters the detrimental action of a full-length RAGE, making it a promising therapeutic target in ALS treatment.[11] Our previous studies demonstrated that RAGE deficiency improves post injury sciatic nerve regeneration

in type 1 diabetic mice, at least in part by reducing tissue-damaging inflammation at the injury site.[12] We also showed that in SOD1 93A mice, a common animal model of ALS, daily administration of soluble RAGE delays the onset and prolongs the lifespan of ALS mice, further underscoring its potential in ALS therapy. The results of our earlier studies with human and murine ALS-affected spinal cords revealed an overexpression of RAGE and its ligands in motor neurons, microglia and astrocytes.[13,14]

Our most recent studies further underscored the importance of RAGE-signaling, uncovering that the conditional RAGE knockout in microglia cells in ALS spinal cord extends the lifespan of SOD1 93A mice.[15] We also revealed time-depended changes in the expression of RAGE and its ligands in ALS mice over the course of the disease.[16]

Based on our previous results and collective evidence on the role of RAGE in ALS mouse models, we aimed to determine the levels of soluble RAGE, AGE and AOPP in donated blood surplus of early-stage ALS patients admitted to the Department of Neurology of Jagiellonian University Medical College (Cracow, Poland).

Materials and methods

Blood samples

Forty-six donated, coded and anonymized surplus plasma samples of ALS patients aged 30–73 (median age 55): 14 women of average age 55.7 ±6.3 years and 32 men of average age 55 ±10 years, and 11 non-neurological controls, aged 31–47 (median age 36) treated at the regional hospital were used in the study. All ALS patients were evaluated using Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS), scoring on average 46–48 points, and were classified as early-stage ALS. Samples were categorized according to the onset of the disease: spinal or bulbar. All patients met clinical and electrophysiological criteria of ALS as set in El Escorial criteria (EEC). Amyotrophic lateral sclerosis and control blood was donated and coded at the time of diagnosis. The study was designed in accordance with the tenets of the Declaration of Helsinki and approved by the Institutional Ethics Committee at the Jagiellonian University (approval No. 501/NKL/32/L).

RAGE, AGE and AOPP level measurement

The plasma levels of RAGE, AGE and AOPP were measured using enzyme-linked immunosorbent assay (ELISA) commercially available kits, i.e., Human RAGE Quantikine ELISA Kit (R&D Systems, Minneapolis, USA), OxiSelect™ AGE Competitive ELISA Kit and OxiSelect™ AOPP Assay Kit (Cell Biolabs Inc., San Diego, USA). Statistical evaluation of data was performed using one-way non-parametric analysis of variance (ANOVA) with Kruskal–Wallis post hoc test using GraphPad software (GraphPad Prism, San Diego, USA).

Results

Changes in the levels of various plasma proteins are often used as a diagnostic and/or prognostic tool in detecting a specific disease and assessing its progression. The clinical validity of plasma protein measurement has been established in routine testing for bone marrow disorders, liver and kidney diseases, leukemia, or cardiovascular disease (CVD).[17] However, the validity of using plasma markers in diagnosis and/or assessment of progression in neurodegenerative disease have been scarce and mainly limited to testing only for Alzheimer's disease (AD).[18,19] A number of studies offering insight into the use of plasma markers in ALS have been limited. Therefore, based on our previous research and research conducted by others, we attempt to fill in the gap by investigating the potential value of RAGE, AGE and AOPP plasma levels as prognostic markers of ALS.

RAGE

A significant reduction of soluble RAGE levels was detected in spinal and bulbar plasma samples as compared to the control (Fig. 1). This indicates the loss of neuroprotective, soluble form of RAGE already at the early stage of the disease. The mean values plus SEM were as follows: control – 720.5 ±81.66 pg/mL, spinal ALS – 365.2 ±43.66 pg/mL and bulbar ALS 257.7 ±96.46 pg/mL.

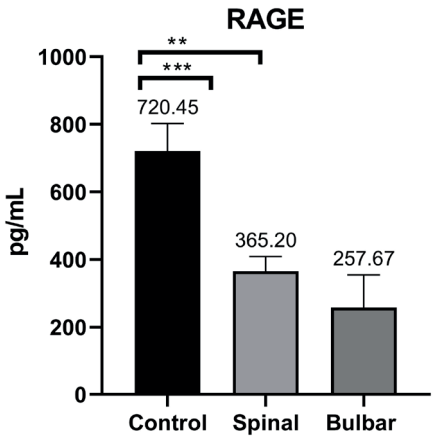

Fig. 1. Soluble plasma levels of receptor for advanced glycation end products (RAGE). The levels of soluble RAGE were significantly decreased in both groups of patients with ALS as compared to controls and, on average, the observed reduction was more noticeable, but not reaching statistical significance, in the bulbar group as compared to the spinal group; ** denotes $p < 0.01$ and *** denotes $p < 0.001$; bars represent standard error of the mean (SEM), mean value for each group is given above the corresponding bar

AGEs

An increasing trend in AGE plasma concentration was observed in spinal and bulbar plasma samples as compared to the control group (Fig. 2). This trend may reflect an uptake of pro-inflammatory AGE production already at the early stage of the disease. The mean values plus

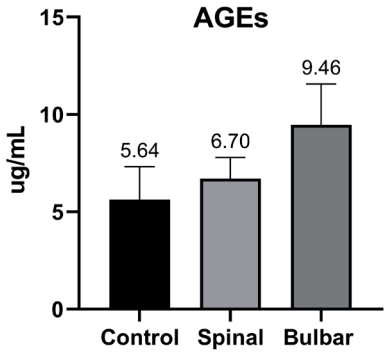

Fig. 2. Plasma levels of advanced glycation end product (AGE). While an increasing trend, signifying increased AGE activity, was noticed in both amyotrophic lateral sclerosis (ALS) groups, the increase was not yet statistically significant. The increasing trend more pronounced in plasma of the bulbar group was likely reflecting higher production of AGEs as compared to the spinal onset and to the control group; bars represent standard error of the mean (SEM), mean value for each group is given above the corresponding bar

SEM were as follows: control – 5.64 ±1.68 μg/mL, spinal ALS – 7.8 ±1.09 μg/mL and bulbar ALS 9.46 ±2.1 μg/mL.

AOPPs

Plasma analysis revealed high basal concentrations of AOPPs in all studied groups, on average 2.5 times higher as compared with the corresponding AGE levels. Similarly to AGEs, at the early stage of the disease, we observed an increasing trend in AOPP concentration in spinal and bulbar ALS as compared to controls (Fig. 3). The mean values plus SEM were as follows: control – 19.11 ±7.4 μmol/L, spinal ALS – 23.36 ±5.87 μmol/L and bulbar ALS 26.82 ±12.77 μmol/L.

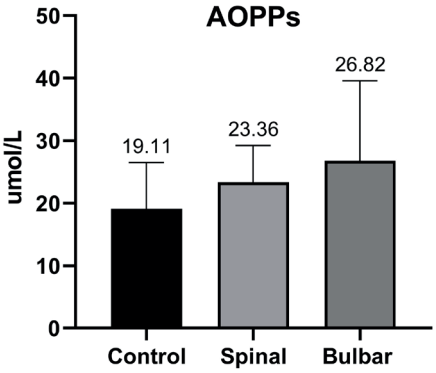

Fig. 3. Plasma levels of advanced oxidation protein products (AOPPs). Similarly to advanced glycation end products (AGEs), the levels of AOPPs were also increased in both amyotrophic lateral sclerosis (ALS) groups as compared to controls, with highest values observed in the bulbar groups. However, the observed increase was not statistically significant at this stage of the disease; bars represent standard error of the mean (SEM), mean value for each group is given above the corresponding bar

Discussion

Our present study focused on determining the plasma levels of soluble RAGE, AGE and AOPP at the early stage of ALS. We found that while the levels of soluble RAGE, a physiological antagonist of a full-length RAGE, were already significantly reduced, the levels of AGE and AOPP showed increased activity level compared to controls.

The results of our study are consistent with the study by Iłżecka,[20] who reported significantly reduced expression of soluble RAGE in ALS patients. Numerous studies show the correlation between the level of soluble RAGE on the onset and/or during progression of various neurodegenerative or metabolic diseases.[20–24] Reports from diabetes and atherosclerosis studies have revealed correlations between the levels of soluble RAGE and the severity of the pathological processes in a given disease.[25–27] Similarly, clinical reports from studies of neuroinflammatory disorders such as multiple sclerosis (MS) or Guillain–Barré syndrome have reported low levels of circulating soluble RAGE in both diseases compared to the controls, which correlated with the disease severity.[28–30]

However, in patients with MS who underwent CinnoVex (IFNβ-1a) or fingolimod (sphingosine-1-phosphate receptor modulator) treatments, the levels of soluble RAGE were increased, which corresponded to a positive treatment response, emphasizing the possible role of soluble RAGE in reducing neuroinflammation in affected tissue, thus diminishing the signs and symptoms of the disease.[31,32]

Despite the fact that studies on the use of soluble RAGE as a specific marker of ALS progression are still in the early stages, based on evidence derived from our and other recent translational studies demonstrating the involvement of RAGE in the pathogenesis of ALS, we might assume that measuring RAGE level, especially in conjunction with AGE and AOPP, could be a prognostic marker of ALS progression. Our study demonstrated that blocking RAGE signaling with soluble RAGE delays the progression of the disease and slows down the motor function decline. A recent study by Liu et al. supports our results, revealing that RAGE inhibition by soluble RAGE-like inhibitors, such as RAGE antagonist peptide (RAP) and FPS-ZM1, has a positive effect on motor neuron survival and reduced gliosis, effectively improving motor function in ALS mice, but not extending lifespan.[14,33]

Taken together, these results indicate that the role of RAGE in ALS progression might extend beyond secondary involvement, making it a potential marker and therapeutic target.

Opposing the observed reduction in plasma levels of soluble RAGE, we noted that the levels of AGEs and AOPP were trending toward higher values in patients with ALS as compared to controls. The increasing trend of AGE levels in plasma of ALS-affected patients is consistent with our own previous data showing an increase in tissue bound full-length RAGE and CML – one of the AGE representatives, in the spinal cord tissue of patients with ALS.[13]

Advanced glycation end products are naturally occurring products of nonenzymatic glycation and oxidation of proteins or lipids, presenting in low levels in plasma of healthy,

young and middle-aged individuals, and increasing during physiological aging and pathological processes such as atherosclerosis, diabetes, inflammation, and neurodegeneration.[34] Some studies showed that the excessive production and accumulation of AGEs exacerbates the inflammatory response, disturbs cellular metabolism, and increases cellular toxicity, leading to cellular malfunction, increased apoptosis and accelerated disease progression.[35,36] In neurodegenerative disorders, AGEs accumulation have been linked to an AGE-depended direct toxicity excreted on neuronal cells in cortex of patients with AD;[37] similarly, in Parkinson's disease, AGE-triggered cytotoxicity has been observed, affecting neuronal cell metabolism and reducing neuronal viability.[38] In ALS, significantly increased level of AGEs have been also observed in cerebrospinal fluid of patients with ALS and lower motor neuron disease compared to controls, but also to patients with AD, underscoring its potential as a diagnostic/prognostic tool in ALS.[39]

As with AGEs, AOPPs are the products of excessive oxidation, yet another of the pathological processes in which full-length RAGE plays a prominent role. Advanced oxidation protein products have recently gained attention as reliable markers of oxidation-triggered protein damage, including ALS.[40,41] It has been noted that RAGE, involved in the production of reactive oxygen species (ROS), plays a significant role in cellular damage and apoptosis driven by oxidation.[42]

Furthermore, the formation of AOPP through ROS triggers RAGE's detrimental role on cellular metabolism and long-term disfunction. Increased levels of AOPPs were first shown in the patients with chronic uremia.[43] However, subsequent studies demonstrated that their increase was not unique for uremic patients and they are also elevated in a wide array of pathological conditions, most notably, osteoporosis,[44] rosacea – which is a chronic inflammatory dermatosis,[45] psoriasis – yet another immunological disorder,[46] myasthenia gravis,[47] breast cancer,[48] and HIV.[49] Furthermore, a study by Siciliano et al. on antioxidant capacity and protein oxidation in ALS demonstrated a significantly increased presence of AOPPs in both plasma and cerebrospinal fluid of ALS patients, underscoring the importance of gaining a better insight into the role of oxidative stress in ALS pathogenesis and progression.[50] Consistent with these results, we also report elevated levels of AOPP in ALS and demonstrate that, similarly to AGEs, AOPPs are likely involved in ALS pathogenesis, signifying increased oxidative stress and oxidation-related cellular damage even in the early stages of the disease.

Conclusions

We report a decline of soluble RAGE concurrent with a rise of AGEs and AOPPs in plasma of ALS patients at the early stage of the disease. The results presented here suggest that a longer, longitudinal study of RAGE, AGE and AOPP plasma levels in ALS patients at different stage of the disease would be beneficial, outlining the dynamics between RAGE and its ligand levels as the disease progresses, thus making them likely diagnostic tools and potential therapeutic targets.

The results also indicate that further studies evaluating not only the plasma, but also cerebrospinal fluid levels of these substances would be warranted, providing new insights into their role in ALS pathogenesis and progression.

ORCID iDs

Judyta Juranek https://orcid.org/0000-0001-8665-6606
Adam Osowski https://orcid.org/0000-0002-0544-5653
Joanna Wojtkiewicz https://orcid.org/0000-0003-0856-3644
Marta Banach https://orcid.org/0000-0002-4656-3656

References

1. Chiò A, Logroscino G, Traynor BJ, et al. Global epidemiology of amyotrophic lateral sclerosis: A systematic review of the published literature. *Neuroepidemiology*. 2013;41(2):118–130. doi:10.1159/000351153
2. Wijesekera LC, Nigel Leigh P. Amyotrophic lateral sclerosis. *Orphanet J Rare Dis*. 2009;4(1):3. doi:10.1186/1750-1172-4-3
3. Barber SC, Mead RJ, Shaw PJ. Oxidative stress in ALS: A mechanism of neurodegeneration and a therapeutic target. *Biochim Biophys Acta Mol Basis Dis*. 2006;1762(11–12):1051–1067. doi:10.1016/j.bbadis.2006.03.008
4. Barber SC, Shaw PJ. Oxidative stress in ALS: Key role in motor neuron injury and therapeutic target. *Free Radic Biol Med*. 2010;48(5):629–641. doi:10.1016/j.freeradbiomed.2009.11.018
5. Blokhuis AM, Groen EJN, Koppers M, Van Den Berg LH, Pasterkamp RJ. Protein aggregation in amyotrophic lateral sclerosis. *Acta Neuropathol*. 2013;125(6):777–794. doi:10.1007/s00401-013-1125-6
6. Van Den Bosch L, Van Damme P, Bogaert E, Robberecht W. The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. *Biochim Biophys Acta Mol Basis Dis*. 2006;1762(11–12):1068–1082. doi:10.1016/j.bbadis.2006.05.002
7. Nowicka N, Juranek J, Juranek JK, Wojtkiewicz J. Risk factors and emerging therapies in amyotrophic lateral sclerosis. *Int J Mol Sci*. 2019;20(11):2616. doi:10.3390/ijms20112616
8. Juranek J, Mukherjee K, Kordas B, et al. Role of RAGE in the pathogenesis of neurological disorders. *Neurosci Bull*. 2022;38(10):1248–1262. doi:10.1007/s12264-022-00878-x
9. Ray R, Juranek JK, Rai V. RAGE axis in neuroinflammation, neurodegeneration and its emerging role in the pathogenesis of amyotrophic lateral sclerosis. *Neurosci Biobehav Rev*. 2016;62:48–55. doi:10.1016/j.neubiorev.2015.12.006
10. Nogueira-Machado J, De Oliveira Volpe C. HMGB-1 as a target for inflammation controlling. *Recent Pat Endocr Metab Immune Drug Discov*. 2012;6(3):201–209. doi:10.2174/187221412802481784
11. Yan SF, Ramasamy R, Schmidt AM. Soluble RAGE: Therapy and biomarker in unraveling the RAGE axis in chronic disease and aging. *Biochem Pharmacol*. 2010;79(10):1379–1386. doi:10.1016/j.bcp.2010.01.013
12. Juranek JK, Geddis MS, Song F, et al. RAGE deficiency improves postinjury sciatic nerve regeneration in type 1 diabetic mice. *Diabetes*. 2013;62(3):931–943. doi:10.2337/db12-0632
13. Juranek JK, Daffu GK, Wojtkiewicz J, Lacomis D, Kofler J, Schmidt AM. Receptor for advanced glycation end products and its inflammatory ligands are upregulated in amyotrophic lateral sclerosis. *Front Cell Neurosci*. 2015;9:485. doi:10.3389/fncel.2015.00485
14. Juranek JK, Daffu GK, Geddis MS, et al. Soluble RAGE treatment delays progression of amyotrophic lateral sclerosis in SOD1 mice. *Front Cell Neurosci*. 2016;10:117. doi:10.3389/fncel.2016.00117
15. MacLean M, Juranek J, Cuddapah S, et al. Microglia RAGE exacerbates the progression of neurodegeneration within the SOD1G93A murine model of amyotrophic lateral sclerosis in a sex-dependent manner. *J Neuroinflammation*. 2021;18(1):139. doi:10.1186/s12974-021-02191-2

16. Nowicka N, Szymańska K, Juranek J, et al. The involvement of RAGE and its ligands during progression of ALS in SOD1 G93A transgenic mice. *Int J Mol Sci*. 2022;23(4):2184. doi:10.3390/ijms23042184
17. Hemalatha T, UmaMaheswari T, Krithiga G, Sankaranarayanan P, Puvanakrishnan R. Enzymes in clinical medicine: An overview. *Indian J Exp Biol*. 2013;51(10):777–788. PMID:24266101.
18. Tucker R, Pedro A. Blood-derived non-extracellular vesicle proteins as potential biomarkers for the diagnosis of early ER+ breast cancer and detection of lymph node involvement. *F1000Res*. 2018;7:283. doi:10.12688/f1000research.14129.3
19. Udeh-Momoh C, Zheng B, Sandebring-Matton A, et al. Blood derived amyloid biomarkers for Alzheimer's disease prevention. *J Prev Alzheimers Dis*. 2021;9(1):12–21. doi:10.14283/jpad.2021.70
20. Iłżecka J. Serum-soluble receptor for advanced glycation end product levels in patients with amyotrophic lateral sclerosis. *Acta Neurol Scand*. 2009;120(2):119–122. doi:10.1111/j.1600-0404.2008.01133.x
21. Huang M, Que Y, Shen X. Correlation of the plasma levels of soluble RAGE and endogenous secretory RAGE with oxidative stress in pre-diabetic patients. *J Diabetes Complications*. 2015;29(3):422–426. doi:10.1016/j.jdiacomp.2014.12.007
22. Wagner NB, Weide B, Reith M, et al. Diminished levels of the soluble form of RAGE are related to poor survival in malignant melanoma. *Int J Cancer*. 2015;137(11):2607–2617. doi:10.1002/ijc.29619
23. Fuller KNZ, Miranda ER, Thyfault JP, Morris JK, Haus JM. Metabolic derangements contribute to reduced sRAGE isoforms in subjects with Alzheimer's disease. *Mediators Inflamm*. 2018;2018:2061376. doi:10.1155/2018/2061376
24. Yokota C, Minematsu K, Tomii Y, et al. Low levels of plasma soluble receptor for advanced glycation end products are associated with severe leukoaraiosis in acute stroke patients. *J Neurol Sci*. 2009;287(1–2): 41–44. doi:10.1016/j.jns.2009.09.013
25. Di Pino A, Urbano F, Zagami RM, et al. Low endogenous secretory receptor for advanced glycation end-products levels are associated with inflammation and carotid atherosclerosis in prediabetes. *J Clin Endocrinol Metab*. 2016;101(4):1701–1709. doi:10.1210/jc.2015-4069
26. Gurecká R, Koborová I, Csongová M, Šebek J, Šebeková K. Correlation among soluble receptors for advanced glycation end-products, soluble vascular adhesion protein-1/semicarbazide-sensitive amine oxidase (sVAP-1) and cardiometabolic risk markers in apparently healthy adolescents: A cross-sectional study. *Glycoconj J*. 2016;33(4):599–606. doi:10.1007/s10719-016-9696-9
27. Grossin N, Wautier MP, Meas T, Guillausseau PJ, Massin P, Wautier JL. Severity of diabetic microvascular complications is associated with a low soluble RAGE level. *Diabetes Metab*. 2008;34(4):392–395. doi:10.1016/j.diabet.2008.04.003
28. Glasnović A, Cvija H, Stojić M, et al. Decreased level of sRAGE in the cerebrospinal fluid of multiple sclerosis patients at clinical onset. *Neuroimmunomodulation*. 2014;21(5):226–233. doi:10.1159/000357002
29. Sternberg Z, Weinstock-Guttman B, Hojnacki D, et al. Soluble receptor for advanced glycation end products in multiple sclerosis: A potential marker of disease severity. *Mult Scler*. 2008;14(6):759–763. doi:10.1177/1352458507088105
30. Zhang DQ, Wang R, Li T, et al. Reduced soluble RAGE is associated with disease severity of axonal Guillain–Barré syndrome. *Sci Rep*. 2016;6(1):21890. doi:10.1038/srep21890
31. Rahimi M, Aghabozorg Afjeh SS, Omrani MD, et al. Soluble receptor for advanced glycation end products (sRAGE) is up-regulated in multiple sclerosis patients treated with interferon β-1a. *Cell Physiol Biochem*. 2018;46(2):561–567. doi:10.1159/000488622
32. Sternberg Z, Kolb C, Chadha K, et al. Fingolimod anti-inflammatory and neuroprotective effects modulation of RAGE axis in multiple sclerosis patients. *Neuropharmacology*. 2018;130:71–76. doi:10.1016/j.neuropharm.2017.11.047
33. Liu L, Killoy KM, Vargas MR, Yamamoto Y, Pehar M. Effects of RAGE inhibition on the progression of the disease in hSOD1 ^{G93A} ALS mice. *Pharmacology Res Perspect*. 2020;8(4):e00636. doi:10.1002/prp2.636
34. Juranek J, Ray R, Banach M, Rai V. Receptor for advanced glycation end-products in neurodegenerative diseases. *Rev Neurosci*. 2015; 26(6):691–698. doi:10.1515/revneuro-2015-0003
35. Ramasamy R, Vannucci SJ, Yan SSD, Herold K, Yan SF, Schmidt AM. Advanced glycation end products and RAGE: A common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology*. 2005;15(7):16R–28R. doi:10.1093/glycob/cwi053
36. Li J, Liu D, Sun L, Lu Y, Zhang Z. Advanced glycation end products and neurodegenerative diseases: Mechanisms and perspective. *J Neurol Sci*. 2012;317(1–2):1–5. doi:10.1016/j.jns.2012.02.018
37. Takeuchi M, Kikuchi S, Sasaki N, et al. Involvement of advanced glycation end-products (AGEs) in Alzheimer's disease. *Curr Alzheimer Res*. 2004;1(1):39–46. doi:10.2174/1567205043480582
38. Chen L, Wei Y, Wang X, He R. Ribosylation rapidly induces α-synuclein to form highly cytotoxic molten globules of advanced glycation end products. *PLoS One*. 2010;5(2):e9052. doi:10.1371/journal.pone.0009052
39. Kaufmann E, Boehm BO, Süssmuth SD, et al. The advanced glycation end-product Nε-(carboxymethyl)lysine level is elevated in cerebrospinal fluid of patients with amyotrophic lateral sclerosis. *Neurosci Lett*. 2004;371(2–3):226–229. doi:10.1016/j.neulet.2004.08.071
40. Wu Q, Zhong Z, Pan Y, et al. Advanced oxidation protein products as a novel marker of oxidative stress in postmenopausal osteoporosis. *Med Sci Monit*. 2015;21:2428–2432. doi:10.12659/MSM.894347
41. Pasquinelli A, Chico L, Pasquali L, et al. Gly482Ser *PGC-1α* gene polymorphism and exercise-related oxidative stress in amyotrophic lateral sclerosis patients. *Front Cell Neurosci*. 2016;10:102. doi:10.3389/fncel.2016.00102
42. Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J Biol Chem*. 1997;272(28):17810–17814. doi:10.1074/jbc.272.28.17810
43. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int*. 1996;49(5):1304–1313. doi:10.1038/ki.1996.186
44. Zhou Q, Zhu L, Zhang D, et al. Oxidative stress-related biomarkers in postmenopausal osteoporosis: A systematic review and meta-analyses. *Dis Markers*. 2016;2016:7067984. doi:10.1155/2016/7067984
45. Erdogan HK, Bulur I, Kocaturk E, Saracoglu ZN, Alatas O, Bilgin M. Advanced oxidation protein products and serum total oxidant/antioxidant status levels in rosacea. *Adv Dermatol Allergol*. 2018;35(3): 304–308. doi:10.5114/ada.2018.76228
46. Yazici C, Köse K, Utaş S, Tanrikulu E, Taşlidere N. A novel approach in psoriasis: First usage of known protein oxidation markers to prove oxidative stress. *Arch Dermatol Res*. 2016;308(3):207–212. doi:10.1007/s00403-016-1624-0
47. Adamczyk-Sowa M, Bieszczad-Bedrejczuk E, Galiniak S, et al. Oxidative modifications of blood serum proteins in myasthenia gravis. *J Neuroimmunol*. 2017;305:145–153. doi:10.1016/j.jneuroim.2017.01.019
48. Tesarová P, Kalousová M, Trnková B, et al. Carbonyl and oxidative stress in patients with breast cancer: Is there a relation to the stage of the disease? *Neoplasma*. 2007;54(3):219–224. PMID:17447853.
49. Morimoto HK, Simão ANC, Almeida ERDD, et al. Role of metabolic syndrome and antiretroviral therapy in adiponectin levels and oxidative stress in HIV-1 infected patients. *Nutrition*. 2014;30(11–12):1324–1330. doi:10.1016/j.nut.2014.03.017
50. Siciliano G, Piazza S, Carlesi C, et al. Antioxidant capacity and protein oxidation in cerebrospinal fluid of amyotrophic lateral sclerosis. *J Neurol*. 2007;254(5):575–580. doi:10.1007/s00415-006-0301-1

The influence of selected polymers on the surface tension of solutions developed for the preparation of eye drops

Wpływ wybranych polimerów na napięcie powierzchniowe roztworów przeznaczonych do przygotowania kropli doocznych

Monika Gasztych[1,A–E], Katarzyna Burdzy[1,B,C], Arleta Dołowacka-Jóźwiak[1,C,E], Witold Musiał[1,A,C,E,F]

[1] Department of Physical Chemistry and Biophysics, Faculty of Pharmacy, Wroclaw Medical University, Poland
[2] Department of Drug Form Technology, Faculty of Pharmacy, Wroclaw Medical University, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Polymers in Medicine, ISSN 0370-0747 (print), ISSN 2451-2699 (online) *Polim Med*. 2023;53(2):111–116

Address for correspondence
Witold Musiał
E-mail: witold.musial@umw.edu.pl

Funding sources
This research was funded by Wroclaw Medical University, grant No. SUBZ.D060.23.025.

Conflict of interest
None declared

Received on November 7, 2023
Reviewed in November 21, 2023
Accepted on November 21, 2023

Published online on December 6, 2023

Abstract

Background. Many substances are used to increase the viscosity of eye drops and reduce their surface tension. Their function is to prolong the persistence of the product on the surface of the eyeball and to increase the bioavailability of the pharmacologically active ingredient.

Objectives. To investigate the surface tension of substances added to the eye drops, with the main aim of modulating properties of the preparation.

Materials and methods. Five substances contained in solutions proposed for the development of eye drops were studied: sodium hyaluronate macromolecular (H-Na W), sodium hyaluronate ultramolecular (H-Na UM), hyaluronic acid 4% (K-H), methylcellulose (MC), and polyacrylic acid (PA). The main method was to study the surface tension using the du Noüy ring tensiometer.

Results. The research presented in this paper shows the various effects of different eye drop ingredients on the surface tension of the solutions. The surface tension values of PA solutions are in the range of 48.89–56.03 mN/m, of MC in the range of 68.94–89.32 mN/m, of K-H 54.54–65.66 mN/m, of H-Na UM 67.18–70.97 mN/m, and of H-Na W 67.09–71.73 mN/m.

Conclusions. The use of different polymers affects the surface tension of model solutions proposed for use in ophthalmic preparations. Compounds containing carboxyl groups and anionic polymers have a similar effect on reducing the surface tension of the solution as classical surfactant compounds.

Key words: eye drops, surface tension, hyaluronic acid, polyacrylic acid, methylcellulose

Cite as
Gasztych M, Burdzy K, Dołowacka-Jóźwiak A, Musiał W. The influence of selected polymers on the surface tension of solutions developed for the preparation of eye drops. *Polim Med*. 2023;53(2):111–116. doi:10.17219/pim/175643

DOI
10.17219/pim/175643

Streszczenie

Wprowadzenie. Aby zwiększyć lepkość kropli do oczu, a także zmniejszyć ich napięcie powierzchniowe, stosuje się wiele substancji. Ich funkcją jest przedłużenie trwałości preparatu na powierzchni gałki ocznej oraz zwiększenie biodostępności substancji farmakologicznie czynnej.

Cel. Celem badań było zbadanie napięcia powierzchniowego substancji dodawanych do kropli do oczu, których głównym celem była modulacja właściwości preparatu.

Materiały i metody. Zbadano pięć substancji zawartych w roztworach proponowanych do opracowania kropli do oczu: hialuronian sodu wielkocząsteczkowy, hialuronian sodu ultramolekularny, kwas hialuronowy 4%, metylocelulozę i kwas poliakrylowy. Główną metodą było badanie napięcia powierzchniowego za pomocą tensjometru z wykorzystaniem pierścienia du Noüye.

Wyniki. Badania przedstawione w artykule pokazują zróżnicowany wpływ różnych substancji przeznaczonych do kropli do oczu na napięcie powierzchniowe roztworów. Wartości napięcia powierzchniowego roztworów PA mieszczą się w przedziale 48,89–56,03 mN/m, MC w przedziale 68,94–89,32 mN/m, K-H 54,54–65,66 mN/m, H-Na UM 67,18–70,97 mN/m, oraz dla H-Na W 67,09–71,73 mN/m.

Wnioski. Zastosowanie różnych polimerów wpływa na napięcie powierzchniowe modelowych roztworów do stosowania w okulistyce. Związki zawierające grupy karboksylowe – polimery anionowe, wpływają na obniżenie napięcia powierzchniowego roztworu podobnie jak klasyczne związki powierzchniowo czynne.

Słowa kluczowe: krople do oczu, napięcie powierzchniowe, kwas hialuronowy, kwas poliakrylowy, metyloceluloza

Background

Eye drops are the most popular method of administering ocular medications, not only for the treatment but also for the prevention and relief of symptoms of many diseases. They are a relatively safe way of delivering many groups of drugs, including antibiotics and anti-inflammatory drugs, to the ocular surface. During manufacture, a number of excipients are added to increase eye drops bioavailability, ensure optimal storage conditions, and prolong the action and persistence of the active ingredients at the site of application.[1]

To increase the bioavailability of eye drops, a number of substances are used to increase the viscosity of the drops at the site of application and to reduce their surface tension. Their function is to prolong the persistence of the product on the ocular surface.[2]

There are forces between the molecules of a liquid that determine its cohesion, i.e., surface tension forces. They are the result of asymmetric forces acting on the surface of liquids. In the depths of the liquid, the are no forces acting on the particles because all the particles surround each other and are in equilibrium. However, at the surface, at a thickness of about 10 nm, the forces are not balanced because there are not enough interactions from the free surface. The unbalanced force is called the surface tension or surface tension coefficient, and is defined as the force exerted by a single free surface of a liquid per unit length of its circumference. The value of the surface tension is expressed in [N/m] and [J/m^2]. Surface tension measurements are mainly used to characterize solutions containing amphiphilic substances.[3,4]

The reduction of surface tension values can be critical in assessing the quality and manufacture of many dosage forms, including emulsions, inhalation preparations, and eye drops. The substances that enable this phenomenon to occur are surfactants, tensides, and emulsifiers. They are characterized by an amphiphilic structure, which has both lipophilic and hydrophilic parts in its structure. This allows the surfactant molecules to properly align on the surface of the solution and form micelles, which affect the reduction of the surface activity value. Surfactants have the ability to reduce this value to a certain limit, which cannot be exceeded despite increasing the surfactant concentration. This limit is called the critical micellar concentration.[5,6]

The addition of most compounds to a pure solution results in a reduction in surface tension. In particular, insoluble organic compounds have good surfactant properties due to their structure and high content of -CH_2- groups. These include lipids, which are often used in the manufacture of medicines and in everyday life. Surfactants can be divided into cationic (containing an amine or ammonium group in their structure), anionic (with a carboxyl group), and non-ionic (made up of sugar molecules or oligooxyethylene groups). The substances most commonly used to reduce surface tension in the manufacture of eye drops are Tween 80 and some preservatives such as benzalkonium salts.[6,7]

The study focused on investigating the surface tension of substances added to eye drops whose primary purpose is not to modulate the surface tension of the formulation. The aim of the study was to investigate the effect of the diversity of raw material composition of eye drops on their surface tension value.

Materials and methods

Sodium hyaluronate macromolecular (H-Na W) of molecular weight of 1.15 MDa (Escent, Szczecin, Poland), sodium hyaluronate ultramolecular (H-Na UM) of molecular weight of 4.5 kDa (Escent), hyaluronic acid 4% (K-H)

(Escent), polyacrylic acid Carbopol®980 NF (PA) (Lubrizol, Wickliffe, USA), methylcellulose (MC) (Sigma-Aldrich, St. Louis, USA), and deionized water were obtained from the ionic column according to monography of the purified water from the European Pharmacopoeia; this type of water was used in all studies.

Composition of prepared solutions

Appropriate amounts of substances and deionized water were weighed out on an analytical balance. The solids of PA, MC, H-Na W, and H-Na UM were then combined with deionized water. The prepared solutions were allowed to dissolve completely at 8°C under a cover. Polyacrylic acid-based solutions were neutralized with an appropriate amount of NaOH. Previously prepared solutions of H-Na W and H-Na UM mixtures were used to prepare solutions of these substances. Ten milliliters of each solution was weighed on an analytical balance and mixed with deionized water in a ratio of 1:1. All solutions were stored under a cover at 8°C. The prepared solutions shown in Table 1.

To measure the surface tension of prepared solutions, the formulations were placed in a dry measuring vessel, the du Noüy ring was immersed in the liquid, and the platform was slowly lowered. This method tested the force required to completely detach the ring from the surface of the solution. The schematic design of the tensiometer is shown in Fig. 1. Individual results have been calculated directly using D-MT1A.exe software (Polon Izot, Warsaw, Poland)using the following formulas (Eq. 1):

$$S = \frac{K \times f \times F_{max}}{4\pi R} \times 1000 \quad (1)$$

S – surface tension in [mN/m];
F_{max} – force [N];
K – calibration factor automatically determined by the program;
R – ring radius [mm];

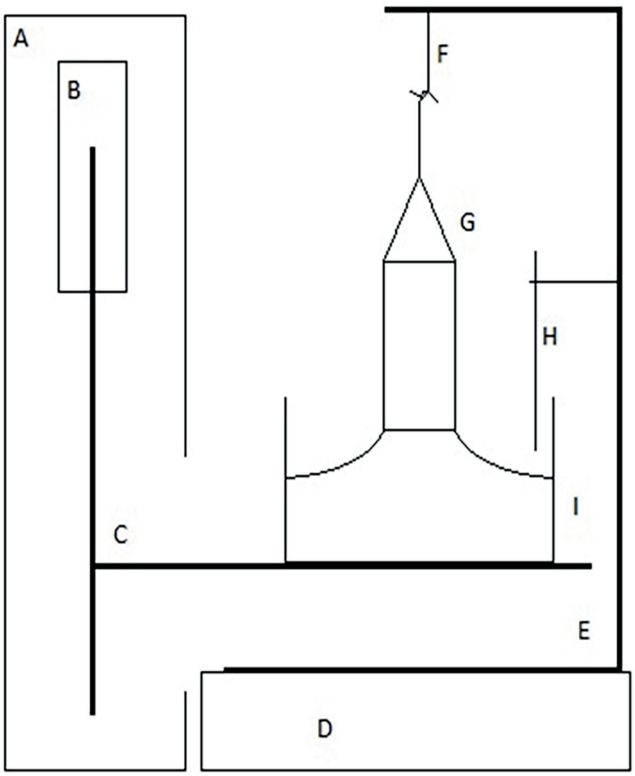

Fig. 1. Schematic design of the tensiometer. A. lift; B. lift mechanism; C. mobile platform; D. balance pan; E. measurement stand; F. ring holder; G. du Noüy ring; H. temperature sensor; I. tested solution

f – correlation coefficient determined according to the Zuidema–Waters formula (Eq. 2):

$$f = 0.725 + \left[\frac{0.03678 \times S_{POM}}{R^2 \times \Delta\rho} + 0.04534 - 1.679 \times \left(\frac{R}{r}\right)\right]^{0.5}$$

S_{POM} – surface tension value [mN/m];
R – radius of the ring [mm];
r – wire radius [mm];
Δρ – difference in density between the tested liquid and air [g/mm^3].

Table 1. Quantitative composition of prepared solutions.

% concentration of the solution	Substrates					
	H_2O [g]	H-Na W [g]	H-Na UW [g]	4% K-H [g]	PA [g]	MC [g]
1.00	49.500	0.500	0.500	0.500	0.500	0.500
0.75	49.625	0.375	0.375	0.375	0.375	0.375
0.50	49.750	0.250	0.250	0.250	0.250	0.250
0.40	49.800	0.200	0.200	0.200	-	-
0.30	49.850	0.150	0.150	0.150	-	-
0.25	49.875	0.125	0.125	0.125	0.125	0.125
0.20	49.900	0.100	0.100	0.100	-	-
0.15	49.925	0.075	0.075	0.075	-	-
0.10	49.950	0.050	0.050	0.050	-	-

Results

Changes in the surface tension values of different concentrations of PA are shown in Fig. 2A. The highest surface tension was observed for the 0.25% solution. The decrease in surface tension is clearly visible in solutions with higher concentrations except for the 1.0% solution. The surface tension values are in the range of 48.89–56.03 mN/m. In Fig. 2A, we can observe clear differences in the surface tension of MC solutions. The surface tensions of solutions with concentrations from 0.25% to 0.75% have an increasing trend, while the tension of the 1.00% solution is lower than the previous one by 2.96 mN/m. Figure 2B–D shows the surface tension values of K-H, H-Na UM, and H-Na W solutions. The surface tension values of K-H decrease with increasing concentration, oscillating in the range of 54.54–65.66 mN/m. In the H-NA UM solutions, the lowest surface tension values were recorded for concentrations of 0.30% and 0.40%. The minimum surface tension result was 67.18 mN/m and the maximum was 70.97 mN/m. Pointing out the surface tension of H-Na W, it can be observed that clearly the highest surface tension is shown by the 0.25% and 1.00% solutions: 71.43 mN/m and 71.73 mN/m, respectively. The smallest values are shown by the 0.10% solution: 67.09 mN/m.

Discussion

The research presented in this paper shows different effects of selected polymers on the surface tension of eye drop formulations. The experiments were carried out using deionized water and solutions of PA, MC, K-H, and their salts of different molecularities at different concentrations. The structural forms of the polymers used are shown in Fig. 3. During the measurement of the surface tension with the tensiometric method using the du Noüy ring, the recorded values of the surface tension increased up to a certain maximum.[8] Over time, they reached an extreme, after which they decreased, and the reading at the highest point was the correct value of this voltage. The shape of the curve obtained in the experiment results from the interactions between individual molecules of the substance. The strength of these interactions depends on many factors, including temperature and small differences in composition, such as the absorption of CO_2 from the air by the solution. Intermolecular interactions in water solutions are mainly based on the forces of repulsion and attraction between molecules, the strongest of which are the forces between ions in electrolyte solutions and the van der Waals forces. It is these interactions, which can be perturbed by the phenomena of physical adsorption and chemical adsorption, that are important in surface tension measurements. Polymers such as PA and MC are used as thickeners and viscosity enhancers

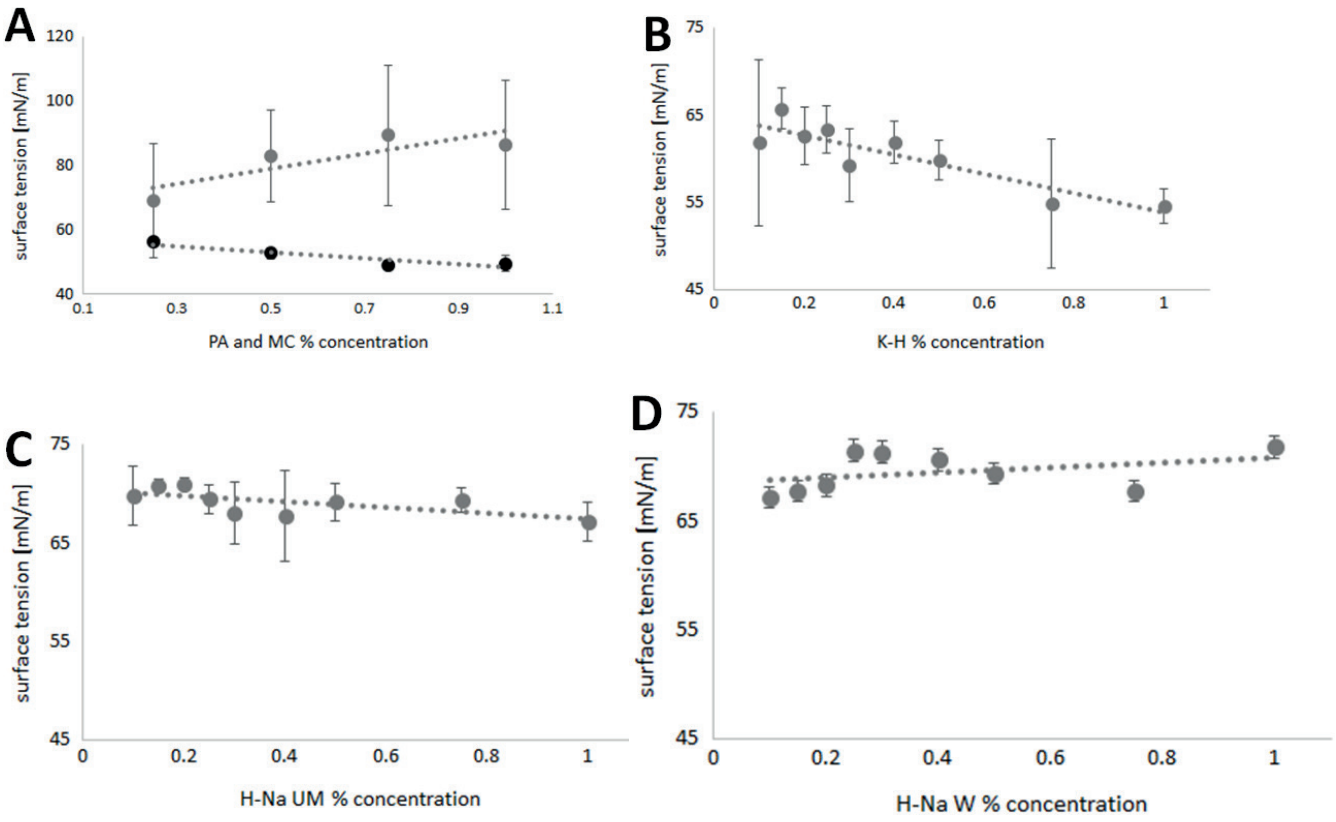

Fig. 2. Surface tension of polyacrylid acid (PA) and methylcellulose (MC) solutions (grey dots are for MC and black dots are for PA) (A), and surface tension of hyaluronic acid 4% (K-H) (B), sodium hyaluronate ultramolecular (H-Na UM) (C), and sodium hyaluronate macromolecular (H-Na W) (D). The Y-bars represent standard deviation

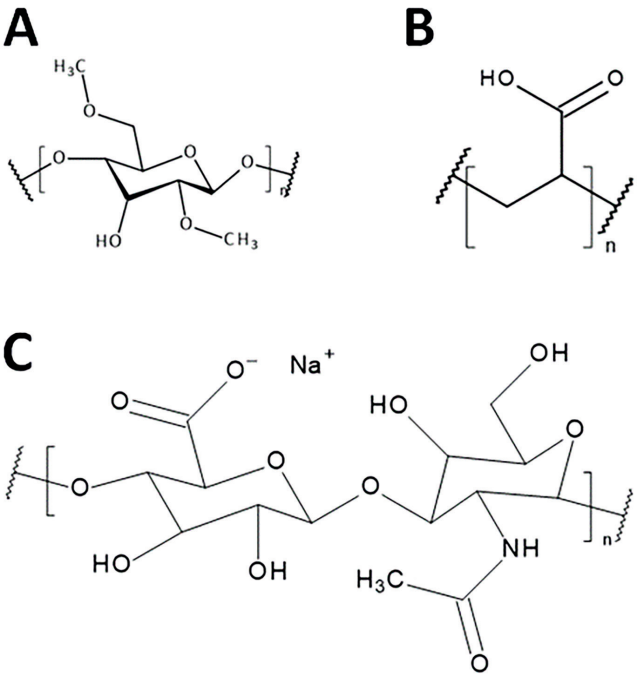

Fig. 3. Structural forms of used polymers. A. methylcellulose; B. polyacrylic acid; C. sodium hyaluronate

in ophthalmic products.[9] The acrylic acid polymer, which contains free carboxyl groups, has a clear effect in reducing the surface tension of the system. In contrast, in the case of the non-ionic polymer shown in Fig. 2, an increase in surface tension with increasing polymer concentration is observed over a relatively wide range of concentrations.

The presence of carboxyl and hydrophobic groups may promote a reduction in the surface tension of the PA solution in a manner analogous to that of surfactants. In the case of MC, the absence of ionized groups suggests that cohesion forces between linear polymer chains are responsible for the measured results. These forces increase with increasing concentration and the number of polymer chains present in the solution.[10,11] There are many scientific papers in which K-H molecules of different molecular weight and their salts have been characterized using traditional structural methods such as Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), scanning electron microscopy (SEM), or X-ray diffraction.[12–14] Surface tension is not a commonly studied parameter in solutions of K-H and its salts, but it is very useful in the treatment of certain diseases, especially those associated with dry eye syndrome.[15] In the case of pure K-H, the surface tension values shown in Fig. 2B tend to decrease with increasing concentration. It is likely that in this case the interactions of the polymer with the solvent took place on a similar basis as in the case of PA. Polymers containing carboxyl groups can be compared with surfactants containing the corresponding ionized group and aliphatic chain.[16] Unfortunately, the sodium salt of K-H did not give clear results on the effect of its concentration in solution on the surface tension in the cited study. This knowledge was used to evaluate the interactions between the molecules. The results confirm that the addition of sodium ions is likely to have a major effect in stabilizing the surface tension. Compared to MC and PA, the system containing the sodium salt of K-H is slightly more complex. In this system, the surface tension is influenced not only by the polysaccharide chain with its carboxyl groups, but also by the sodium ions.[17] The result obtained from the surface tension measurements may be a result of the influence of the above factors. This is confirmed by the fact that the surface tension values of H-Na UM and H-Na W are directly independent of the polymer concentration. In the case of a short chain polymer, the observed surface tension values initially tend to increase, after which the surface tension decreases and then increases again. The same is true for H-Na W, but probably due to interactions between the polymer chains, the values are slightly shifted.[15] In the next stage of the planned research, it is advisable to carry out viscosity tests, as the surface tension of liquids depends on many factors, including viscosity.[18] In 1966, Pelfosky presented the first possible relationships between the natural logarithm of surface tension values and the reciprocal of viscosity.[19] Today, his research is constantly being refined and extended, allowing the introduction of more variables. Many scientists are also developing their own methods for studying these relationships, as evidenced by the publication of Vaziri et al.[20] who presented 2 new methods for calculating surface tension values based on surface tension relationship with particle size, density, and viscosity of the solution. They showed that in the ionic solutions studied, the surface tension decreases as the length of the cationic alkyl chain increases. This is due to a decrease in van der Waals forces and the aliphatic nature of the solution as the length of the anion increases. This has the effect of reducing electrostatic forces in the solution and increasing dispersion,[19] which confirms the importance of intermolecular forces, cohesion, and adhesion in the formation of surface tension.

Conclusions

1. Measurement conditions using the du Noüy ring have a significant effect on the recorded surface tension.
2. Higher surface tension values and higher standard deviations were observed for MC compared to PA.
3. Hyaluronic acid 4% surface tension values decreased significantly with increasing solution concentration, which was not observed for K-H salts.

ORCID IDs

Monika Gasztych https://orcid.org/0000-0002-4868-8727
Katarzyna Burdzy https://orcid.org/0009-0007-8603-4090
Arleta Dołowacka-Jóźwiak https://orcid.org/0000-0003-0947-8847
Witold Musiał https://orcid.org/0000-0001-5695-5998

References

1. Mofidfar M, Abdi B, Ahadian S, et al. Drug delivery to the anterior segment of the eye: A review of current and future treatment strategies. *Int J Pharm*. 2021;607:120924. doi:10.1016/j.ijpharm.2021.120924
2. Irimia T, Ghica M, Popa L, Anuţa V, Arsene AL, Dinu-Pîrvu CE. Strategies for improving ocular drug bioavailability and corneal wound healing with chitosan-based delivery systems. *Polymers (Basel)*. 2018; 10(11):1221. doi:10.3390/polym10111221
3. Fung SY, Keyes C, Duhamel J, Chen P. Concentration effect on the aggregation of a self-assembling oligopeptide. *Biophys J*. 2003;85(1):537–548. doi:10.1016/S0006-3495(03)74498-1
4. Matušová D, Gluštíková P, Špaglová M, Krchňák D. Evaluation of properties of dexamethasone eye drops [published online as ahead of print on August 24, 2023]. *Eur Pharm J*. 2023. doi:10.2478/afpuc-2023-0009
5. Mandal A, Bisht R, Rupenthal ID, Mitra AK. Polymeric micelles for ocular drug delivery: From structural frameworks to recent preclinical studies. *J Control Release*. 2017;248:96–116. doi:10.1016/j.jconrel.2017.01.012
6. Ibrahim SS. The role of surface active agents in ophthalmic drug delivery: A comprehensive review. *J Pharm Sci*. 2019;108(6):1923–1933. doi:10.1016/j.xphs.2019.01.016
7. Van Santvliet L, Ludwig A. Determinants of eye drop size. *Surv Ophthalmol*. 2004;49(2):197–213. doi:10.1016/j.survophthal.2003.12.009
8. Lee BB, Chan ES, Ravindra P, Khan TA. Surface tension of viscous biopolymer solutions measured using the du Noüy ring method and the drop weight methods. *Polym Bull*. 2012;69(4):471–489. doi:10.1007/s00289-012-0782-2
9. Gupta B, Mishra V, Gharat S, Momin M, Omri A. Cellulosic polymers for enhancing drug bioavailability in ocular drug delivery systems. *Pharmaceuticals (Basel)*. 2021;14(11):1201. doi:10.3390/ph14111201
10. Manglik RM, Wasekar VM, Zhang J. Dynamic and equilibrium surface tension of aqueous surfactant and polymeric solutions. *Exp Therm Fluid Sci*. 2001;25(1–2):55–64. doi:10.1016/S0894-1777(01)00060-7
11. Nasatto P, Pignon F, Silveira J, Duarte M, Noseda M, Rinaudo M. Methylcellulose, a cellulose derivative with original physical properties and extended applications. *Polymers (Basel)*. 2015;7(5):777–803. doi:10.3390/polym7050777
12. Chen H, Qin J, Hu Y. Efficient degradation of high-molecular-weight hyaluronic acid by a combination of ultrasound, hydrogen peroxide, and copper ion. *Molecules*. 2019;24(3):617. doi:10.3390/molecules24030617
13. Al-Sibani M, Al-Harrasi M, Neubert R. Characterization of linear and chemically cross-linked hyaluronic acid using various analytical techniques including FTIR, ESI-MS, H1 NMR, and SEM. *J Biochem Anal Stud*. 2018;3(1). doi:10.16966/2576-5833.115
14. Sionkowska A, Kaczmarek B, Michalska M, Lewandowska K, Grabska S. Preparation and characterization of collagen/chitosan/hyaluronic acid thin films for application in hair care cosmetics. *Pure Appl Chem*. 2017;89(12):1829–1839. doi:10.1515/pac-2017-0314
15. Snetkov P, Zakharova K, Morozkina S, Olekhnovich R, Uspenskaya M. Hyaluronic acid: The influence of molecular weight on structural, physical, physico-chemical, and degradable properties of biopolymer. *Polymers (Basel)*. 2020;12(8):1800. doi:10.3390/polym12081800
16. Di Mola A, Landi MR, Massa A, D'Amora U, Guarino V. Hyaluronic acid in biomedical fields: New trends from chemistry to biomaterial applications. *Int J Mol Sci*. 2022;23(22):14372. doi:10.3390/ijms232214372
17. Smith P, Ziolek RM, Gazzarrini E, Owen DM, Lorenz CD. On the interaction of hyaluronic acid with synovial fluid lipid membranes. *Phys Chem Chem Phys*. 2019;21(19):9845–9857. doi:10.1039/C9CP01532A
18. Szewczyk E, Karlowicz-Bodalska K, Han S, Musial W. Influence of liquid paraffin, white soft paraffin and initial hydration on viscosity of corticosteroid cream. *Trop J Pharm Res*. 2014;13(8):1233. doi:10.4314/tjpr.v13i8.6
19. Li X, Tian J, Mulero A. Empirical correlation of the surface tension versus the viscosity for saturated normal liquids. *Fluid Phase Equilibria*. 2013;352:54–63. doi:10.1016/j.fluid.2013.05.003
20. Vaziri N, Khordad R, Rezaei G. Effect of potential attraction term on surface tension of ionic liquids. *Phys B Condens Matter*. 2018;533:50–57. doi:10.1016/j.physb.2017.12.068

Drug delivery applications and future prospects of microbial exopolysaccharides

Tioluwani Ibukun Adegbolagun[1,B,D–E], Olubusola Ayoola Odeniyi[2,A,D–F], Michael Ayodele Odeniyi[1,A,D–F]

[1] Department of Pharmaceutics & Industrial Pharmacy, University of Ibadan, Nigeria
[2] Department of Microbiology, University of Ibadan, Nigeria

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Polymers in Medicine, ISSN 0370-0747 (print), ISSN 2451-2699 (online) *Polim Med*. 2023;53(2):117–127

Address for correspondence
Michael Ayodele Odeniyi
E-mail: deleodeniyi@gmail.com

Funding sources
None declared

Conflict of interest
None declared

Received on October 20, 2023
Reviewed on December 1, 2023
Accepted on December 8, 2023

Published online on December 19, 2023

Abstract

Over the years, exopolysaccharides (EPSs) have been utilized in various areas of research, including health, industry, environment, and agriculture, due to their flexible physical, chemical and structural properties that can be readily modified to suit desired purposes. Current research trends have shown that EPS production is dependent on numerous factors which can be combined to varying extent to optimize production yields. Although the majority of research is directed towards their industrial and medicinal uses, these chemical substances possess peculiar characteristics which are also exploited for biomedical research, where they are being used as drug delivery systems, some of which include their abundance in nature, biocompatibility, biodegradability, non-toxicity, and ability to efficiently encapsulate sensitive bioactive agents. However, despite the numerous beneficial prospects of microbial EPSs in drug delivery, there are limitations to the commercial production and industrial applications of these biopolymers. These limitations have inspired revolutionary research into the cost-effective production of safe EPSs polymers. In this review, we classify EPSs and discuss their methods of extraction and characterization. We also summarized current drug delivery applications and discussed limitations to extensive industrial commercialization of EPSs, while highlighting prospects for the utilization of microbial EPSs and implications for research.

Key words: exopolysaccharides, drug delivery, biopolymers

Cite as
Adegbolagun TI, Odeniyi OA, Odeniyi MA. Drug delivery applications and future prospects of microbial exopolysaccharides. *Polim Med*. 2023;53(2):117–127. doi:10.17219/pim/176590

DOI
10.17219/pim/176590

Introduction

Exopolysaccharides

Exopolysaccharides (EPS) are extracellular metabolic by-products excreted by bacteria, yeast, fungi, etc.1 They are high-molecular-weight polymeric biomolecules with glycosidic linkages connecting monosaccharide units that are synthesized by microbial cells and secreted to the outside of the cells, where they perform beneficial functions, including protection from extreme pH and temperature.[2] Exopolysaccharides are crucial components that determine the structural and functional integrity of biofilms, which provide microorganisms with protection from harsh environments.[3,4] For example, xanthan, alginate and cellulose form a biofilm on bacterial cell surfaces that serves as a protective cover.[5] Along with these biological functions, EPSs have distinct chemical and physical properties that make them suitable industrial raw materials.[6]

In agriculture, EPSs have been used to improve the flow of pesticides and uniformly disperse solid components in formulations; the improved rheology of the formulation also ensures surface cling.[7] Exopolysaccharides are also used as gelling agents for culture media, paints and ink, and detergents.

Pharmacologically, EPSs have shown peculiar biological bioactivities which have found therapeutic applications in animal and human medicine.[8] These activities have demonstrated potential for use in prophylactic and antibiotic therapies. Some of them include; anti-inflammatory activities, antimicrobial and anti-tumor activities, hypocholesterolemic activities, immunomodulatory activities, and antidiabetic activities.[9,10] Through technological advancement in immunological studies, EPSs have been used to develop capsular polysaccharide-based vaccines for infectious illnesses.[11] Microbial EPSs have been extensively studied for their applications in vaccine development. They show prospects as antigen delivery systems and as antigenic materials (as adjuvants) to provide more robust immune responses.[12]

Due to their non-toxic nature, EPSs are being used to refashion international treatment models by modifying drug therapy, surgical therapy and disease diagnosis; this forms the basis of their biomedical applications.[13] They are versatile biomedical applications because they have flexible physical, chemical and structural properties that can be readily modified to suit desired purposes. Furthermore, they have published and standardized methods of fermentation and isolation, which makes them suitable to meet global demands. As a result, EPSs have found numerous applications in tissue engineering and drug delivery. They have also been used as surgical sealants and coating materials for medical devices.[14]

The most notable biomedical application of EPSs is in the delivery of sensitive drug molecules and biomolecules. Exopolysaccharides have shown potential as drug delivery materials due to their capacity to effectively entrap bioactive agents. Through this application, they are used to improve the therapeutic efficacy of drugs and increase the drug shelf life and duration of pharmacological action.[14,15] The versatile applications of EPSs in drug delivery applications are owed to their abundance in nature, biocompatibility, biodegradability, and non-toxicity.[1]

Classifications and structure of exopolysaccharides

Due to differences in their chemical components which influence their physical, biological, chemical and functional properties, EPSs are a diverse and complex class of biomolecules.[6] Based on their chemical composition, EPSs are primarily categorized according to their monomeric content into homopolysaccharides (HoPs), which contain one type of sugar monomer unit, and (HePs) which have several recurring subunits of different sugars from disaccharides to heptasaccharides, monosaccharide derivatives and/or substitutes.[16,17] This classification is summarized in Fig. 1.

To make further distinctions within the major categorizations, EPSs are subclassified based on the nature of their monomeric units, linkages and the degree of branching.[16]

Homopolysaccharides

Homopolysaccharides are identified based on monosaccharide units and classified as glucans, fructans and galactans, according to the presence of sugars (D-glucose, D-fructose and D-galactose) in their backbones.[17,18] Glucans exhibit varying degrees of branching and monomeric linkages; they are further classified into 1) α-D-glucans (alternan, reuteran, dextran, and mutan), where D-glucose residues are linearly or non-linearly interlinked by α-l-2, α-l-3, α-l-4 and/or α-l-6 glycosidic bonds; and 2) β-D-glucans (cellulose and curdlan), comprising of D-glucose connected by β-l-3 bonds and β-l-2 branching.[6] Due to these different linkage patterns, glucans exhibit varying degrees of water solubility and viscosity. Fructans are water-soluble and are grouped into 1) levan with β-2-6 bonds and 2) inulin with β-2-1 bonds.[19] Galactans, although less abundant, are also water-soluble and consist of α-1-6 linked galactose monomers.[17]

Heteropolysaccharides

Heteropolysaccharides comprise repeating sugar units at varying ratios which may include D-xylose, D-ribose and D-arabinose, which are pentose sugars, and D-galactose, D-mannose and D-glucose, which are hexose sugars. The HePs also include uronic acids such as D-galacturonic acids and N-glucuronic acid. Other HePs are acetylated monosaccharide units, including N-acetyl-glucosamine and N-acetyl-galactosamine.[20,21] They may also be branched or unbranched, and contain non-sugar substituents (organic or inorganic) such as phosphate, acetyl and glycerol.[22]

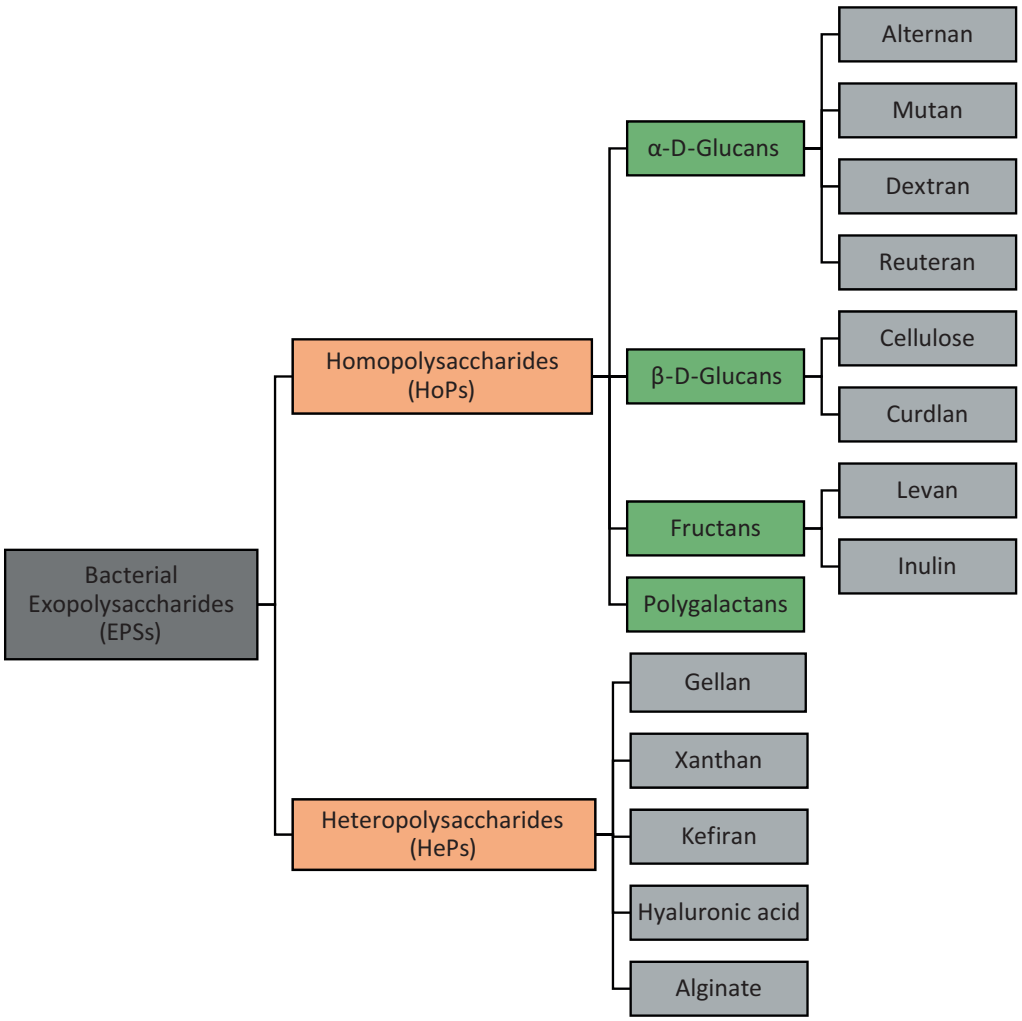

Fig. 1. Classifications and examples of bacterial exopolysaccharides[14]

The linkages between monomeric units in polymers are rigid 1,2-α- or 1,6-α- and 1,4-β- or 1,3-β- linkages, which are more flexible.[16] Examples include gellan, xanthan, kefiran, hyaluronic acid, and alginate.[1,18]

Exopolysaccharides can also be identified by their degree of association with the microbial cell wall and where their synthesis occurs. Based on the degree of attachment, they can be broadly grouped as: 1) capsular EPSs, which are firmly attached to the cell surface, creating a resistance mechanism against attacks from phagocytes and bacteriophages, and protection against excessive water loss and osmotic stress; and 2) slime EPSs, which are loosely associated with the cell surface and can be easily isolated. Depending on the location of synthesis, extracellular EPSs are produced outside the cell envelope, whereas intracellular polysaccharides are accumulated inside the cells and released outside the cells.[1,23]

Microorganisms producing exopolysaccharides

Microorganisms that produce EPSs are abundant in nature and have been identified in numerous environments in the ecosystem, especially those with increased carbon–nitrogen ratios.[24,25] Exopolysaccharides can therefore be extracted from bacterial, fungal and archaebacterial sources (such as algae).[13,26]

Some bacteria genera that produce EPSs are *Acetobacter* (cellulose), *Agrobacterium* (curdlan), *Bacillus* (levan), *Brenneria, Geobacillus, Gluconacetobacter* (cellulose, levan), *Halomonas* (levan), *Lactobacillus* (dextran, kefiran), *Rhizobium* (curdlan), *Saccharomyces, Sarcina, Pseudomonas* (alginate, gellan, cellulose, gellan), *Streptococcus* (levan, hyaluronic acid), *Xanthomonas* (xanthan), *and Zymomonas* (levan).[18,27] Some probiotic bacteria, such as *Lactobacillus* (dextran), *Leuconostoc* (dextran), *Lactococcus, Bifidobacterium* (hyaluronic acid), *Streptococcus* (levan, hyaluronic acid), *and Enterococcus* have also been used to synthesize EPSs for numerous medical and biomedical applications.[1,14] Other bacterial sources include marine bacteria such as *Vibrio diabolicus* (hyaluronian-like EPS) and *Arthrospira platensis*, and extremophiles such as *Bacillus thermantarcticus* and *Geobacillu sthermodenitrifican*.[13,16]

Due to their cellular and structural complexity, fungi are considered to be major non-bacterial sources of EPSs. Major fungi from which EPSs are isolated include *Aspergillus fumigatus* (galactosaminogalactan), *Candida albicans*

(chitosan, chitin) and *Zygosaccharomyces rouxii* (chitosan, chitin). Other examples of EPS-producing fungi are *Botryosphaeria* sp. (botryosphaeran), *Pleurotus ostreatus* (pleuran), *Aureobasidium pullulans* (pullulan), *Schizophyllum commun* (schizophyllan), *Phellinus linteus, Ganoderma lucidium*, *Fusarium* sp., and *Inonotus obliquus*.[13] Exopolysaccharides have also been extracted from surface biofilms of archaea that grow at extreme temperatures and salt concentrations: *Thermococcus, Sulfolobus, Archaeglobus fulgidus*, and *Thermococcus litoralis*.[28]

Production of exopolysaccharides

Biosynthesis of exopolysaccharides

Among various species of EPS-producing microorganisms, the biosynthetic pathways involved in EPS biosynthesis are notably similar, regardless of their different characteristics.[6] In bacteria, the synthesis of EPS occurs through intricate pathways that consist of specialized enzymes, carriers and transporter proteins[29]: They are the intracellular and extracellular pathways.[1] Depending on their natures, bacteria EPSs can either be produced entirely outside by extracellular bacterial enzymes, or synthesized inside the cells and released into the cell surrounding.[30]

Through the intracellular biosynthetic pathway, extracellular sugar residues are taken into the cells, broken down into various monomers, polymerized and transported out of the cells through a membrane-bound lipid carrier protein.[31] The extracellular biosynthetic pathway occurs outside the cell when sugar molecules are lysed into uniform monomeric units which are assembled, with glycosyl transferases enzymes or fructosyl transferases, into polymers that are secreted into the surrounding environment.[1,19] Although the extracellular pathway is majorly utilized in the production of HoPs, either of both biosynthetic pathways are involved in the synthesis of HePs.[32,33]

In general, there are 4 mechanisms involved in the biosynthesis of EPSs in bacterial cells[14]: the Wzx/Wzy- dependent pathway; the ABC transporter-dependent pathway; the synthase-dependent pathway; and extracellular biosynthesis by sucrase protein.[34–36] These mechanisms are illustrated in Fig. 2.

1. Wzy/Wzy-dependent pathway: At the inner membrane, imported monomeric sugar molecules are converted into sugar nucleotides and linked to a lipid carrier.[37] Via the addition of more sugar units by glycosyltransferases, the sugar chain is extended and transported, as an oligosaccharide unit, through the membrane by Wzx flippase.[38] The unit is them enzymatically modified, and the Wzy protein polymerizes the altered oligosaccharide into polysaccharides, which are released into the cell exterior by outer membrane polysaccharide transporters.[14,39]

2. ABC transporter dependent pathway: This pathway is employed in the synthesis of capsular EPSs[40] and it is similar to the Wzy/Wzy-pathway but excludes the activities

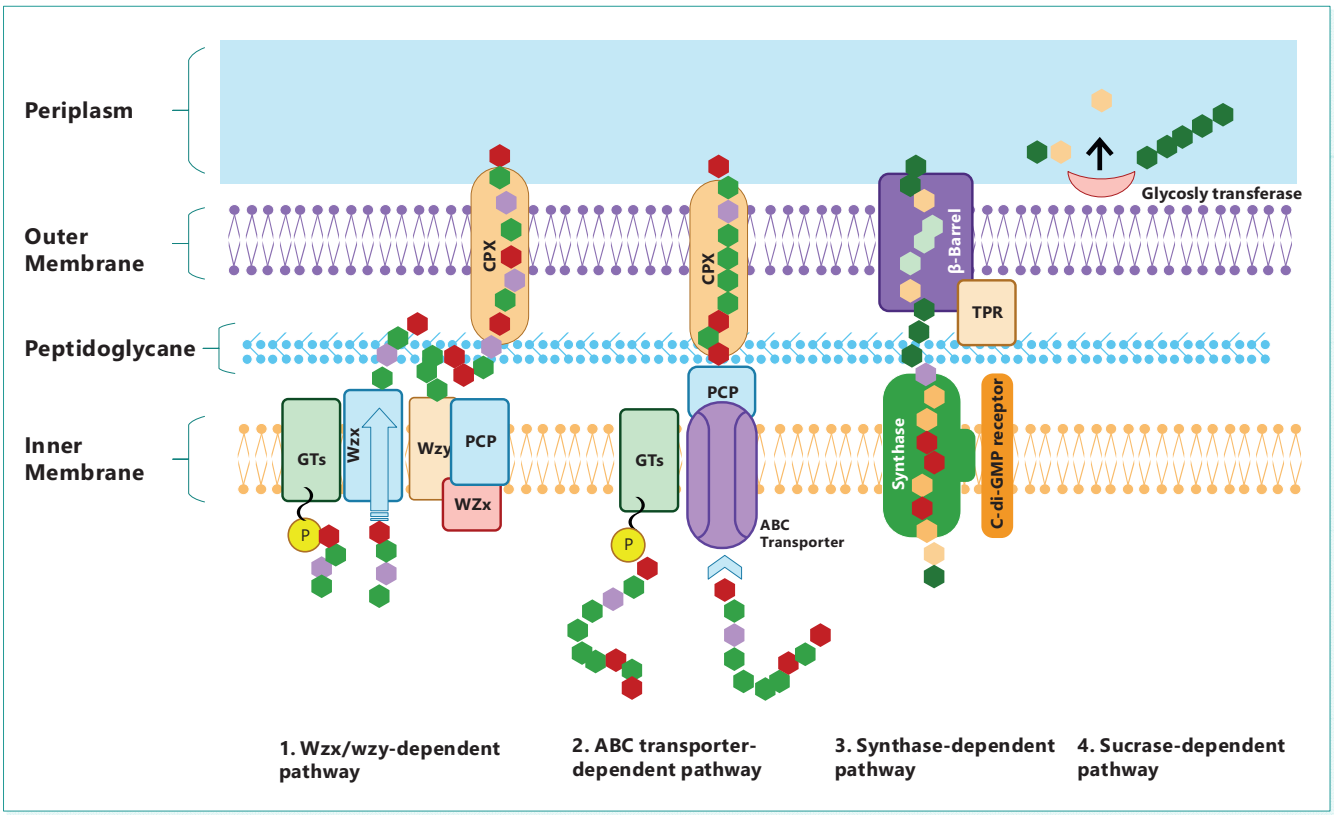

Fig. 2. Four major biosynthetic pathways of bacterial exopolysaccharides[14]

PCP – polysaccharide co-polymerase; OPX – outer membrane polysaccharide export; TPR – tetratricopeptide repeat protein; GTs – glycosyltransferase.

Table 1. Optimized exopolysaccharide production medium and incubation conditions for some of the microorganisms and the methods of optimization employed

Microorganism	Production medium	Incubation condition			Method of optimization	Reference
		temp. [°C]	pH	time		
Alkalibacillus sp. *w3*	Horikoshi I broth media	35	10	7 days	OFAT PBD	44
Lactobacillus acidophilus	MRS	30	6	48 h	PBD BBD	45
Lactobacillus plantarum R301	MRS medium	27	7.4	24 h	OFAT PBD CCD	46
Enterobacter cloacae MBB8	modified minimal medium	28	7.2	24 h	PBD	48
Tuber borchii	yeast extract medium	25	7	28 days	OFAT	49
Fusarium proliferatum	malt extract broth	25	6	9 days	PBD	50
Pseudomonas aeruginosa MTCC 1688	sterile nutrient broth medium	32	6	96 h	OFAT	51

OFAT – one-factor-at-a-time; PBD – Plackett-Burman design; BBD – Box-Behnken design; CCD – central composite design; MRS – De Man–Rogosa–Sharpe medium.

of Wzx flippase and Wzy protein. Polysaccharide chains synthesized by glycosyltransferases from nucleotide sugar units are transported across the inner membrane via a tripartite efflux pump complex – the (ATP)-binding cassette (ABC) transporter.[41]

3. Synthase-dependent pathway: In this pathway, same-type monosaccharide units are arranged into undecaprenyl phosphate glucose units by synthase protein located in the membrane.[42]

4. Extracellular synthesis by sucrase enzyme: This extracellular pathway occurs in the periplasm where sucrose is converted, outside the outer membrane, into its monosaccharide components (glucose or fructose) by sucrase enzymes. The monomer units are polymerized (with varying degrees of branching) by glycosyltransferases to form glucans or fructans – glucose and fructose units, respectively.[14,43]

Heteropolysaccharides are mainly produced through the ABC transporter-dependent and Wzx/Wzy-dependent pathways, while HoPs – via the extracellular production and synthase-based pathways.[1]

Fermentation of microorganisms: Production media and incubation conditions

The quality and quantity of EPSs are determined by several factors; bacteria strains, nutrient level and makeup of the growth/production media (salt concentration and the carbon:nitrogen composition) and incubation conditions (such as time, pH and temperature).[14,44] Consequently, EPS production is subject to numerous non-microbial factors which can be combined and permutated to varying degrees to achieve different production yields and characteristics of EPSs. This complicates the selection process of production media and incubation conditions for EPS production, inspiring the research into predicting the best combinations and arrangement of culture conditions (variables) to maximize production yields using one-factor-at-a-time (OFAT) optimizations and statistical experiment designs such as response surface methodology (RSM).[44,45] The OFAT optimizations involve testing each variable, one at a time while holding other variables constant, to determine which variable has the most effect on EPS production. The RSM statistically analyzes several independent variables to predict their relationship with each other and their effects on production yield[45]; it is being extensively employed by researchers to optimize the production of EPSs in microbes using experimental designs including Plackett–Burman Design (PBD), Box–Behnken Design (BBD) and Central Composite Design (CCD).[46,47] Table 1 summarizes some findings of recent research works on the optimization of production factors to improve EPS production from microbial sources.[44–46,48–51]

Extraction and recovery of exopolysaccharides

The recovery process of EPSs includes concentration, isolation and purification. After EPSs are concentrated in the fermentation broths, they are isolated from microorganisms by centrifugation.[45] More extreme methods are boiling in saline solution, sonication and autoclaving. Isolated EPSs are mostly extracted from isolated mixtures by solvent precipitation via the addition of a polar organic (such as ethanol) that reduces the solubility of EPSs in water.[45,46] Desalting and deproteination treatments may be further carried out to purify precipitated polysaccharides. The resultant precipitate is collected by centrifugation, filtration or sedimentation and dried under pressure.[24,25]

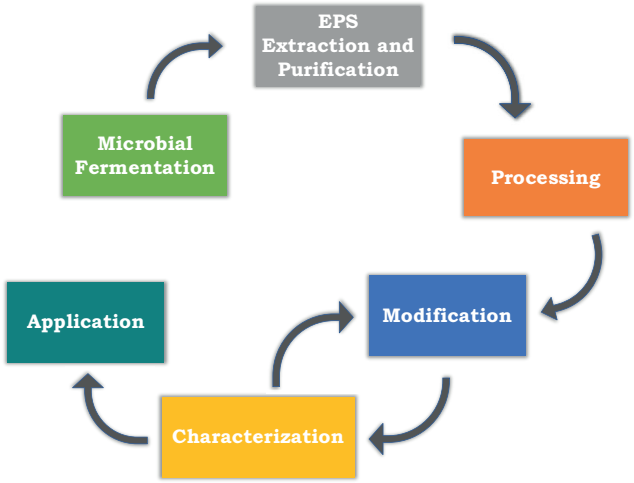

Fig. 3. Schematic process flowchart of microbial exopolysaccharides

To further separate sugars and minute proteins, the collected precipitate may undergo membrane filtration after dissolution in water. The filtrate is then freeze-dried to get the EPS powder which is packaged for use. [17,52,53] Anion exchange chromatography and size exclusion anion exchange are additional steps of purification employed in advance separation processes.[54,55]

Oftentimes, extracted EPSs are utilized in the same form as they are extracted (native form). They may also be physically or chemically treated before their use.[24] These treatments are applied as a means to modify the characteristics of these polysaccharides, thereby optimizing their suitability for pharmaceutical, food and biomedical purposes (Fig. 3).

Characterization of exopolysaccharides

Extensive characterization of extracted EPSs requires the evaluation of monosaccharide content, ring formations, extent of branching, and molecular weight. However, because these parameters cannot be accurately evaluated with singular methods, multiple methods and techniques are employed in combination.[6] Some of these parameters and respective determination methods are summarized in Table 2.[34,44,45,48,49,56,57]

Drug delivery applications of exopolysaccharides

The EPSs have shown potential as drug delivery materials for the delivery of sensitive drug molecules and biomolecules due to their bioactive function and capacity to hold bioactive agents effectively. Although they have similar functions as drug delivery systems, they are easier to apply as drug carriers than to produce as biological scaffolds of viable cells. This is because they can be readily modified to improve therapeutic efficacy by controlling and targeting the release of drugs in body tissues and protecting drug molecules from harsh physical and biological environments to increase the shelf life and duration of pharmacological action.[14,15] In addition to their abundance in nature, biocompatibility, biodegradability and non-toxicity are the main factors responsible for the ubiquitous drug delivery applications of EPSs.[1]

Nano- and micro-delivery systems

The development of nano- and microparticles is arguably the most significant application of EPSs in the delivery of drugs. The benefits of these include increased efficacy and decreased toxicity of encapsulated drugs. Levan nanoparticles exhibit decreased intestinal toxicity of selenium, iron and cobalt.[58] Serum bovine encapsulated by Sezer et al. exhibited zero-order drug release kinetics.[59] Hydrogel nanoparticles of curdlan derivatives have shown potential in the delivery of anticancer drugs.[60] Because EPSs are bio-compatible and non-toxic, they offer more benefits in the green synthesis of silver nanoparticles compared to synthetic polymers.[61]

Transdermal drug delivery systems

Exopolysaccharides are also utilized in the development of transdermal drug delivery systems (TDDS). Researchers postulate hyaluronic acid is the most commonly used marine EPS drug delivery via the transdermal route.[62] Alginate is also frequently applied in TDDS to prepare microneedles (which have been used to deliver vaccines, bovine serum

Table 2. Some of the parameters used to characterize exopolysaccharides and methods of determination

Parameter	Determination methods	Reference
Molecular weight	gel permeation chromatography ion exclusion chromatography	49 56
Monosaccharide composition	high-performance liquid chromatography-refractive index high-performance ion exchange chromatography-pulse amperometry detection gas chromatography-mass spectrometry	34 45 48
Bond types and chemical groups	Fourier transform infrared spectroscopy X-ray diffraction	44 49
Glycosidic linkages and ring and anomeric formation	nuclear magnetic resonance	57
Extraction yield	phenol-sulfuric acid method gravimetric analysis	44 45

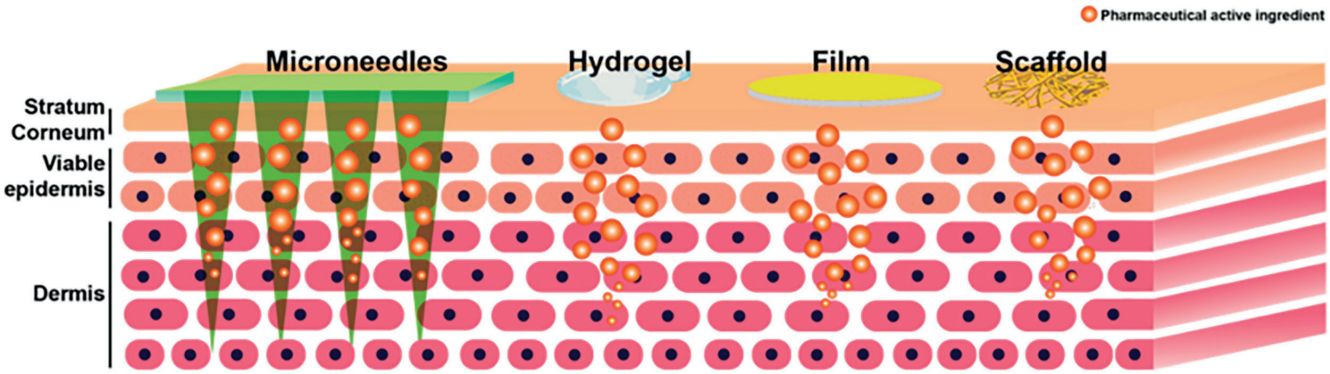

Fig. 4. Four types of recent transdermal drug delivery vehicles[66]

proteins and insulin) and nanoparticles (in combination with chitosan) for target antibiotic therapy of cutaneous pathogens.[63–65] Transdermal drug delivery systems made with EPSs are constantly evolving. Recent TDDSs which are illustrated in Fig. 4 vehicles include[66]:

1. Hydrogels: These are hydrophilic, polymeric networks which are 3-dimensional and have large water-holding capacities. A notable use is "smart" hydrogels that regulate molecule release by responding to changes in external stimuli.[66]
2. Films: These are thought to be a good substitute for topical patches and traditional topical dosage forms due to their flexibility, transparency, non-occlusivity, and ease of use because they are non-invasive. They also demonstrate higher drug loading capacity and prolonged drug release and drug retention on the skin.[67,68] The film-forming ability of EPSs makes them particularly suitable candidates in the formulation of these delivery vehicles.
3. Microneedles: These are efficient, minimally invasive vehicles of transdermal drug delivery, which typically create micro-channels through the layers of the skin and release the drugs, thereby localizing drug delivery and drug effects.[69] Biodegradable polymers such as hyaluronic acid, alginate and cellulose are more beneficial as microneedle components than silicon and metals because they are non-biodegradable.[70]
4. Tissue scaffolds: Polysaccharides are used to enhance the mechanism of drug release and improve the performance of tissue scaffolds. Hydrogels are frequently used as tissue engineering scaffolds, and those based on alginate exhibit better mechanical strength and controlled drug release.[71]

Modifications to improve drug delivery

Bacterial EPSs can be modified in a variety of ways to customize drug delivery properties and enhance the functionality of the delivery systems. Altering the side chain and functional group composition via chemical reactions of cross-linking reactions is one way of modification.[72] Partial sulfation of curdlan at the O-6 portion improves the aqueous solubility of curdlan hydrogel nanoparticles; however, the extent of substitution affects the thermal stability and ability to cross-link with polycytidylic acid.[60,73] Exopolysaccharides are also combined with other polysaccharides, biopolymers or synthetic polymers to optimize desirable material characteristics of these polymers and improve upon their limitations. This combination results in polymer blends with high-performance characteristics capable of being tailored toward various novel drug delivery applications. A study by Inoue et al. demonstrated the ability of cellulose membrane to increase the release of chlorhexidine (which complexed with β-cyclodextrin) by 10-fold; this was postulated to be a result of rigid chemical interactions between cellulose and the chlorhexidine-β-cyclodextrin complex.[74] In vitro studies of ciprofloxacin microspheres demonstrated adequate gastric protection of ciprofloxacin by kefiran-alginate polymer blends.[75] Similarly, xanthan hydrogels modified with succinic anhydride extended the release of gentamycin over 9 days.[76]

Future prospects for the use of microbial exopolysaccharides

The spectrum of future EPS applications is as wide as the extent to which their functionality can be improved. Consequently, EPS-based drug delivery systems show promise in revolutionizing the paradigm of drug delivery.[12] Diverse works of research have been dedicated to unearthing the untapped drug delivery potentials of EPSs.

Temperature- and pH-sensitive formulations

In transdermal delivery systems, they are capable of being modified with nanotechnology to prepare "smart" formulations which release entrapped drug molecules in response to temperature and pH changes at target delivery sites.[66] Skin conditions induced by pH disparity have been treated with isoliquiritigenin-loaded hydroxyethyl cellulose – hyaluronic acid pH-sensitive hydrogels.[77] In combination, hydrogels sensitive to temperature and pH have been developed; gallic acid loaded within Pluronic® F-127 bi-responsive hydrogels of chitosan oligosaccharide lactate and hyaluronic acid nano-conjugates have been used to resolve atopic dermatitis.[78] In addition to the potential of improved clinical therapy in preventing infections and

improving wound healing, these "smart" formulations have the benefit of being cost-effective.[66]

Simultaneously, in systemic delivery systems, EPSs show promise as substrates for "Intelligent Drug Delivery Systems".[79] The fungal EPS pullulan which, due to its recurring hydroxyl groups, is especially amenable to derivatization through chemical means, has been grafted on the backbones of poly-(N-isopropyl-acrylamide)-co-acrylamide and succinic carboxyl ether groups in modulating the release of lysozyme protein using pH and temperature.[80] Similarly, carboxy-methyl pullulan modified with polyetheramine has demonstrated sol–gel transition at physiological temperature, providing assurance for thermally regulated sustained drug delivery.81

From current research in drug delivery, microbial EPSs show potential for application in targeted gastrointestinal delivery to the intestines. A typically useful feature of EPSs is their swelling and shrinking tendencies in response to changes in their environment; swelling increases the porosity of the polymers, thus triggering the release of contained drugs. Due to this sensitivity, restriction of drug release to the upper section of the small intestine is possible. This is because the majority of EPSs that are sensitive to pH shrink in mediums with low pH and swell in environments with high pH.[82] Therefore, such oral delivery systems withhold the release of the drugs till the upper intestine, where swelling occurs due to the high pH.[83] Exopolysaccharides are also being modified to improve target drug release even in an acidic medium. Similarly, non-starch EPSs that are highly stable and resistant to digestion all through the gastrointestinal tract (GIT) remain intact till the colon, where the colonic bacterial flora degrades them. This implies a possibility for target drug delivery to the colon.[84]

Interpenetrating polymer network

Exopolysaccharide hydrogels may also have the potential for molecular-scale penetration and drug delivery.[12] This is demonstrated by the entrapment and the extended-release properties shown by the interpenetrating polymer network (IPN) of alginate and polymers with an anti-inflammatory agent (indomethacin), an antibiotic (gatifloxacin), anticancer agent (5-fluorouracil), and an anticoagulant (heparin).[85] Steroid hormones are bioactive agents that stand to benefit significantly from this potential, as they are hardly mentioned in recent EPS research. Micro- and nano-transdermal delivery systems can bypass the side effects associated with their oral administration to improve their therapeutic efficacy.[12]

Discussion

Despite the numerous beneficial prospects of microbial EPSs and their composites in drug delivery, there are limitations to the commercial production and industrial applications. The most apparent of these limitations is the high cost associated with production and extraction, which is non-commensurate with their commercial value.[16] This is consequent to the complexities of their cost-intensive fermentation, isolation, purification, and characterization processes; reconciliation of these with the often-minimal production output of EPSs shows the poor economic feasibility and sustainability of large-scale EPS production. Another limitation is the relative paucity of knowledge about the interconnection between structure and biological functions.[9] This is particularly complicated for bacterial EPS by the realization that the structure and biological effects of EPSs vary with every strain of bacteria.[14] There is also the concern of health risks associated with certain microbial sources. For instance, the most studied microbial source of alginate is *Pseudomonas aeruginosa*, a bacteria linked to numerous infections with severity ranging from mild to severe. Although genetically engineered *P. aeruginosa* strains that are not pathogenic have been developed by researchers such as Valentine et al.,[86] there is still a significant proportion of un-engineered microbial sources. Bacterial capsular EPSs have been found to possess surface antigens that elicit immunogenic responses. This contributes to the pathogenicity risks of microbial EPSs.[87] Therefore, it would not be quixotic to envision a revolution in the production processes of EPSs as a prospect of microbial EPSs. The main objective of the revolution would be to enhance commercialization by optimizing the production yield and quality of microbial EPSs while minimizing their cost of production. There should be more research studies on approaches to achieve this, such as methods to optimize industrial-scale fermentation and extraction processes,[14] as well as on developing methods of artificial chemical synthesis to aid the modification and enhancement of structural and functional properties of EPSs,[88] utilization of less expensive production materials (living and non-living), development of manipulation methods (mutagenesis, genetic and metabolic) of producing microbial strains with higher yields,[38] and less pathogenicity. All these will ultimately result in a significant improvement in the commercial value of these biomaterials and, consequently, their availability for various industrial and biomedical applications.

Specifically, these developments promise a significant impact in Africa by improving the current perspective on the utilization potentials of EPSs. A random preliminary study by Osemwegie et al. to evaluate the course of global research on EPSs over a period of 18 years was used to provide an overview of African research on EPS applications. The review showed that Africa ranked low among the continents with prominent research in microbial EPS research and application. These authors related the small number of publications to the low interest of African countries in the numerous industrial benefits of EPSs despite the possibility of Africa being one of the best repositories of EPSs in the world.[13]

Conclusions

As the commercial value and industrial applications of microbial EPSs increase, there is sure to be a corresponding paradigm shift in African research on the potential applications and benefits of these biomaterials.

ORCID iDs

Tioluwani Ibukun Adegbolagun https://orcid.org/ 0000-0002-9442-2639
Olubusola Ayoola Odeniyi https://orcid.org/0000-0002-0826-791X
Michael Ayodele Odeniyi https://orcid.org/0000-0002-9918-4377

References

1. Angelin J, Kavitha M. Exopolysaccharides from probiotic bacteria and their health potential. *Int J Biol Macromol*. 2020;162:853–865. doi:10.1016/j.ijbiomac.2020.06.190
2. Manjanna K, Shivakumar B, Pramodkumar T. Natural exopolysaccharides as novel excipients in drug delivery: A review. *Arch Appl Sci Res*. 2009;1(2):230–253. https://sphinxsai.com/sphinxsaiVol_2No.1/ChemTech_Vol_2No.1/ChemTech_Vol_2No.1PDF/CT=82%20(509-525).pdf. Accessed September 15, 2023
3. Flemming H, Wingender J, Griebe T, Mayer C. Physico-chemical properties of biofilms. In: Evans LV, ed. *Biofilms: Recent Advances in Their Study and Control*. Boca Raton, USA: CRC Press; 2000:19–34. doi:10.1201/9781482284157
4. Fulaz S, Vitale S, Quinn L, Casey E. Nanoparticle–biofilm interactions: The role of the EPS matrix. *Trends Microbiol*. 2019;27(11):915–926. doi:10.1016/j.tim.2019.07.004
5. Kodali VP, Das S, Sen R. An exopolysaccharide from a probiotic: Biosynthesis dynamics, composition and emulsifying activity. *Food Res Int*. 2009;42(5–6):695–699. doi:10.1016/j.foodres.2009.02.007
6. Jurášková D, Ribeiro SC, Silva CCG. Exopolysaccharides produced by actic acid bacteria: From biosynthesis to health-promoting properties. *Foods*. 2022;11(2):156. doi:10.3390/foods11020156
7. Majee S, Avlani D, Biswas G. Rheological behavior and pharmaceutical applications of bacterial exopolysaccharides. *J App Pharm Sci*. 2017;7(9):224–232. doi:10.7324/JAPS.2017.70931
8. Lima Santos F, Modesto De Amorim G. Biotechnological challenges and perspectives of using exopolysaccharides. *J Anal Pharm Sci*. 2018;7(3):264–266. doi:10.15406/japlr.2018.07.00236
9. Hussain A, Zia KM, Tabasum S, et al. Blends and composites of exopolysaccharides: Properties and applications. A review. *Int J Biol Macromol*. 2017;94:10–27. doi:10.1016/j.ijbiomac.2016.09.104
10. Patel S, Majumder A, Goyal A. Potentials of exopolysaccharides from lactic acid bacteria. *Indian J Microbiol*. 2012;52(1):3–12. doi:10.1007/s12088-011-0148-8
11. Flemming HC, Neu TR, Wozniak DJ. The EPS matrix: The "house of biofilm cells." *J Bacteriol*. 2007;189(22):7945–7947. doi:10.1128/JB.00858-07
12. Moscovici M. Present and future medical applications of microbial exopolysaccharides. *Front Microbiol*. 2015;6:1012. doi:10.3389/fmicb.2015.01012
13. Osemwegie OO, Adetunji CO, Ayeni EA, et al. Exopolysaccharides from bacteria and fungi: Current status and perspectives in Africa. *Heliyon*. 2020;6(6):e04205. doi:10.1016/j.heliyon.2020.e04205
14. Mohd Nadzir M, Nurhayati RW, Idris FN, Nguyen MH. Biomedical applications of bacterial exopolysaccharides: A review. *Polymers (Basel)*. 2021;13(4):530. doi:10.3390/polym13040530
15. Rahbar Saadat Y, Yari Khosroushahi A, Pourghassem Gargari B. A comprehensive review of anticancer, immunomodulatory and health beneficial effects of the lactic acid bacteria exopolysaccharides. *Carbohydr Polym*. 2019;217:79–89. doi:10.1016/j.carbpol.2019.04.025
16. Nwodo U, Green E, Okoh A. Bacterial exopolysaccharides: Functionality and prospects. *Int J Mol Sci*. 2012;13(12):14002–14015. doi:10.3390/ijms131114002
17. Torino MI, Font De Valdez G, Mozzi F. Biopolymers from lactic acid bacteria: Novel applications in foods and beverages. *Front Microbiol*. 2015;6:834. doi:10.3389/fmicb.2015.00834
18. Chaisuwan W, Jantanasakulwong K, Wangtueai S, et al. Microbial exopolysaccharides for immune enhancement: Fermentation, modifications and bioactivities. *Food Biosci*. 2020;35:100564. doi:10.1016/j.fbio.2020.100564
19. Zannini E, Waters DM, Coffey A, Arendt EK. Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. *Appl Microbiol Biotechnol*. 2016;100(3):1121–1135. doi:10.1007/s00253-015-7172-2
20. Daba GM, Elnahas MO, Elkhateeb WA. Contributions of exopolysaccharides from lactic acid bacteria as biotechnological tools in food, pharmaceutical, and medical applications. *Int J Biol Macromol*. 2021;173:79–89. doi:10.1016/j.ijbiomac.2021.01.110
21. Mozzi F, Vaningelgem F, Hébert EM, et al. Diversity of heteropolysaccharide-producing lactic acid bacterium strains and their biopolymers. *Appl Environ Microbiol*. 2006;72(6):4431–4435. doi:10.1128/AEM.02780-05
22. Ruas-Madiedo P, Hugenholtz J, Zoon P. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *Int Dairy J*. 2002;12(2–3):163–171. doi:10.1016/S0958-6946(01)00160-1
23. Ruas-Madiedo P, De Los Reyes-Gavilán CG. Invited review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *J Dairy Sci*. 2005;88(3):843–856. doi:10.3168/jds.S0022-0302(05)72750-8
24. Suresh Kumar A, Mody K, Jha B. Bacterial exopolysaccharides: A perception. *J Basic Microbiol*. 2007;47(2):103–117. doi:10.1002/jobm.200610203
25. Morin A. Screening of polysaccharide-producing microorganisms, factors influencing the production and recovery of microbial polysaccharides. In: Dumitriu S, ed. *Polysaccharides: Structural Diversity and Functional Versatility*. 2nd ed. Boca Raton, USA: CRC Press; 2004:275–296. doi:10.1201/9781420030822
26. Rodríguez-Carmona E, Villaverde A. Nanostructured bacterial materials for innovative medicines. *Trends Microbiol*. 2010;18(9):423–430. doi:10.1016/j.tim.2010.06.007
27. Yildiz H, Karatas N. Microbial exopolysaccharides: Resources and bioactive properties. *Process Biochem*. 2018;72:41–46. doi:10.1016/j.procbio.2018.06.009
28. Poli A, Di Donato P, Abbamondi GR, Nicolaus B. Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by archaea. *Archaea*. 2011;2011:693253. doi:10.1155/2011/693253
29. Patten DA, Laws AP. *Lactobacillus*-produced exopolysaccharides and their potential health benefits: Z review. *Beneficial Microbes*. 2015;6(4):457–471. doi:10.3920/BM2014.0117
30. Lynch KM, Coffey A, Arendt EK. Exopolysaccharide producing lactic acid bacteria: Their techno-functional role and potential application in gluten-free bread products. *Food Res Int*. 2018;110:52–61. doi:10.1016/j.foodres.2017.03.012
31. Madhuri K, Prabhakar K. Microbial exopolysaccharides: Biosynthesis and potential applications. *Orient J Chem*. 2014;30(3):1401–1410. doi:10.13005/ojc/300362
32. Yother J. Capsules of *Streptococcus pneumoniae* and other bacteria: Paradigms for polysaccharide biosynthesis and regulation. *Annu Rev Microbiol*. 2011;65(1):563–581. doi:10.1146/annurev.micro.62.081307.162944
33. Li J, Wang N. The *gpsX* gene encoding a glycosyltransferase is important for polysaccharide production and required for full virulence in *Xanthomonas citri* subsp. *citri*. *BMC Microbiol*. 2012;12(1):31. doi:10.1186/1471-2180-12-31
34. Rana S, Upadhyay LSB. Microbial exopolysaccharides: Synthesis pathways, types and their commercial applications. *Int J Biol Macromol*. 2020;157:577–583. doi:10.1016/j.ijbiomac.2020.04.084
35. Ates O. Systems biology of microbial exopolysaccharides production. *Front Bioeng Biotechnol*. 2015;3:200. doi:10.3389/fbioe.2015.00200
36. Schmid J. Recent insights in microbial exopolysaccharide biosynthesis and engineering strategies. *Curr Opin Biotechnol*. 2018;53:130–136. doi:10.1016/j.copbio.2018.01.005
37. De Vuyst L, De Vin F, Vaningelgem F, Degeest B. Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *Int Dairy J*. 2001;11(9):687–707. doi:10.1016/S0958-6946(01)00114-5

38. Rehm BHA. Bacterial polymers: Biosynthesis, modifications and applications. *Nat Rev Microbiol*. 2010;8(8):578–592. doi:10.1038/nrmicro2354
39. Islam ST, Lam JS. Synthesis of bacterial polysaccharides via the Wzx/Wzy-dependent pathway. *Can J Microbiol*. 2014;60(11):697–716. doi:10.1139/cjm-2014-0595
40. Huszczynski SM, Hao Y, Lam JS, Khursigara CM. Identification of the *Pseudomonas aeruginosa* O17 and O15 O-specific antigen biosynthesis loci reveals an ABC transporter-dependent synthesis pathway and mechanisms of genetic diversity. *J Bacteriol*. 2020;202(19):e00347-20. doi:10.1128/JB.00347-20
41. Cuthbertson L, Kos V, Whitfield C. ABC transporters involved in export of cell surface glycoconjugates. *Microbiol Mol Biol Rev*. 2010;74(3):341–362. doi:10.1128/MMBR.00009-10
42. Krasteva PV, Bernal-Bayard J, Travier L, et al. Insights into the structure and assembly of a bacterial cellulose secretion system. *Nat Commun*. 2017;8(1):2065. doi:10.1038/s41467-017-01523-2
43. Ua-Arak T, Jakob F, Vogel RF. Fermentation pH modulates the size distributions and functional properties of *Gluconobacter albidus* TMW 2.1191 levan. *Front Microbiol*. 2017;8:807. doi:10.3389/fmicb.2017.00807
44. Arayes MA, Mabrouk MEM, Sabry SA, Abdella B. Exopolysaccharide production from *Alkalibacillus* sp. *w3*: Statistical optimization and biological activity. *Biologia*. 2022;78(1):229–240. doi:10.1007/s11756-022-01233-1
45. Abo-Zaid GA, Kenawy AM, El-Deeb NM, Al-Madboly LA. Improvement and enhancement of oligosaccharide production from *Lactobacillus acidophilus* using statistical experimental designs and its inhibitory effect on colon cancer. *Microb Cell Fact*. 2023;22(1):148. doi:10.1186/s12934-023-02153-8
46. Wang J, Zhang J, Guo H, et al. Optimization of exopolysaccharide produced by *Lactobacillus plantarum* R301 and its antioxidant and anti-inflammatory activities. *Foods*. 2023;12(13):2481. doi:10.3390/foods12132481
47. Sharma S, Roy T, Kashyap Y, et al. Characterizing and demonstrating the role of *Klebsiella* SSN1 exopolysaccharide in osmotic stress tolerance using neutron radiography. *Sci Rep*. 2023;13(1):10052. doi:10.1038/s41598-023-37133-w
48. Prakash Shyam K, Rajkumar P, Ramya V, Sivabalan S, Kings AJ, Miriam LRM. Exopolysaccharide production by optimized medium using novel marine *Enterobacter cloacae* MBB8 isolate and its antioxidant potential. *Carbohydr Polym Technol Appl*. 2021;2:100070. doi:10.1016/j.carpta.2021.100070
49. Chen CC, Nargotra P, Kuo CH, Liu YC. High-molecular-weight exopolysaccharides production from *Tuber borchii* cultivated by submerged fermentation. *Int J Mol Sci*. 2023;24(5):4875. doi:10.3390/ijms24054875
50. Behera S, Kumari Panda A, Kumar Behera S, Gupta N. Media optimization, extraction, purification and characterization of exopolysaccharide from *Fusarium proliferatum*: A novel source bioactive polysaccharide. *Results Chem*. 2023;5:100923. doi:10.1016/j.rechem.2023.100923
51. Chug R, Mathur S, Kothari SL, Harish, Gour VS. Maximizing EPS production from *Pseudomonas aeruginosa* and its application in Cr and Ni sequestration. *Biochem Biophys Rep*. 2021;26:100972. doi:10.1016/j.bbrep.2021.100972
52. Petry S, Furlan S, Crepeau MJ, Cerning J, Desmazeaud M. Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. *Appl Environ Microbiol*. 2000;66(8):3427–3431. doi:10.1128/AEM.66.8.3427-3431.2000
53. Robitaille G, Moineau S, St-Gelais D, Vadeboncoeur C, Britten M. Detection and quantification of capsular exopolysaccharides from *Streptococcus thermophilus* using lectin probes. *J Dairy Sci*. 2006;89(11):4156–4162. doi:10.3168/jds.S0022-0302(06)72460-2
54. Barcelos MCS, Vespermann KAC, Pelissari FM, Molina G. Current status of biotechnological production and applications of microbial exopolysaccharides. *Crit Rev Food Sci Nutr*. 2020;60(9):1475–1495. doi:10.1080/10408398.2019.1575791
55. Wu J, Zhang Y, Ye L, Wang C. The anti-cancer effects and mechanisms of lactic acid bacteria exopolysaccharides in vitro: A review. *Carbohydr Polym*. 2021;253:117308. doi:10.1016/j.carbpol.2020.117308
56. Loeffler M, Hilbig J, Velasco L, Weiss J. Usage of in situ exopolysaccharide-forming lactic acid bacteria in food production. Meat products: A new field of application? *Comp Rev Food Sci Food Safe*. 2020;19(6):2932–2954. doi:10.1111/1541-4337.12615
57. Zhang G, Zhang W, Sun L, et al. Preparation screening, production optimization and characterization of exopolysaccharides produced by *Lactobacillus sanfranciscensis* Ls-1001 isolated from Chinese traditional sourdough. *Int J Biol Macromol*. 2019;139:1295–1303. doi:10.1016/j.ijbiomac.2019.08.077
58. Bondarenko OM, Ivask A, Kahru A, et al. Bacterial polysaccharide levan as stabilizing, non-toxic and functional coating material for microelement-nanoparticles. *Carbohydr Polym*. 2016;136:710–720. doi:10.1016/j.carbpol.2015.09.093
59. Sezer AD, Kazak H, Öner ET, Akbuğa J. Levan-based nanocarrier system for peptide and protein drug delivery: Optimization and influence of experimental parameters on the nanoparticle characteristics. *Carbohydr Polym*. 2011;84(1):358–363. doi:10.1016/j.carbpol.2010.11.046
60. Bhatia S. Microbial polysaccharides as advance nanomaterials. In: Bhatia S. *Systems for Drug Delivery*. Cham, Switzerland: Springer International Publishing; 2016:29–54. doi:10.1007/978-3-319-41926-8_2
61. Kanmani P, Lim ST. Synthesis and structural characterization of silver nanoparticles using bacterial exopolysaccharide and its antimicrobial activity against food and multidrug resistant pathogens. *Process Biochem*. 2013;48(7):1099–1106. doi:10.1016/j.procbio.2013.05.011
62. Kim KS, Kim H, Park Y, et al. Noninvasive transdermal vaccination using hyaluronan nanocarriers and laser adjuvant. *Adv Funct Mater*. 2016;26(15):2512–2522. doi:10.1002/adfm.201504879
63. Wu M, Zhang Y, Huang H, et al. Assisted 3D printing of microneedle patches for minimally invasive glucose control in diabetes. *Mater Sci Eng C Mater Biol Appl*. 2020;117:111299. doi:10.1016/j.msec.2020.111299
64. Arshad MS, Fatima S, Nazari K, et al. Engineering and characterisation of BCG-loaded polymeric microneedles. *J Drug Target*. 2020;28(5):525–532. doi:10.1080/1061186X.2019.1693577
65. Friedman AJ, Phan J, Schairer DO, et al. Antimicrobial and anti-inflammatory activity of chitosan–alginate nanoparticles: A targeted therapy for cutaneous pathogens. *J Invest Dermatol*. 2013;133(5):1231–1239. doi:10.1038/jid.2012.399
66. Li J, Xiang H, Zhang Q, Miao X. Polysaccharide-based transdermal drug delivery. *Pharmaceuticals (Basel)*. 2022;15(5):602. doi:10.3390/ph15050602
67. Chen C, Zhou P, Huang C, et al. Photothermal-promoted multi-functional dual network polysaccharide hydrogel adhesive for infected and susceptible wound healing. *Carbohydr Polym*. 2021;273:118557. doi:10.1016/j.carbpol.2021.118557
68. Tripathi K, Singh A. Chitin, chitosan and their pharmacological activities: A review. *Int J Pharm Sci Res*. 2018;9(7):2626–2635. doi:10.13040/IJPSR.0975-8232.9(7).2626-35
69. Serrano-Castañeda P, Escobar-Chavez JJ, Rodriguez-Cruz IM, Melgoza LM, Martinez-Hernandez J. Microneedles as enhancer of drug absorption through the skin and applications in medicine and cosmetology. *J Pharm Pharm Sci*. 2018;21:73–93. doi:10.18433/jpps29610
70. Shelke NB, James R, Laurencin CT, Kumbar SG. Polysaccharide biomaterials for drug delivery and regenerative engineering. *Polym Adv Technol*. 2014;25(5):448–460. doi:10.1002/pat.3266
71. Zare P, Pezeshki-Modaress M, Davachi SM, et al. Alginate sulfate-based hydrogel/nanofiber composite scaffold with controlled Kartogenin delivery for tissue engineering. *Carbohydr Polym*. 2021;266:118123. doi:10.1016/j.carbpol.2021.118123
72. Freitas F, Alves VD, Torres CAV, et al. Fucose-containing exopolysaccharide produced by the newly isolated *Enterobacter* strain A47 DSM 23139. *Carbohydr Polym*. 2011;83(1):159–165. doi:10.1016/j.carbpol.2010.07.034
73. Koumoto K, Umeda M, Numata M, et al. Low Mw sulfated curdlan with improved water solubility forms macromolecular complexes with polycytidylic acid. *Carbohydr Res*. 2004;339(1):161–167. doi:10.1016/j.carres.2003.09.022
74. Inoue BS, Streit S, Dos Santos Schneider AL, Meier MM. Bioactive bacterial cellulose membrane with prolonged release of chlorhexidine for dental medical application. *Int J Biol Macromol*. 2020;148:1098–1108. doi:10.1016/j.ijbiomac.2020.01.036
75. Blandón LM, Noseda MD, Islan GA, et al. Optimization of culture conditions for kefiran production in whey: The structural and biocidal properties of the resulting polysaccharide. *Bioact Carbohydr Diet Fibre*. 2018;16:14–21. doi:10.1016/j.bcdf.2018.02.001

76. Wang B, Han Y, Lin Q, et al. In vitro and in vivo evaluation of xanthan gum–succinic anhydride hydrogels for the ionic strength-sensitive release of antibacterial agents. *J Mater Chem B*. 2016;4(10):1853–1861. doi:10.1039/C5TB02046H
77. Kwon SS, Kong BJ, Park SN. Physicochemical properties of pH-sensitive hydrogels based on hydroxyethyl cellulose–hyaluronic acid and for applications as transdermal delivery systems for skin lesions. *Eur J Pharm Biopharm*. 2015;92:146–154. doi:10.1016/j.ejpb.2015.02.025
78. Chatterjee S, Hui PCL, Wat E, Kan CW, Leung PC, Wang W. Drug delivery system of dual-responsive PF127 hydrogel with polysaccharide-based nano-conjugate for textile-based transdermal therapy. *Carbohydr Polym*. 2020;236:116074. doi:10.1016/j.carbpol.2020.116074
79. Patel D, Patel D, Patel B, Patel C. An overview on intelligent drug delivery systems. *Int J Adv Pharm Rev*. 2011;2:57–63.
80. Fundueanu G, Constantin M, Ascenzi P. Preparation and characterization of pH- and temperature-sensitive pullulan microspheres for controlled release of drugs. *Biomaterials*. 2008;29(18):2767–2775. doi:10.1016/j.biomaterials.2008.03.025
81. Dulong V, Mocanu G, Picton L, Le Cerf D. Amphiphilic and thermosensitive copolymers based on pullulan and Jeffamine®: Synthesis, characterization and physicochemical properties. *Carbohydr Polym*. 2012;87(2):1522–1531. doi:10.1016/j.carbpol.2011.09.049
82. Andhare P, Kishor C, Margi D, Hilor P. Chapter 0311. Microbial exopolysaccharides: Advances in applications and future prospects. In: Tewari R, ed. *Biotechnology Volume 3: Microbial Biotechnology*. New Delhi, India: Studium Press LLC; 2014:25–51. doi:10.13140/RG.2.1.3518.4484
83. Manjanna K, Kumar T, Shivakumar B. Natural polysaccharide hydrogels as novel excipients for modified drug delivery systems: A review. *Int J Chemtech Res*. 2010;2(1):509–525. https://www.researchgate.net/profile/Dr-Manjanna-Km/publication/215538020_Natural_polysaccharide_hydrogels_as_novel_excipients_for_modified_drug_delivery_systems_A_review/links/551bf1960cf2909047b98045/Natural-polysaccharide-hydrogels-as-novel-excipients-for-modified-drug-delivery-systems-A-review.pdf. Accessed August 10, 2023.
84. Chavhan S, Shinde S, Sapkal S, Shrikhande V. Herbal excipients in novel drug delivery systems. *Asian J Pharm Res*. 2017;7(2):111. doi:10.5958/2231-5691.2017.00019.3
85. Matricardi P, Di Meo C, Coviello T, Hennink WE, Alhaique F. Interpenetrating Polymer Networks polysaccharide hydrogels for drug delivery and tissue engineering. *Adv Drug Deliv Rev*. 2013;65(9):1172–1187. doi:10.1016/j.addr.2013.04.002
86. Valentine ME, Kirby BD, Withers TR, et al. Generation of a highly attenuated strain of *Pseudomonas aeruginosa* for commercial production of alginate. *Microb Biotechnol*. 2020;13(1):162–175. doi:10.1111/1751-7915.13411
87. Looijesteijn PJ, Van Casteren WHM, Tuinier R, Doeswijk-Voragen CHL, Hugenholtz J. Influence of different substrate limitations on the yield, composition and molecular mass of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* in continuous cultures. *J Appl Microbiol*. 2000;89(1):116–122. doi:10.1046/j.1365-2672.2000.01082.x
88. Oerlemans MMP, Akkerman R, Ferrari M, Walvoort MTC, De Vos P. Benefits of bacteria-derived exopolysaccharides on gastrointestinal microbiota, immunity and health. *J Funct Foods*. 2021;76:104289. doi:10.1016/j.jff.2020.104289

Properties of scaffolds as carriers of mesenchymal stem cells for use in bone engineering

Właściwości membran jako nośników komórek macierzystych wykorzystywanych w inżynierii tkanki kostnej

Piotr Grzegorz Grelewski[A–F], Monika Kwaśnicka[B,D,F], Julia Krystyna Bar[A,D–F]

Department of Immunopathology and Molecular Biology, Wroclaw Medical University, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Polymers in Medicine, ISSN 0370-0747 (print), ISSN 2451-2699 (online) *Polim Med*. 2023;53(2):129–139

Address for correspondence
Piotr Grzegorz Grelewski
E-mail: piotr.grelewski@umw.edu.pl

Funding sources
None declared

Conflict of interest
None declared

Received on March 14, 2023
Reviewed on May 24, 2023
Accepted on May 24, 2023

Published online on June 29, 2023

Abstract

Tissue engineering has become one of the most studied medical fields and appears to be promising for the regeneration of injured bone tissues. Even though the bone has self-remodeling properties, bone regeneration may be required in some cases. Current research concerns materials employed to develop biological scaffolds with improved features as well as complex preparation techniques. Several attempts have been made to achieve compatible and osteoconductive materials with good mechanical strength in order to provide structural support. The application of biomaterials and mesenchymal stem cells (MSCs) is a promising prospect for bone regeneration. Recently, various cells have been utilized alone or in combination with biomaterials to accelerate bone repair in vivo. However, the question of what cell source is the best for use in bone engineering remains open. This review focuses on studies that evaluated bone regeneration using biomaterials with MSCs. Different types of biomaterials for scaffold processing, ranging from natural and synthetic polymers to hybrid composites, are presented. These constructs demonstrated an enhanced ability to regenerate the bone in vivo using animal models. Additionally, future perspectives in tissue engineering, such as the MSC secretome, that is the conditioned medium (CM), and the extracellular vesicles (EVs), are also described in this review. This new approach has already shown promising results for bone tissue regeneration in experimental models.

Key words: tissue engineering, stem cells, materials, secretome

Cite as
Grelewski PG, Kwaśnicka M, Bar JK. Properties of scaffolds as carriers of mesenchymal stem cells for use in bone engineering. *Polim Med*. 2023;53(2):129–139. doi:10.17219/pim/166471

DOI
10.17219/pim/166471

Streszczenie

Inżynieria tkankowa stała się jedną z najlepiej rozwijających się dziedzin medycyny i przypuszczalnie wysoce obiecującym rozwiązaniem w regeneracji uszkodzonych tkanek kostnych. Pomimo tego, że kość ma właściwości auto-modelujące, w niektórych przypadkach może być wymagana wspomagająca regeneracja kości. W obecnie prowadzonych badaniach w inżynierii tkankowej stosuje się materiały naturalne oraz syntetyczne do tworzenia rusztowań dla komórek z wykorzystaniem różnych technik ich przygotowania. Stosowane membrany wykazują strukturę zbliżoną do tkanki kostnej oraz dobrą wytrzymałość mechaniczną zapewniającą mechaniczne wsparcia dla komórek. Obiecujące wyniki w regeneracji kości uzyskano stosując membrany i mezenchymalne komórki macierzyste (MSCs). W inżynierii tkankowej wykorzystywane są komórki macierzyste różnego pochodzenia, które wprowadza się do miejsc ubytku bezpośrednio lub w połączeniu z materiałami w celu przyspieszenia naprawy kości in vivo. Brak jest odpowiedzi na pytanie, jakie źródło komórek macierzystych najlepiej nadaje się do wykorzystania w inżynierii kości. Niniejsza praca koncentruje się na badaniach oceniających regenerację kości przy użyciu różnych materiałów w połączeniu z MSCs. Przedstawiono różne rodzaje materiałów – od naturalnych i syntetycznych do hybrydowych polimerów stosowanych w przygotowaniu rusztowań dla komórek macierzystych. Omawiane rusztowania wykazywały wysoką zdolność do regeneracji kości w modelach in vivo. W tym artykule omówiono również perspektywy rozwoju inżynierii tkankowej opartej na połączeniu membran z czynnikami wydzielanymi przez komórki macierzyste, określanymi jako „sekretome", czyli pożywkę kondycjonowaną (CM), oraz pozakomórkowe pęcherzyki (EVs). To nowe podejście metodyczne dało obiecujące wyniki w zakresie regeneracji tkanki kostnej w modelach eksperymentalnych.

Słowa kluczowe: komórki macierzyste, inżynieria tkankowa, materiały, czynniki wydzielnicze komórek macierzystych

Introduction

Millions of bone grafting surgical procedures for the partial excision of bone are performed worldwide.[1,2] In general, autograft transplantation and allograft transplantation are the standard clinical procedures.[1] However, they are associated with postoperative complications and availability problems.[1] In this context, tissue engineering is a potential alternative for tissue transplants and constitutes a novel approach in regenerative medicine that involves using natural and synthetic materials in combination with stem cells to repair the damaged areas.[3,4] The bone tissue engineering strategy consists of 3 essential components: scaffold, mesenchymal stem cells (MSCs) and bioactive growth factors. Mesenchymal stem cells are cultured on 2D or 3D scaffolds to induce the growth of new bone tissue through osteoinductivity.[1,3,5] Studies have focused on the use of MSCs seeded on a scaffold due to their differentiation potential and paracrine/autocrine effects, which are important for tissue regeneration. The main source of cells used in tissue engineering is the bone marrow (BM).[1] The procedure of stem cell isolation from the BM is highly invasive and painful, which limits their application in tissue engineering, thus, new sources of MSCs are urgently needed. Adult stem cells such as adipose-derived stem cells (ASCs), umbilical cord blood mesenchymal stem cells (CB-MSCs) and oral mesenchymal stem cells (OMSCs) are among the candidates for bone tissue engineering applications.[1,3] The second component is the membrane used as a platform for MSCs. The scaffold can be a substrate that forms a fibrous network, which is a prerequisite for later bone formation.[5,6] Many parameters are involved in scaffold design that affects the mechanical properties and biological function of the scaffold while directly affecting the rate of bone regeneration.[6,7] The desirable parameters of a scaffold as a platform for MSCs are biodegradability, biocompatibility, imitation of the microenvironment, incorporation of different extracellular matrices (ECMs), stability, porosity, non-immunogenicity, interconnectivity, safety (low or no toxicity), and alignment.[5] Designing scaffolds made from natural and synthetic components for bone engineering involves several parameters, such as physical and mechanical properties, chemical composition, and biological activities that affect scaffold properties and stem cell behavior.[3,5] The viability and differentiation of MSCs towards osteoblasts were found to depend on the intrinsic properties of the material and the interactions of specific chemical components of materials with stem cells.[3,5,7] Several hybrid systems in the form of 2D and 3D natural and synthetic scaffolds have been fabricated and used for bone engineering; however, a good scaffold has not been developed yet.[1,4,7,8] Many papers discussed the advantages and drawbacks of different types of scaffolds with relevant examples of the impact of scaffold on stem cell biological behavior during stem cell differentiation and growth.[3–5,7,9] Despite the advanced techniques and the variety of substrates used for the production of scaffolds, there is currently no construct with ideal parameters for tissue engineering. Consequently, one of the greatest challenges in bone tissue engineering is designing a scaffold with optimal architecture for cell growth and differentiation. This review summarizes the current developments in scaffold design as well as the effect of scaffolds on stem cell growth and the interaction between stem cells and materials in the context of bone engineering.

Selection and parameters of scaffolds used for bone tissue engineering

The selection of a suitable scaffold is crucial in bone engineering. Ideally, scaffolds should be similar to native tissue structure and degrade in a controlled manner

consistent with the formation of new tissue.[1–4] The structure of a scaffold should allow the stem cells to release many particles affecting the surroundings of the damaged tissue.[10] Conductivity, porosity, tortuosity, scaffold architecture, and financial cost should be considered for scaffold fabrication.1,4,7 The chemical content of the scaffold also has a major effect on osteogenic cell differentiation and should support the osteoinduction, osteoconduction and osteointegration of the stem cells seeded on it.[11,12] The components used in scaffold design can be classified into natural or synthetic.

Natural biomaterials

For scaffold design of natural biomaterials, different proteins such as collagen (Col), fibrin, gelatin, silk, and polysaccharides (agarose, alginate (Alg), hyaluronan, and chitosan), which are found in the ECM, have been used.[1,3,5] These bioactive molecules usually contain sites for cellular adhesion, such as the arginyl–glycyl–aspartic acid (Arg–Gly–Asp) binding sequences, and can release non-toxic substances while degrading.[9] Natural scaffolds are often more biocompatible than synthetic ones, and have the advantage of providing specific cell interactions.[4,8] However, the disadvantages of natural scaffolds include the difficulty in obtaining large amounts of the material, risk of transmitting pathogens and limited mechanical properties.[9] Nonetheless, these proteins are very important for tissue structure and function, which justifies their choice in tissue engineering.[5] Collagen is the crucial structural protein in human tissues that contains sites for cell adhesion. Moreover, Col builds the tissue basement membrane and has natural properties similar to those of soft human tissue.[9] The main feature of Col is a unique structure rich in amino acids, such as glycine, proline and hydroxyproline.[13] Collagen is non-toxic, non-immunogenic and expands when exposed to water in order to better fill the cavities in the damaged tissue and support the structural integrity of organs and tissues.[11] The disadvantages of Col as a natural biopolymer include a high probability of deformation and low mechanical stability.[14] Collagens mixed with nano-inorganic materials are used to prepare scaffolds more widely than an ECM imitation of the bone in bone repair.[15] Many attempts have been made to modify Col to achieve a good form for use in bone regeneration.[15] Hyaluronic acid (HAc) is a natural polymer that consists of disaccharides, such as D-glucuronic and N-acetyl-D-glucosamine connected by β(1-4) and β(1-3) bonds within its polymeric structure.[14] Hyaluronic acid is classified as a glycosaminoglycan. It has a highly consistent structure, except for deacetylated glucosamine residues, which occur occasionally.[14] Molecular weight was found to play an important role in the biological, chemical, physical, and degradable properties of HAc.[16] The pivotal physicochemical properties of HAc, such as biodegradability, biocompatibility, non-inflammatory, non-toxic and non-immunogenic behavior, allow for its wide application in drug delivery bioengineering and biomedicine, e.g., endoprosthesis of the joint fluid, wound dressings and polymeric scaffolds.[16,17] Chitosan is obtained from the N-deacetylation of chitin and consists of 2 D-glucosamine units: the deacetylated unit and the acetylated unit such as N-acetyl D-glucosamine. These units are randomly distributed within the polymer and linked by β-(1-4)-glycosidic bonds. Moreover, their derivatives showed several biological properties, including anti-inflammatory, antitumoral, antimicrobial, and antioxidant activities.[18,19] Chitosan is biocompatible and thermo-responsive, has excellent strength and stability, and is often being used as a gelling agent.[18] Many tissues such as cartilage, bone, skin, and blood vessels have been regenerated by CS or CS in combination with ceramics or other biopolymers.[18]

Synthetic biomaterials

An alternative to natural materials is synthetic biomaterials for bone tissue engineering. Synthetic biomaterials include biodegradable polymers, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly-ε-caprolactone (PCL), poly(ethylene glycol) (PEG), polyvinyl alcohol (PVA), polyurethane (PU), poly(lactic-co-glycolic acid) (PLGA), and ceramics-based biomaterials such as hydroxyapatite (HA) and bioactive glass. Scaffolds can be fabricated from these materials in controlled conditions to optimize the chemical and physical parameters of a scaffold.[1,3–5,20] These materials have many advantages over their natural counterparts, including reproducibility and the possibility of controlling their mechanical properties. Likewise, the degradation rate can be controlled independently of shape.[3,5,20,21]

Chemical and physical modification of the polymer surfaces can increase their biological properties.[5] Polymers derived from natural sources, especially those derived from the ECMs, are very good materials for tissue engineering due to their intrinsic bioactivity and self-assembling ability.[4] A major drawback of natural polymers is the batch-to-batch variability, which makes it difficult to process at a huge scale because of changes in chemical composition and mechanical properties.[6] Due to the lack of cell adhesion properties, many synthetic biomaterials must be chemically modified to allow stem cell adhesion and growth.[5,8,9] Moreover, another disadvantage of polymers is their inadequate vascularization compared to natural bones.[11] Poly(lactic acid) is an aliphatic polyester and a hydrophobic polymer that naturally degrades through the process of hydrolysis: the ester bonds are broken down by water molecules, with the ester bonds determining the polymer structure.[22] Poly(lactic acid) has good tensile strength and occurs in various forms, such as poly(D-lactic acid) (PDLA) and poly(L-lactic acid) (PLLA).[21,22] Unfortunately, PLA has some disadvantages, such as high fragility and lack of reactive side chain groups, which causes

chemical inertness and low cell affinity due to its hydrophobicity.[23] Poly(lactic acid) has good potential for bone regeneration, but there are still certain problems, such as the mechanical modulus and the strength of bone scaffolds based on PLA, which is much weaker than cortical bone, particularly when PLA is produced with high porosity.[24] Poly-ε-caprolactone is a synthetic material with a melting temperature ranging between 59°C and 64°C (i.e., above body temperature) and a glass transition temperature of –60°C.[25] Poly-ε-caprolactone scaffolds have good elasticity and ductility, which shows that PCL has great potential for application in tissue engineering.[25] The presence of 5 hydrophobic –CH2 moieties in PCL repeating units makes it degrade the slowest among all the polyesters.[26] Poly-ε-caprolactone exhibits promising mechanical properties with good flexibility and substantial elongation, conducive to the construction of scaffolds for craniofacial bone repair.[25,26]

Bioceramics can be classified into 3 main types: bioinert (alumina and zirconia), bioactive (HA and Bioglass®) and biodegradable (calcium sulfate and tricalcium phosphate (TCP)).[27] Ceramics are bioinert, bioactive, fragile, bioresorbable, and characterized by good biocompatibility and high toughness. However, they are vulnerable to tensile stress and can be damaged due to high mechanical stress.[5] Hydroxyapatite and TCP are widely used materials.[1,5] The presence of both, known as biphasic calcium phosphate (BCP), promotes the formation of apatite on the surface of the scaffold, which integrates with the host bone after in vivo implantation.[1,5] Hydroxyapatite has been used in tissue engineering because of its chemical similarity to the mineral component of bone tissue.[28] The size of the molecules of the compounds used to build the scaffolds is also an important parameter.[11] Scaffolds made of hydroxyapatite showed good stability in animal models and sustained their mechanical properties for a long period.[4]

Architecture of the scaffold

A different issue critical to scaffold success is the creation of a suitable microenvironment for the stem cells to differentiate into osteoblasts and form a new bone. Other problems concern the vascular supply upon bioimplant implantation or even the high density of seeded cells on the scaffold.[4] In a porous scaffold, the density and size of pores affect cell migration and adherence to the scaffold, as well as nutrient and oxygen diffusion.[4,29] Too small pores may inhibit cell migration to the central layers of the scaffold, whereas the surface available for stem cell attachment in large pores may be limited. Moreover, the connection between the cells may not be enough to fill the gaps between the pores.[3,4,7,29] Tortuosity is another factor that affects mass transport, in turn affecting the migration of nutrients and cells within the scaffold structure.[7,30] Tortuosity refers to the path that the culture medium has to take through the pores to move from one point within the scaffold to another.[7,30] Surface roughness is a key parameter to take into account when designing scaffolds for tissue engineering. Roughness regulates the biological reaction of tissues in contact with the bioimplant. In vitro and in vivo studies showed that cellular morphology, proliferation rate and cell phenotype depend on the surface roughness of the material.[1,31] Technologies for scaffold preparation used in bone tissue engineering are very important because they affect cell behavior. The fabrication technique should be selected based on the architectural structure of the scaffold, which depends on the type of engineered tissue. Electrospinning is one of the new processing technologies to obtain suitable membranes for tissue engineering.[7,11] Freeze-drying is used to produce porous scaffolds through sublimation.[11] The main advantage of scaffold fabrication using the freeze-drying technique is that it does not require high temperatures.[11] Gas foaming is a technique that can produce highly porous scaffolds using high-pressure carbon dioxide gas.[22] Additive manufacturing includes selective laser sintering (SLS) and 3D printing, which enables independent control over pore sizes, shapes, porosity, and roughness during scaffold designing.[32] This technology allows different types of materials to be used, ranging from ceramics and polymers to hybrid composites. Moreover, additive manufacturing allows scaffolds to be designed for a wide range of tissue applications.[32–36]

Source of stem cells used in bone engineering

Mesenchymal stem cells obtained from different parts of the body, such as the BM (BM-MSCs), fat cells (ASCs) or dental pulp (dental pulp stem cells (DPSCs)), show the capability to differentiate into various types of cells and are the closest to the stem cells involved in the reconstruction of bone damage.[10–12,37] However, as presented in Table 1, the aforementioned subpopulations of cells used in bone defect repair have both advantages and disadvantages.[10,12,37] The BM-MSCs have a high osteogenic potential and may possess proangiogenic paracrine effects involved in vascular reconstruction.[32,37] The BM-MSCs are recognized based on specific criteria: fibroblastic morphology, the ability to adhere and form clusters, and the potential to differentiate into 3 lineages (endoderm, mesoderm and ectoderm).[32] Many successful bone healing procedures have been performed using BM-MSCs (through direct injection or in combination with 3D scaffolds) in both calvarial and long bones in different animal models.[32] The DPSCs are stem cells harvested from the dental tissue of both adults and children.[10,12] The DPSCs easily differentiate into osteoblasts (Fig. 1).[10,12] In recent years, DPSCs have been considered applicable in bone engineering. These cells are osteogenic-specific markers (e.g., osteopontin,

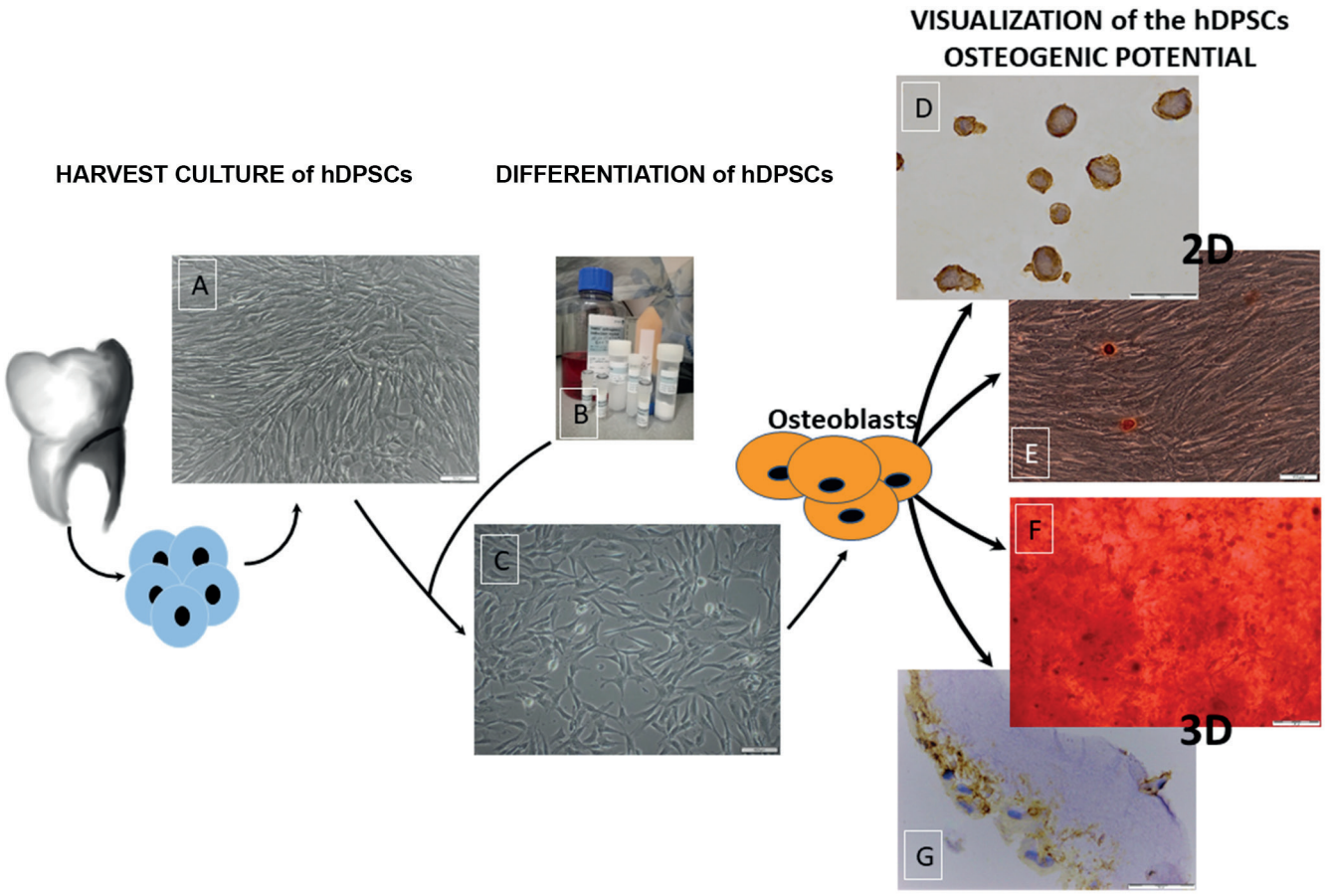

Fig. 1. Dental pulp stem cell (DPSC) differentiation into osteoblasts. A. Culturing of human DPSCs (hDPSCs) to increase the number of cells; B. Set of the differentiation reagents; C. Differentiation of hDPSCs into osteoblasts; D,E. Visualization of hDPSC differentiation into osteoblasts in 2D. High osteocalcin expression in differentiated hDPSCs (D). Mineralized nodules are visible in differentiated hDPSCs stained with Alizarin Red S (E); F,G. Visualization of hDPSC differentiation into osteoblasts in 3D. Mineral deposits on poly(L-lactide-co-caprolactone) (PLCL)/hDPSC scaffold are visible after osteogenic differentiation stained with Alizarin Red S (F). High osteocalcin expression is found on cells grown on the poly-ε-caprolactone (PCL) scaffold after osteogenic differentiation (G) (scale bar = 100 µm (A,C,E) and 50 µm (D,F,G))

Table 1. Advantages and disadvantages of stem cells used in bone tissue engineering

Stem cells	Advantages	Disadvantages
Bone marrow-derived mesenchymal stem cells (BM-MSCs)	1. High osteogenic potential 2. Vascular reconstruction 3. Chemotaxis to bone defect site	1. Harvesting of cells is painful 2. Poor performance 3. Faster aging
Dental pulp stem cells (DPSCs)	1. Harvesting of cells is painless and not invasive 2. Easy to differentiate to osteoblasts	1. Weak angiogenesis potential and chemotaxis to bone defect site
Adipose-derived stem cells (ASCs)	1. Large quantities of cells from waste material 2. Easy to differentiate to osteoblasts	1. Weak angiogenesis potential and chemotaxis to bone defect site

osterix and osteocalcin) and can form bone-like nodules in vitro.[37] The ASCs have been targeted as useful cells for bone engineering due to their ability to differentiate into several different types of cell lineages, ease of accessibility, immunogenic capabilities, and stability in long-term cell cultures.[38] Contrary to BM-MSCs, ASCs do not lead as quickly to senescence and easily differentiate into osteoblasts.[12] Moreover, they have high vascularization properties, which benefit bone reconstruction.[12] The ASCs are also a rich source of biologically active factors that are secreted via exosomes, which can help in the signaling pathways that differentiate cells into bone.[39]

Impact of different scaffolds on the osteogenic potential of stem cells in bone engineering

Until now, 235 ongoing clinical studies involving MSCs have been used for several medical conditions (https://www.clinicaltrials.gov/). However, some limitations of MSC therapy have been observed, particularly the short MSC survival time after injection, the right doses and the best route of administration are unclear.[40] To avoid such complications, MSC-based tissue engineering

approaches have been applied.[40] In bone engineering, scaffolds work as a temporary supporting platform to (a) ensure that the tissue regeneration area is suitable for bone formation and remodeling, (b) provide and maintain mechanical functionality during tissue regeneration, and (c) facilitate growth and angiogenesis during tissue regeneration.[29] The mechanism of bone regeneration induced by scaffolds is determined through the scaffold's chemical and biological environment, where the stem cells differentiate and grow into osteoblasts.[29] However, the mechanisms by which scaffold-induced bone regeneration takes place are not fully described, and many problems must still be explained and solved. For example, one problem is the lack of a functional vascular supply after MSC implantation, even for scaffolds possessing good biocompatibility with regenerated tissue.[3,5,7] A crucial and challenging requirement that an appropriately designed scaffold must meet is mimicking the dynamic nature of the native tissue.[4] Scaffolds for bone tissue engineering should imitate the natural matrix. Natural and synthetic polymers, ceramics, and composites are the most frequently used materials.[3–5,7] Among natural scaffolds, the Col sponge is the most commonly used material in bone repair because Col is the important component of the ECM and widely occurs in the bone.[8,9,41] Great efforts have been made to design Col-based scaffolds that exhibit similarity to the native environment.[42] Collagen is frequently added to synthetic scaffolds to increase their bioactivity and allow stem cells to attach to the scaffold surface.[9] Modification of gelatin methacryloyl (GelMa) can serve as a valuable membrane for MSCs in bone tissue engineering.[9] Recently, heparin-modified mineralized Col scaffolds with seeded BM-MSCs have been successfully used in bone repair because of naturally occurring bioactive factor mixtures promoting cell proliferation, migration and vascularization.[43] Furthermore, Wang et al. found that Alg–Col composite hydrogels seeded with BM-MSCs promoted proliferation and osteogenic differentiation as well as enhanced new bone formation in a critical-sized calvarial defect animal model.[44]

Hyaluronan scaffolds combined with MSCs have been used in cartilage and bone tissue engineering.[45] Human DPSCs (hDPSCs) were seeded on a hyaluronan-based biomaterial and tested for bone repair.[34] The Woodhouse group identified that using adipose substitutes from stem cells in bone engineering demonstrated better effect in the new bone tissue and blood vessel formation than using only the scaffold.[46] New approaches in tissue engineering are based on hydrogels/scaffolds combining materials, small molecules and stem cells for bone engineering.[36] Liu et al. modified CS hydrogels by incorporating catechol (CA) and zeolitic imidazolate framework-8 nanoparticles (ZIF-8 NP) to enhance the adhesive properties of the hydrogel. The modified hydrogels exhibited antibacterial properties and induced new vessel formation and osteogenesis.[33] Interesting data were reported by Sancilio et al., who combined hDPSCs with an Alg/HA scaffold and found that the scaffold enhanced hDPSC proliferation and differentiation towards osteoblasts and was capable of promoting successful tissue regeneration and calcium deposition, as well as sustaining natural bone formation.[35] Fouad et al. observed that the porous calcium sulfate–HA (CS–HA) coating of the scaffold with seeded BM-MSCs not only improved the attachment and proliferation of BM-MSCs but also enhanced the process of osteointegration in the therapy of critical-sized calvarial bone defects.[47] Another report found that a highly interconnected porous Alg/HA scaffold coated with lactose-modified CS (CTL) and cultured with hDPSCs increases the proliferation rate and osteogenic differentiation of hDPSCs.[48] Gutiérrez-Quintero et al. demonstrated that a HA matrix and a PLGA (HA/PLGA) scaffold seeded with hDPSCs induced angiogenesis and bone regeneration in rabbit bilateral mandibular critical-sized defects more effectively than a scaffold without hDPSCs.[49] Tschon et al. showed that a scaffold with zoledronate and HA nanocrystals (HAZOL) combined with human BM-MSCs (hBM-MSCs) not only enhanced stem cell adherence and movement into the scaffold but also increased the expression of bone-related proteins.[50] Some reports showed that a HA–Col scaffold created a microenvironment that was conducive to the osteogenic differentiation of dental stem cells and may be the optimal design for maxillofacial and alveolar bone regeneration.[51] Promising results were observed when BM-MSCs were seeded on a HA–Col scaffold.[51] In this study, a commercially available HA–Col scaffold was used to culture DPSCs.[51] Furthermore, this bioactive material induced osteogenic differentiation of hDPSCs and may be used for maxillofacial and alveolar bone regeneration.[51] When BM-MSCs were seeded on an HA–Col scaffold, no biological toxicity was observed. Moreover, the HA–Col scaffold promoted BM-MSC adhesion, proliferation and differentiation into osteoblasts.[52] Alginate and nano-sized HA (nHA) scaffolds (Alg/nHA) were also tested for bone regeneration.[35] Dental pulp stem cells seeded onto a scaffold based on Alg and nHA expressed osteogenic differentiation-related markers and promoted calcium deposition. Moreover, it can efficiently sustain natural bone regeneration.[53] Lorusso et al. found that the synthetic scaffolds in combination with DPSCs showed high ability to support both osteoblast and osteoclast generation in new bone formation in an animal model, and could be useful for bone regeneration procedures.[54] The authors described different scaffolds such as hydrogels, nanofibers, βTCP, and HA combined with the DPSCs for bone regeneration. Furthermore, they identified that the advantage posed by scaffold use is determined by the physical space-maintaining capability of the regenerative area, the three-dimensional structure of the scaffold and bioactive factor, and the cellular osteogenic response of the host.[44,54] Interesting data were presented when bioceramics, such as HA and TCP, were modified to increase the osteogenic potential of stem cells within 3D scaffolds.[55]

The nHA features of the scaffold generate better cellular responses compared to micron-sized particles (mHA).[55] The nanophase of the ceramics revealed the strong adsorption of vitronectin, which promotes osteoblast migration, adhesion and enhanced osteoclast-like cell function compared to conventional HA.[1]

Several authors have developed scaffolds combining calcium phosphate and HA with Col, Alg and CS.[56] In this context, Col has been extensively used with TCP and HA for both in vitro and in vivo studies, supporting both woven and lamellar bone formation and growth.[54,57] In an experimental study investigating a hybrid scaffold consisting of type I Col and HA, the adhesion and proliferation of MSCs and human periodontal ligament stem cells increased.[22] The proposed scaffold was a composite structure made with ceramic (HA, HA-magnesium (Mg) and HA-silicon (Si)) and Col, and demonstrated better mechanical stability and improved MSC proliferation activity.[58] Marine spongin combined with HA accelerated material degradation and improved new bone formation.[59] In mouse critical-sized defects, a bioceramic material composed of dicalcium phosphate and HA showed a better regenerative effect than an implant made of BCP with Col.[60] This type of bioimplant revealed other advantages, such as early resorption of material and high bone formation.[60] In the treatment of a large critical-sized bone defect in rabbits, the addition of MSCs on silica-coated calcium–HA scaffolds induced higher osteogenesis than that observed with the scaffold alone.[46] When bioactive factors were introduced to the scaffold–MSC construct, the bone healing process was increased.[46] The use of the composite biomaterial poly(3-hydroxybutyrate)/HA/Alg with the addition of MSCs enhanced the regeneration process and facilitated bone formation in a critical-sized defect about fourfold.[61] Bernardo et al. fabricated 3D-printed porous scaffolds composed of PLA/HA with loaded BM-MSCs for bone engineering. The authors showed that pure PLA scaffolds moderately induced an osteoconductive effect, whereas PLA/HA scaffolds efficiently enhanced osteogenic differentiation of MSCs even in the absence of any typical osteogenic stimuli.[62] In the author's opinion, 3D-printed PLA scaffolds supplemented by high concentrations of HA are most suitable for applications in bone tissue engineering.[62] Comparable data were also obtained by other researchers for scaffolds fabricated using PLLA with different concentrations of nHA. These scaffolds were tested to reveal their impact on human ASCs (hASCs), morphology, proliferation rate, and adhesion.[63] Poly(L-lactic acid) functionalized with 10% of nHA positively influenced hASC biological features such as attachment and proliferation. Furthermore, an nHA/PLLA scaffold might be considered in bone tissue engineering.[63] Another study found that HA incorporated on PCL-based scaffolds showed good cytocompatibility and high osteogenic potential of hDPSCs, and PCL/HA scaffolds might be used for bone tissue engineering.[64] Moreover, another study reported that 21 days after CB-MSCs were seeded onto a scaffold composed of Geistlich Bio-Oss® transplantation, significant bone repair was found in a cranial defect.[65] Synthetic polymers include PLA and PGA, which exhibit good mechanical properties of the fibre scaffold and suitable porosity, tortuosity and roughness, are non-inflammatory, biodegrade, and can support stem cell adhesion to the scaffold.[11] A nanofibrous scaffold composed of PCL and Mg oxide (MgO) nanoparticles was used for bone tissue engineering.[66] The MgO nanoparticles added into PCL nanofibers improved the mechano-chemical properties and enhanced cell adhesion and viability of the seeded cells.[66] Before MSC-based applications are adopted, several challenges associated with cell therapy, such as standardization and quality of stem cell culture, regulatory approval, and the sources of stem cells, should be further investigated. Recently, to overcome the abovementioned limitations associated with stem cell therapy, research has focused on the approaches based on the MSC-derived secretome, such as the use of extracellular vesicles (EVs) and the cytokines and growth factors released by MSCs. Although promising valuable data have been reported, tissue engineering has faced problems related to the standardization and validation of the manufacturing processes.[12]

Scaffold modification using the secretome in bone engineering

Stem cells have a paracrine effect and release a wide range of macromolecules to the extracellular area as the conditioned medium, including proteins, lipids and nucleic acids, especially RNA.[10,12,67–70] Moreover, MSCs secrete EVs in the form of microvesicles (MVs), exosomes (EXs), apoptotic bodies, and microRNA.[37,69] Extracellular vesicles are important mediators of intracellular communication that enable the transmission of biological signals between cells.[37,71] As presented in Table 2, there are many reports examining bone defect repair using an MSC-derived secretome combined with a scaffold. Exosomes derived from hASCs were immobilized on polydopamine (pDA)-coated PLGA (PLGA/pDA) scaffolds. Then, the scaffolds were inserted into critical-sized calvarial defects in mice. The results demonstrated that the EXs enhanced the migration, proliferation and osteogenic differentiation of hBM-MSCs and promoted bone regeneration in the calvarial defects in an in vitro model.[72] Khojasteh et al. compared the ability to repair mandibular bone defects in dogs using MSCs and endothelial progenitor cells (EPCs) seeded on βTCP scaffolds coated with PLGA microspheres that partially released the vascular endothelial growth factor (VEGF).[73] Bone formation was found to be most effective in the MSC/VEGF scaffold and then in the MSC/EPC/

Table 2. Summary of the scaffold-based regenerative effect combined with human mesenchymal stem cell (MSC) enrichment with MSC-derived secretome in bone repair

Author (year)	Source of human stem cells	Scaffolds	Secretome and/or CM	Results
Li et al. (2018)[72]	ASCs	PLGA/pDA	exosomes	enhanced migration, proliferation and osteogenic differentiation of BMSCs in vitro
Khojasteh et al. (2017)[73]	MSCs and EPC	b-TCP-PLGA microspheres	VEGF secreted	bone formation in bilateral mandibular body defects in beagles
Liu et al. (2021)[74]	PDLCs	hydrogel	BMSC-sEVS	enhanced migration, proliferation and osteogenic differentiation in periodontitis rat model
Chew et al. (2019)[75]	MSCs	collagen sponge	exosomes	new bone formation in rat periodontal defects model
Takeuchi et al. (2019)[76]	BM-MSCs	atelocollagen sponge	exosomes	enhanced osteogenic and angiogenesis effect in calvaria bone defects rat model
Hiraki et al. (2020)[77]	SHED	–	conditioned medium	osteogenesis and angiogenesis in a mouse calvarial bone defects model
Ogisu et al. (2020)[78]	BM-MSCs and PLFs	collagen sponge	conditioned medium	bone regeneration and angiogenesis in a mouse calvarial bone defects model
Qiu et al. (2020)[79]	PDLSCs and GMSCs	–	conditioned medium	improved rat periodontal defect regeneration
Giuliani et al. (2020)[70]	PDLSCs and GMSCs	collagen membrane and PLA	conditioned medium and EVs	better osteogenic capacity in PLA/hGMSC/CM samples
Merckx et al. (2020)[37]	DPSCs and BM-MSCs	in ovo	EVs and CM	BM-MSC CM increased angiogenesis in ovo

ASCs – adipose-derived stem cells; EPC – endothelial progenitor cell; PDLCs – periodontal ligament cells; BM-MSCs – bone marrow-derived mesenchymal stem cells; SHED – stem cells derived from human exfoliated deciduous teeth; PLFs – periodontal ligament fibroblasts; PDLSCs – periodontal ligament stem cells; GMSCs – gingival MSCs; DPSCs – dental pulp stem cells; PLGA – poly-lactic-co-glycolic acid; pDA – polydopamine; PLA – poly(lactic acid); VEGF – vascular endothelial growth factor; EV – extracellular vesicle; hGMSCs – human GMSCs; BMSC-sEVS – BM-MSC-derived small EVs; b-TCP-PLGA – beta-tricalcium phosphate-poly lactic co-glycolic acid; CM – conditioned medium.

VEGF scaffold.[73] An experimental study presented by Liu et al. showed that the human periodontal ligament cells (hPDLCs) co-cultured with BM-MSC-derived small EVs (BMSC-sEVs) increased the osteogenic potential of hPDLCs.[74] Another study demonstrated that EXs obtained from MSCs and loaded onto a Col sponge increased new bone formation in a rat periodontal defect model.[75] Takeuchi et al. showed that the scaffold-MSCs-EX construct enhanced osteogenesis and the accumulation of vascular endothelial cells in a rat calvarial bone defect model.[76] The results obtained by Hiraki et al. proved that the bone regeneration of stem cells derived from human exfoliated deciduous teeth (SHED) and conditioned medium (CM) was associated with angiogenesis and osteogenesis in a mouse calvarial bone defect model.[77] The CM collected from cultured hBM-MSCs and human periodontal ligament fibroblasts (HPLFs) under cyclic stretching was implanted on a Col sponge to repair mouse calvarial bone defects.[78] The authors suggested that this construct was involved in bone regeneration and angiogenesis.[78] The CM obtained from periodontal ligament stem cells (PDLSCs) and gingival MSCs (GMSCs) and then used for periodontal defect regeneration demonstrated the regenerative potential of these cells.[79] Another study evaluated the regenerative ability of human PDLSCs (hPDLSCs) and GMSCs (hGMSCs) and their secretome. Moreover, CM and EVs seeded on Col or polylactide membranes in a rat calvarial defect model showed that the secretome modified and accelerated bone remodeling, but with different kinetics of mineralization.[70]

Future prospects

A major challenge in bone engineering is using suitable MSCs and materials for bone repair. There are many different biomaterials used in combination with stem cells that have been used for bone reconstruction. In order to mimic the native ECM for bone tissue formation, further efforts are required to improve the interaction between stem cells and the scaffold. Attention should be given to a proper understanding of the kinetics of growth factors released by MSCs during bone regeneration. The construction of an optimal scaffold for bone tissue engineering is an important task because the scaffold must induce the interaction between MSCs and enhance osteogenic differentiation. Overall, a profound scientific knowledge of different types of scaffolds combined with different stem cells is necessary for future clinical usage, which might be important in entirely new applications.

ORCID iDs

Piotr Grzegorz Grelewski https://orcid.org/0000-0002-4955-2370
Monika Kwaśnicka https://orcid.org/0009-0006-3705-7002
Julia Krystyna Bar https://orcid.org/0000-0002-3811-4834

References

1. Yousefi AM, James PF, Akbarzadeh R, Subramanian A, Flavin C, Oudadesse H. Prospect of stem cells in bone tissue engineering: A review. *Stem Cells Int*. 2016;2016:6180487. doi:10.1155/2016/6180487
2. Anitua E, Troya M, Zalduendo M. Progress in the use of dental pulp stem cells in regenerative medicine. *Cytotherapy*. 2018;20(4):479–498. doi:10.1016/j.jcyt.2017.12.011
3. Gugliandolo A, Fonticoli L, Trubiani O, et al. Oral bone tissue regeneration: Mesenchymal stem cells, secretome, and biomaterials. *Int J Mol Sci*. 2021;22(10):5236. doi:10.3390/ijms22105236
4. Lutzweiler G, Ndreu Halili A, Engin Vrana N. The overview of porous, bioactive scaffolds as instructive biomaterials for tissue regeneration and their clinical translation. *Pharmaceutics*. 2020;12(7):602. doi:10.3390/pharmaceutics12070602
5. Willerth S, Sakiyama-Elbert S. Combining stem cells and biomaterial scaffolds for constructing tissues and cell delivery. In: *StemBook*. Cambridge, USA: Harvard Stem Cell Institute; 2008. http://www.ncbi.nlm.nih.gov/books/NBK27050/. Accessed March 1, 2022.
6. Wang S, Suhaimi H, Mabrouk M, Georgiadou S, Ward JP, Das DB. Effects of scaffold pore morphologies on glucose transport limitations in hollow fibre membrane bioreactor for bone tissue engineering: Experiments and numerical modelling. *Membranes*. 2021;11(4):257. doi:10.3390/membranes11040257
7. Paim Á, Tessaro IC, Cardozo NSM, Pranke P. Mesenchymal stem cell cultivation in electrospun scaffolds: Mechanistic modeling for tissue engineering. *J Biol Phys*. 2018;44(3):245–271. doi:10.1007/s10867-018-9482-y
8. Proksch S, Galler KM. Scaffold materials and dental stem cells in dental tissue regeneration. *Curr Oral Health Rep*. 2018;5(4):304–316. doi:10.1007/s40496-018-0197-8
9. Koyyada A, Orsu P. Recent advancements and associated challenges of scaffold fabrication techniques in tissue engineering applications. *Regen Eng Transl Med*. 2021;7(2):147–159. doi:10.1007/s40883-020-00166-y
10. Carluccio M, Ziberi S, Zuccarini M, et al. Adult mesenchymal stem cells: Is there a role for purine receptors in their osteogenic differentiation? *Purinergic Signal*. 2020;16(3):263–287. doi:10.1007/s11302-020-09703-4
11. Battafarano G, Rossi M, De Martino V, et al. Strategies for bone regeneration: From graft to tissue engineering. *Int J Mol Sci*. 2021;22(3):1128. doi:10.3390/ijms22031128
12. Marolt Presen D, Traweger A, Gimona M, Redl H. Mesenchymal stromal cell-based bone regeneration therapies: From cell transplantation and tissue engineering to therapeutic secretomes and extracellular vesicles. *Front Bioeng Biotechnol*. 2019;7:352. doi:10.3389/fbioe.2019.00352
13. Zheng M, Wang X, Chen Y, et al. A review of recent progress on collagen-based biomaterials [published online ahead of print on November 17, 2022]. *Adv Healthc Mater*. 2022:2202042. doi:10.1002/adhm.202202042
14. Cardoso LMDF, Barreto T, Gama JFG, Alves LA. Natural biopolymers as additional tools for cell microencapsulation applied to cellular therapy. *Polymers (Basel)*. 2022;14(13):2641. doi:10.3390/polym14132641
15. Qu H, Fu H, Han Z, Sun Y. Biomaterials for bone tissue engineering scaffolds: A review. *RSC Adv*. 2019;9(45):26252–26262. doi:10.1039/C9RA05214C
16. Snetkov P, Zakharova K, Morozkina S, Olekhnovich R, Uspenskaya M. Hyaluronic acid: The influence of molecular weight on structural, physical, physico-chemical, and degradable properties of biopolymer. *Polymers (Basel)*. 2020;12(8):1800. doi:10.3390/polym12081800
17. Bayer IS. Hyaluronic acid and controlled release: A review. *Molecules*. 2020;25(11):2649. doi:10.3390/molecules25112649
18. Islam MM, Shahruzzaman M, Biswas S, Nurus Sakib M, Rashid TU. Chitosan based bioactive materials in tissue engineering applications: A review. *Bioact Mater*. 2020;5(1):164–183. doi:10.1016/j.bioactmat.2020.01.012
19. Aranaz I, Alcántara AR, Civera MC, et al. Chitosan: An overview of its properties and applications. *Polymers (Basel)*. 2021;13(19):3256. doi:10.3390/polym13193256
20. Yousefzade O, Katsarava R, Puiggalí J. Biomimetic hybrid systems for tissue engineering. *Biomimetics*. 2020;5(4):49. doi:10.3390/biomimetics5040049
21. Li G, Zhao M, Xu F, et al. Synthesis and biological application of polylactic acid. *Molecules*. 2020;25(21):5023. doi:10.3390/molecules25215023
22. Echeverria Molina MI, Malollari KG, Komvopoulos K. Design challenges in polymeric scaffolds for tissue engineering. *Front Bioeng Biotechnol*. 2021;9:617141. doi:10.3389/fbioe.2021.617141
23. Casalini T, Rossi F, Castrovinci A, Perale G. A perspective on polylactic acid-based polymers use for nanoparticles synthesis and applications. *Front Bioeng Biotechnol*. 2019;7:259. doi:10.3389/fbioe.2019.00259
24. Feng P, Jia J, Liu M, Peng S, Zhao Z, Shuai C. Degradation mechanisms and acceleration strategies of poly (lactic acid) scaffold for bone regeneration. *Mater Des*. 2021;210:110066. doi:10.1016/j.matdes.2021.110066
25. Nemati S, Kim SJ, Shin YM, Shin H. Current progress in application of polymeric nanofibers to tissue engineering. *Nano Converg*. 2019;6(1):36. doi:10.1186/s40580-019-0209-y
26. Dwivedi R, Kumar S, Pandey R, et al. Polycaprolactone as biomaterial for bone scaffolds: Review of literature. *J Oral Biol Craniofac Res*. 2020;10(1):381–388. doi:10.1016/j.jobcr.2019.10.003
27. Dixon DT, Gomillion CT. Conductive scaffolds for bone tissue engineering: Current state and future outlook. *J Funct Biomater*. 2021;13(1):1. doi:10.3390/jfb13010001
28. Habibah T, Amlani D, Brizuela M. Hydroxyapatite dental material. In: *StatPearls*. Boca Raton, USA: StatPearls Publishing; 2022. http://www.ncbi.nlm.nih.gov/books/NBK513314/. Accessed March 9, 2023.
29. Poh PSP, Valainis D, Bhattacharya K, Van Griensven M, Dondl P. Optimization of bone scaffold porosity distributions. *Sci Rep*. 2019;9(1):9170. doi:10.1038/s41598-019-44872-2
30. Guerreiro R, Pires T, Guedes JM, Fernandes PR, Castro APG. On the tortuosity of TPMS scaffolds for tissue engineering. *Symmetry*. 2020;12(4):596. doi:10.3390/sym12040596
31. Calore AR, Srinivas V, Groenendijk L, et al. Manufacturing of scaffolds with interconnected internal open porosity and surface roughness. *Acta Biomater*. 2023;156:158–176. doi:10.1016/j.actbio.2022.07.017
32. Kargozar S, Mozafari M, Hamzehlou S, Brouki Milan P, Kim HW, Baino F. Bone tissue engineering using human cells: A comprehensive review on recent trends, current prospects, and recommendations. *Appl Sci*. 2019;9(1):174. doi:10.3390/app9010174
33. Liu Y, Zhu Z, Pei X, et al. ZIF-8-modified multifunctional bone-adhesive hydrogels promoting angiogenesis and osteogenesis for bone regeneration. *ACS Appl Mater Interfaces*. 2020;12(33):36978–36995. doi:10.1021/acsami.0c12090
34. Ferroni L, Gardin C, Sivolella S, et al. A hyaluronan-based scaffold for the in vitro construction of dental pulp-like tissue. *Int J Mol Sci*. 2015;16(3):4666–4681. doi:10.3390/ijms16034666
35. Sancilio S, Gallorini M, Di Nisio C, et al. Alginate/hydroxyapatite-based nanocomposite scaffolds for bone tissue engineering improve dental pulp biomineralization and differentiation. *Stem Cells Int*. 2018;2018:9643721. doi:10.1155/2018/9643721
36. Huo SC, Yue B. Approaches to promoting bone marrow mesenchymal stem cell osteogenesis on orthopedic implant surface. *World J Stem Cells*. 2020;12(7):545–561. doi:10.4252/wjsc.v12.i7.545
37. Merckx G, Hosseinkhani B, Kuypers S, et al. Angiogenic effects of human dental pulp and bone marrow-derived mesenchymal stromal cells and their extracellular vesicles. *Cells*. 2020;9(2):312. doi:10.3390/cells9020312
38. Storti G, Scioli MG, Kim BS, Orlandi A, Cervelli V. Adipose-derived stem cells in bone tissue engineering: Useful tools with new applications. *Stem Cells Int*. 2019;2019:3673857. doi:10.1155/2019/3673857
39. Zhu M, Liu Y, Qin H, et al. Osteogenically-induced exosomes stimulate osteogenesis of human adipose-derived stem cells. *Cell Tissue Bank*. 2021;22(1):77–91. doi:10.1007/s10561-020-09867-8
40. Morgan EF, Unnikrisnan GU, Hussein AI. Bone mechanical properties in healthy and diseased states. *Annu Rev Biomed Eng*. 2018;20(1):119–143. doi:10.1146/annurev-bioeng-062117-121139
41. Sharif S, Ai J, Azami M, et al. Collagen-coated nano-electrospun PCL seeded with human endometrial stem cells for skin tissue engineering applications. *J Biomed Mater Res*. 2018;106(4):1578–1586. doi:10.1002/jbm.b.33966
42. Collignon AM, Castillo-Dali G, Gomez E, et al. Mouse Wnt1-CRE-Rosa Tomato dental pulp stem cells directly contribute to the calvarial bone regeneration process. *Stem Cells*. 2019;37(5):701–711. doi:10.1002/stem.2973

43. Bretschneider H, Quade M, Lode A, Gelinsky M, Rammelt S, Vater C. Chemotactic and angiogenic potential of mineralized collagen scaffolds functionalized with naturally occurring bioactive factor mixtures to stimulate bone regeneration. *Int J Mol Sci*. 2021;22(11):5836. doi:10.3390/ijms22115836
44. Wang J, Wang H, Wang Y, et al. Endothelialized microvessels fabricated by microfluidics facilitate osteogenic differentiation and promote bone repair. *Acta Biomater*. 2022;142:85–98. doi:10.1016/j.actbio.2022.01.055
45. Krieghoff J, Picke AK, Salbach-Hirsch J, et al. Increased pore size of scaffolds improves coating efficiency with sulfated hyaluronan and mineralization capacity of osteoblasts. *Biomater Res*. 2019;23(1):26. doi:10.1186/s40824-019-0172-z
46. Perez JR, Kouroupis D, Li DJ, Best TM, Kaplan L, Correa D. Tissue engineering and cell-based therapies for fractures and bone defects. *Front Bioeng Biotechnol*. 2018;6:105. doi:10.3389/fbioe.2018.00105
47. Fouad H, AlFotawi R, Alothman O, et al. Porous polyethylene coated with functionalized hydroxyapatite particles as a bone reconstruction material. *Materials (Basel)*. 2018;11(4):521. doi:10.3390/ma11040521
48. Porrelli D, Gruppuso M, Vecchies F, Marsich E, Turco G. Alginate bone scaffolds coated with a bioactive lactose modified chitosan for human dental pulp stem cells proliferation and differentiation. *Carbohydr Polym*. 2021;273:118610. doi:10.1016/j.carbpol.2021.118610
49. Gutiérrez-Quintero JG, Durán Riveros JY, Martínez Valbuena CA, Pedraza Alonso S, Munévar J, Viafara-García S. Critical-sized mandibular defect reconstruction using human dental pulp stem cells in a xenograft model: Clinical, radiological, and histological evaluation. *Oral Maxillofac Surg*. 2020;24(4):485–493. doi:10.1007/s10006-020-00862-7
50. Tschon M, Boanini E, Sartori M, et al. Antiosteoporotic nanohydroxyapatite zoledronate scaffold seeded with bone marrow mesenchymal stromal cells for bone regeneration: A 3D in vitro model. *Int J Mol Sci*. 2022;23(11):5988. doi:10.3390/ijms23115988
51. Trivedi S, Srivastava K, Saluja TS, et al. Hydroxyapatite–collagen augments osteogenic differentiation of dental pulp stem cells. *Odontology*. 2020;108(2):251–259. doi:10.1007/s10266-019-00464-0
52. Cai Y, Tong S, Zhang R, Zhu T, Wang X. In vitro evaluation of a bone morphogenetic protein-2 nanometer hydroxyapatite collagen scaffold for bone regeneration. *Mol Med Rep*. 2018;17(4):5830–5836. doi:10.3892/mmr.2018.8579
53. Alipour M, Firouzi N, Aghazadeh Z, et al. The osteogenic differentiation of human dental pulp stem cells in alginate-gelatin/nano-hydroxyapatite microcapsules. *BMC Biotechnol*. 2021;21(1):6. doi:10.1186/s12896-020-00666-3
54. Lorusso F, Inchingolo F, Dipalma G, Postiglione F, Fulle S, Scarano A. Synthetic scaffold/dental pulp stem cell (DPSC) tissue engineering constructs for bone defect treatment: An animal studies literature review. *Int J Mol Sci*. 2020;21(24):9765. doi:10.3390/ijms21249765
55. Chuenjitkuntaworn B, Osathanon T, Nowwarote N, Supaphol P, Pavasant P. The efficacy of polycaprolactone/hydroxyapatite scaffold in combination with mesenchymal stem cells for bone tissue engineering. *J Biomed Mater Res*. 2016;104(1):264–271. doi:10.1002/jbm.a.35558
56. Baheiraei N, Nourani MR, Mortazavi SMJ, et al. Development of a bioactive porous collagen/β-tricalcium phosphate bone graft assisting rapid vascularization for bone tissue engineering applications. *J Biomed Mater Res*. 2018;106(1):73–85. doi:10.1002/jbm.a.36207
57. Schaller B, Fujioka-Kobayashi M, Zihlmann C, et al. Effects of additional collagen in biphasic calcium phosphates: A study in a rabbit calvaria. *Clin Oral Invest*. 2020;24(9):3093–3103. doi:10.1007/s00784-019-03181-8
58. Nitti P, Kunjalukkal Padmanabhan S, Cortazzi S, et al. Enhancing bioactivity of hydroxyapatite scaffolds using fibrous type I collagen. *Front Bioeng Biotechnol*. 2021;9:631177. doi:10.3389/fbioe.2021.631177
59. Parisi JR, Fernandes KR, De Almeida Cruz M, et al. Evaluation of the in vivo biological effects of marine collagen and hydroxyapatite composite in a tibial bone defect model in rats. *Mar Biotechnol*. 2020;22(3):357–366. doi:10.1007/s10126-020-09955-6
60. Chang HH, Yeh CL, Wang YL, et al. Neutralized dicalcium phosphate and hydroxyapatite biphasic bioceramics promote bone regeneration in critical peri-implant bone defects. *Materials (Basel)*. 2020;13(4):823. doi:10.3390/ma13040823
61. Volkov AV, Muraev AA, Zharkova II, et al. Poly(3-hydroxybutyrate)/hydroxyapatite/alginate scaffolds seeded with mesenchymal stem cells enhance the regeneration of critical-sized bone defect. *Mater Sci Eng C Mater Biol Appl*. 2020;114:110991. doi:10.1016/j.msec.2020.110991
62. Bernardo MP, Da Silva BCR, Hamouda AEI, et al. PLA/hydroxyapatite scaffolds exhibit in vitro immunological inertness and promote robust osteogenic differentiation of human mesenchymal stem cells without osteogenic stimuli. *Sci Rep*. 2022;12(1):2333. doi:10.1038/s41598-022-05207-w
63. Smieszek A, Marycz K, Szustakiewicz K, et al. New approach to modification of poly (l-lactic acid) with nano-hydroxyapatite improving functionality of human adipose-derived stromal cells (hASCs) through increased viability and enhanced mitochondrial activity. *Mater Sci Eng C Mater Biol Appl*. 2019;98:213–226. doi:10.1016/j.msec.2018.12.099
64. Biscaia S, Branquinho MV, Alvites RD, et al. 3D printed poly(ε-caprolactone)/hydroxyapatite scaffolds for bone tissue engineering: A comparative study on a composite preparation by melt blending or solvent casting techniques and the influence of bioceramic content on scaffold properties. *Int J Mol Sci*. 2022;23(4):2318. doi:10.3390/ijms23042318
65. Kosinski M, Figiel-Dabrowska A, Lech W, et al. Bone defect repair using a bone substitute supported by mesenchymal stem cells derived from the umbilical cord. *Stem Cells Int*. 2020;2020:1321283. doi:10.1155/2020/1321283
66. Niknam Z, Golchin A, Rezaei-Tavirani M, et al. Osteogenic differentiation potential of adipose-derived mesenchymal stem cells cultured on magnesium oxide/polycaprolactone nanofibrous scaffolds for improving bone tissue reconstruction. *Adv Pharm Bull*. 2020;12(1):142–154. doi:10.34172/apb.2022.015
67. Wang J, Wang T, Zhang F, et al. Roles of circular RNAs in osteogenic differentiation of bone marrow mesenchymal stem cells (review). *Mol Med Rep*. 2022;26(1):227. doi:10.3892/mmr.2022.12743
68. Daneshmandi L, Shah S, Jafari T, et al. Emergence of the stem cell secretome in regenerative engineering. *Trends Biotechnol*. 2020;38(12):1373–1384. doi:10.1016/j.tibtech.2020.04.013
69. Dunn CM, Kameishi S, Grainger DW, Okano T. Strategies to address mesenchymal stem/stromal cell heterogeneity in immunomodulatory profiles to improve cell-based therapies. *Acta Biomater*. 2021;133:114–125. doi:10.1016/j.actbio.2021.03.069
70. Giuliani A, Sena G, Tromba G, et al. Could the enrichment of a biomaterial with conditioned medium or extracellular vesicles modify bone-remodeling kinetics during a defect healing? Evaluations on rat calvaria with synchrotron-based microtomography. *Appl Sci*. 2020;10(7):2336. doi:10.3390/app10072336
71. Driscoll J, Wehrkamp C, Ota Y, Thomas JN, Yan IK, Patel T. Biological nanotherapeutics for liver disease. *Hepatology*. 2021;74(5):2863–2875. doi:10.1002/hep.31847
72. Li W, Liu Y, Zhang P, et al. Tissue-engineered bone immobilized with human adipose stem cells-derived exosomes promotes bone regeneration. *ACS Appl Mater Interfaces*. 2018;10(6):5240–5254. doi:10.1021/acsami.7b17620
73. Khojasteh A, Fahimipour F, Jafarian M, et al. Bone engineering in dog mandible: Coculturing mesenchymal stem cells with endothelial progenitor cells in a composite scaffold containing vascular endothelial growth factor. *J Biomed Mater Res*. 2017;105(7):1767–1777. doi:10.1002/jbm.b.33707
74. Liu L, Guo S, Shi W, et al. Bone marrow mesenchymal stem cell-derived small extracellular vesicles promote periodontal regeneration. *Tissue Eng Part A*. 2021;27(13–14):962–976. doi:10.1089/ten.tea.2020.0141
75. Chew JRJ, Chuah SJ, Teo KYW, et al. Mesenchymal stem cell exosomes enhance periodontal ligament cell functions and promote periodontal regeneration. *Acta Biomater*. 2019;89:252–264. doi:10.1016/j.actbio.2019.03.021
76. Takeuchi R, Katagiri W, Endo S, Kobayashi T. Exosomes from conditioned media of bone marrow-derived mesenchymal stem cells promote bone regeneration by enhancing angiogenesis. *PLoS One*. 2019;14(11):e0225472. doi:10.1371/journal.pone.0225472
77. Hiraki T, Kunimatsu R, Nakajima K, et al. Stem cell-derived conditioned media from human exfoliated deciduous teeth promote bone regeneration. *Oral Dis*. 2020;26(2):381–390. doi:10.1111/odi.13244

78. Ogisu K, Fujio M, Tsuchiya S, et al. Conditioned media from mesenchymal stromal cells and periodontal ligament fibroblasts under cyclic stretch stimulation promote bone healing in mouse calvarial defects. *Cytotherapy*. 2020;22(10):543–551. doi:10.1016/j.jcyt.2020.05.008

79. Qiu J, Wang X, Zhou H, et al. Enhancement of periodontal tissue regeneration by conditioned media from gingiva-derived or periodontal ligament-derived mesenchyml stem cells: A comparative study in rats. *Stem Cell Res Ther*. 2020;11(1):42. doi:10.1186/s13287-019-1546-9

Review on fluoride varnishes currently recommended in dental prophylaxis

Dagmara Piesiak-Pańczyszyn[1,A–D], Wojciech Zakrzewski[2,A–D], Aleksandra Piszko[3,B–D], Paweł J. Piszko[4,B–D], Maciej Dobrzyński[3,E,F]

[1] Department of Conservative Dentistry with Endodontics, Wroclaw Medical University, Poland
[2] Pre-clinical Research Centre, Wroclaw Medical University, Poland
[3] Department of Pediatric Dentistry and Preclinical Dentistry, Wroclaw Medical University, Poland
[4] Faculty of Chemistry, Department of Polymer Engineering and Technology, Wroclaw University of Science and Technology, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Polymers in Medicine, ISSN 0370-0747 (print), ISSN 2451-2699 (online) *Polim Med*. 2023;53(2):141–151

Address for correspondence
Dagmara Piesiak-Pańczyszyn
E-mail: dpanczyszyn@interia.pl

Funding sources
The study was financed by the subsidy from Wroclaw Medical University (grant No. SUBZ.B180.23.054).

Conflict of interest
None declared

Acknowledgements
Figures 1 and 2 were created using BioRender.com.

Received on September 25, 2023
Reviewed on October 11, 2023
Accepted on October 11, 2023

Published online on November 28, 2023

Abstract

In dentistry, fluoride compounds play a very important role in the development of teeth hard tissue. They have been modifying the development of the carious process for many years in accordance with the principles of minimally invasive therapy. Studies have confirmed their effectiveness in the prevention and treatment of carious lesions and erosion of deciduous and permanent teeth, as well as in the dentin hypersensitivity treatment. Typically, each varnish consists of 3 basic components, i.e., a resin usually in the form of mastic, shellac and/or rosin, an alcohol-based organic solvent (usually ethanol) and active agents. In the first-generation varnishes, the active agent is fluorine compounds, most often in the form of 5% NaF, while in second-generation varnishes, the composition is further enriched with calcium and phosphorus compounds in the form of CPP-ACP/CPP-ACPF, ACP, TCP, fTCP, CSPS, TMP, CXP, or CaGP. This influences the bioavailability of fluoride in the oral environment by increasing both its release from the product and its subsequent accumulation in enamel and plaque, promotes more efficient closure of dentinal tubules, and facilitates pH buffering in the oral cavity.

Key words: dental caries, fluoride release, fluoride varnishes

Cite as
Piesiak-Pańczyszyn D, Zakrzewski W, Piszko A, Piszko P, Dobrzyński M. Review on fluoride varnishes currently recommended in dental prophylaxis. *Polim Med*. 2023;53(2):141–151. doi:10.17219/pim/174016

DOI
10.17219/pim/174016

Introduction

Fluoride is a cyclic element widespread in nature, which, due to its high electronegativity and activity, does not occur in elemental form but only in the form of compounds, reacting with almost all other elements, noble gases, and organic and inorganic compounds.[1] It belongs to micronutrients, and can be found in supportive tissue within an organism, in the hard tissues of teeth, in the skin, and in hair. In addition, as an element with high biological activity, it often acts as an inhibitor or, less frequently, an activator of many enzymes, affecting the course of protein biosynthesis processes, carbohydrate metabolism or lipid metabolism.[2]

Fluoride-containing compounds are widely used in minimal invasive therapies in contemporary dentistry. Their effectiveness in preventing caries end erosion of primary teeth and treating hypersensitivity in permanent teeth has been confirmed in many reports.[3–7] The role of varnishes is depicted in Fig. 1. Furthermore, it is suggested that continuous, repeatable fluoride delivery over time is most advantageous for dental treatment.[8–12] It turns out that the amount of fluoride in the fluids flowing around the teeth in the experimental model above 0.03 ppm already initiates a cariostatic effect.

Fluoride varnishes are materials that contain the highest content of this element and are intended for professional use in dental practices. They facilitate prolongation of contact between tooth tissues and fluoride. Their use in dentistry was first reported in 1964 by German researcher Hans Joachim Schmidt. He used 2% sodium fluoride in an alcoholic solution of natural resin as an alternative to preparations that were aqueous. Clinical studies confirming its effectiveness were published 4 years later,[13] resulting in the introduction of the first commercial fluoride varnish to the markets under the name of Duraphat (Woelm Pharma Co., Eshwege, Germany) containing 5% NaF. Promising results contributed to the introduction to the market of newer formulations in subsequent years, i.e., in 1975 Fluor Protector (Vivadent, Schaan, Liechtenstein) with 0.9% difluorosilane, in 1984 Duraflor (AMD Medicom Inc., Montreal, Canada) with 5% NaF, or in 1986 Bifluorid 12 (Voco Chemie GmbH, Cuxhaven, Germany) with 6% NaF.[14] Approved by the FDA in 1994, they initially functioned as hypersensitivity drugs, and over the years have become a permanent part of dental practice. Numerous meta-analyses showed their therapeutic effectiveness in reducing caries at 46% for permanent dentition and 33% for deciduous dentition, provided they were applied 2–4 times a year.[15–19] Similar results were obtained in a study in which the use of fluoride varnish as early childhood caries prophylaxis reduced caries by 25–45%.[20,21]

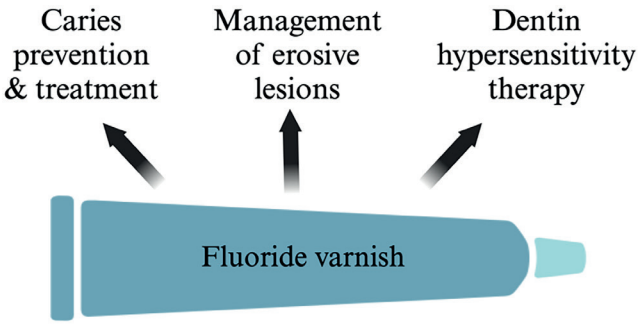

Fig. 1. The role of fluoride varnish applied at the tooth's surface

Structure of fluoride varnishes

Fluoride varnishes are available on the market in a wide variety, differing in chemical composition, particle shape and size, consistency and viscosity, which, according to some researchers, affects different fluoride release patterns.[22–28] Typically, each varnish consists of 3 basic components, i.e., a resin (often in the form of mastic), shellac and/or rosin in an organic solvent (usually ethanol), and of active agents involved in the remineralization process.[29,30] Shellac and mastic are responsible for the formation of an elastic and hard layer on teeth, which prevents rapid dissolution in saliva. On the other hand, rosin obtained from the oleoresin of dead pine wood or talc oil improves the liquidity of the preparation, which translates into better adhesion to enamel, longer contact time with the tooth surface, the ability to flow onto hard-to-reach surfaces and prolonged release of fluoride ions. In turn, the polyvinyl acetate polymer used in varnishes with complex of casein phosphopeptide and amorphous calcium phosphate (CPP-ACP) shows very good solubility in aqueous solutions, which can directly affect the dynamics of fluoride ion release.[31] Alcohol, undergoing rapid evaporation after exposure to air, leaves free volume, which causes easier contact of the varnish with water and accelerates the release of fluoride.[32] The active agents of the first generation of varnishes are usually in the form of neutral or acidified 5% NaF containing 2.26% fluoride ions (22,600 ppm of fluoride), 1% difluorosilane containing 0.1% fluoride ions (1,000 ppm of fluoride) or 6% NaF combined with 6% CaF_2 (56,300 ppm of fluoride). TiF_4-containing varnishes can also be found, while they are still seen as experimental formulations requiring continued in vitro and in vivo studies.[33–35] In the literature, the formation of calcium fluoride after application of 2% neutral NaF has been compared with acidified fluoride preparations, which evidently showed an advantage for the latter in terms of the amount of CaF_2 deposited on the enamel surface.[36,37] This is explained by increased affinity of the released fluoride to the enamel in an acidic environment compared to neutral pH. According to many researchers,[23,38–41] in the oral environment, the amount of available calcium and phosphate ions is insufficient to bind the large amounts of fluoride obtained after fluoride varnish application. For this

reason, so-called second-generation varnishes enriched in Ca^{2+} and PO_4^{3-} ions in the form of, e.g., complex of casein phosphopeptide and amorphous calcium phosphate (CPP-ACP, CPP-ACPF), amorphous calcium phosphate (ACP), tricalcium phosphate (TCP – tricalcium phosphate – $Ca_3(PO4)_2$), active form of tricalcium phosphate (fTCP), sodium-calcium phosphosilicate (CSPS or so-called bioactive glass), sodium trimetaphosphate (TMP), xylitol-coated calcium phosphate (CXP), or calcium glycerophosphate (CaGP).[28,35,42–45] Their presence is believed to have a significant effect on the bioavailability of fluoride in the oral environment by increasing its release from the product, facilitating its accumulation in enamel and plaque, closing dentinal tubules more effectively, and buffering oral pH. However, there is still no conclusive evidence as to which of the aforementioned compounds works best.[25,26,28,37,39,42,43,45–50] According to Karlinsey et al.,[51] the addition of TCP increases the effectiveness of fluoride without compromising its bioavailability. This is explained by the fact that during the manufacturing process, a protective fumar barrier is formed around the Ca^{2+} ions, preventing the ions from being deactivated during storage. Contact limited to saliva causes the barrier to dissolve, resulting in the release of both calcium and fluoride ions, the latter in significantly higher amounts compared to non-enriched varnishes.[48,52,53] Research conducted by Alamoudi et al.[54] showed an increase in the microhardness of the enamel surface treated with varnish from TCP, and Elkassas et al.[52] reported greater remineralization efficiency following reducing its roughness. Another available compound is the CPP-ACP/CPP-ACPF complex, which includes a milk-derived protein that is a source of readily available calcium ions and phosphate ions. Casein phosphopeptides (CPP), with the help of phosphoserine sequences, stabilize amorphous calcium phosphate (ACP) and, after contact with saliva, help it attach to the surface of enamel or plaque.[28,45,55] This was confirmed by in vitro research[38,56] showing CPP-ACP nanocomplexes attached to supragingival plaque, as well as to the surface of *Streptococcus mutans* bacterial cells by means of electron microscopy. By buffering the pH of the biofilm, free Ca^{2+} and PO_4^{2-} ions dissociate, thus not only acting as a reservoir of bioavailable ions, but also maintaining the supersaturated state of saliva relative to the surface of the enamel.[57] Some researchers believe that the enrichment of the varnish with the CPP-ACP complex is the best technology that allows a stable combination of high concentrations of calcium, phosphate and fluoride ions with the tooth surface and plaque.[23,38,39,43,50,58–61] In addition, Bakry et al.[62] observed that the application of MI Varnish (GC, Tokyo, Japan) with CPP-ACP complex is followed by a significant reduction in demineralization and an increase in enamel microhardness. This was confirmed by the study by Świetlicka et al., in which it was shown that 24 h after application, fluoride varnish with CPP-ACP induced partial regeneration of the previously damaged enamel surface, activating the formation of a new honeycomb-like layer.[63] On the other hand, according to Yapp et al.,[64] a better solution is the introduction of CXP (xylitol-coated calcium and phosphate) ions into the varnish, i.e., calcium and phosphate ions coated with xylitol and suspended in a permeable resin, which enables their uniform and prolonged release and enhances the antimicrobial effect. According to the manufacturers,[65] such an enriched varnish can provide 4 times more fluoride compared to others, with the maximum release occurring in the first 4 h after application.

In addition to these 3 main ingredients, manufacturers may also add colorants, sweeteners (e.g., saccharin sodium), stabilizing and adhesion-enhancing agents,[35] as well as xylitol with antimicrobial properties, chlorhexidine with antimicrobial and remineralizing properties, or arginine and chlorhexidine with antibacterial, remineralizing and pH stabilizing properties.[43]

Reviewing the literature, we can find new experimental varnishes that are undergoing clinical evaluation all the time. Pichaiaukrit et al. evaluated in 2019 in vitro the effect of chitosan, a linear copolymer of D-glucosamine and N-acetyl-D-glucosamine, on fluoride release.[66] Comparing fluoride varnishes differing only in the amount of chitosan, it was found that the increase in fluoride release was related to the increase in polymer concentration. The highest values were obtained after 1 h, which significantly decreased after 2 h after application, with slow but steady fluoride release continuing up to 6 h. Unfortunately, fluoride varnishes with chitosan were cytotoxic to human gingival fibroblasts. Table 1 lists examples of first- and second-generation fluoride varnishes with information on their composition.

Mechanism of action of varnishes and fluoride

Fluoride ions released by fluoride varnishes have a direct effect on the processes occurring on the surface of the enamel in the oral environment. More specifically, they inhibit demineralization, promote remineralization and interfere with the adhesion and metabolism of carious bacteria.[12,14,15,29,44,73,74]

Two types of reactions of fluoride with enamel occur depending on the concentration of the preparation used.[75] At low concentrations of no more than 50 ppm, fluorohydroxyapatite is formed with simultaneous acidification of the environment, which follows the reaction described below (Reaction 1).

$$Ca_{10}(PO_4)_6(OH)_2 + F^- + H^+ \rightarrow Ca_{10}(PO_4)_6(OH)F + H_2O$$

The fluorohydroxyapatite thus formed is very strongly bound to the outermost layers of the enamel. In the literature, it is often referred to as bonded fluoride, which can only be lost if the entire tissue is abraded or completely

Table 1. Examples of commercially available fluoride varnishes with respect to their manufacturers and composition

Fluoride varnish	Manufacturer	Active ingredient	Fillers	References
Colgate® Duraphat Varnish – single dose	Colgate Oral Care, Sydney, Australia	5% NaF	30–60% colophonium, 10–30% ethanol, flavor	[67,68]
MiVarnish™	GC, Tokyo, Japan	5% NaF	30–50% polyvinyl acetate, 10–30% hydrogenated rosin, 20–30% ethanol, 1–5% CPP-ACP, 1–5% silicon dioxide, flavor	[43,62]
EmbranceVarnish™	Pulpdent, Watertown, USA	5% NaF	CXP™ – xylitol-coated calcium and phosphate, 10–30% ethanol	[64]
Clinpro White Varnish	3 M ESPE, St. Paul, USA	5% NaF	30–75% pentaerythritol glycerol ester of colophony resin, 10–15% n-hexane, 1–15% ethyl alcohol, 1–5% sodium fluoride, 1–5% flavor enhancer, 1–5% thickener, 1–5% food grade flavor, <5% modified tricalcium phosphate	[23]
Mirafluorid	Hager & Werken, Duisburg, Germany	0.12%NaF cetylaminehydrofluoride bis(hydroxyethyl)-aminopropyl-N-hydroxyethyl-octadecyl-amindihydrofluoride	water-soluble polymer	[67]
Fluor Protector	Vivadent, Schaan, Liechtenstein	0.1% difluorosilan	polyurethane-based transparent resin, ethyl acetate and isoamyl opropionate solution	[69]
Fluor-Opal	Ultradent, South Jordan, USA	5% NaF	ethanol, methyl salicylate, hydrogenated resin	[49]
Bifluorid 12	Voco Chemie GmbH, Cuxhaven, Germany	2.71% as 6%NaF and 2.92% as 6% CaF_2	ethyl acetate, silicates, flavors	[70]
Enamel Pro	Premier Dental Products, Plymouth Meeting, USA	5% NaF	amorphous calcium phosphate (ACP), ethanol	[71]
Novamin	NovaMin® Technology Inc. GlaxoSmithKline, Alachua, USA	5% NaF	calcium sodium phosphosilicate (CSPS), ethanol	[72]

dissolved. If the concentration of fluoride is greater than 100 ppm on the enamel surface, small granules of calcium fluoride form in the plaque and acquired membrane, perceived as loose, unbound fluoride,[14,37,40,42,43,56,74,76–78] as illustrated by the reaction below (Reaction 2).

$$Ca_{10}(PO_4)_6(OH)_2 + 20F^- + 8H^+ \rightarrow 10CaF_2 + 6HPO_4^{2-} + 2H_2O$$

Fluorides react with hydroxyapatites of the enamel by replacing hydroxide ions. As a result of this reaction, some part of hydroxyapatites transform into fluorapatites, which have better crystalline properties, and are more acid-resistant. In the apatite, strong ionic bonds between fluoride and the amine group of the organic enamel matrix are formed, which contributes to greater stability of fluorapatite crystals. Fluoride may also react with apatite by stimulating the growth of fluorapatite crystals. Moreover, it may cause apatite dissolution and forming calcium fluoride. The formation and stimulation of fluorapatite growth may occur during frequent exposure to low fluoride concentration in the solutions (below 0.1%). The role of fluoride delivered through application of varnish at the tooth's surface is showed in Fig. 2.

The bacteriostatic and/or bactericidal effect is achieved by reducing plaque deposition by disrupting bacterial adherence to the acquired membrane and altering bacterial metabolism by inhibiting enolase activity, reducing glucose transport into the cell and storage, interfering with the synthesis of extracellular and intracellular bacterial polysaccharides, decreasing the amount of lactic acid produced, decreasing the activity of cellular phosphatases responsible for the hydrolysis of phosphate esters, and disrupting the transport and accumulation of cations in cells.[9,37,74]

Fluoride varnish application involves the use of high concentrations of fluoride salts suspended in a resin that allows them to persist for some time on the tooth surface. The basic technology involved in the construction of F varnishes is to use very high concentrations of fluoride salts, usually at 50,000 ppm NaF (22,600 ppm of fluoride) in a resin varnish that stays on the tooth surface for several hours. During the dwell time, saliva washes over the varnish and dissolves the fluoride salts, allowing fluoride ions to diffuse out of the varnish and be absorbed into the fluoride reservoirs in oral fluid tissues, plaque and teeth. Over time, fluoride ions are re-released from these reservoirs.[44] Released fluoride ions accumulate in higher concentrations (above 100 ppm) both on the enamel surface and in the enamel itself, as well as in plaque. The presence of fluoride attracts

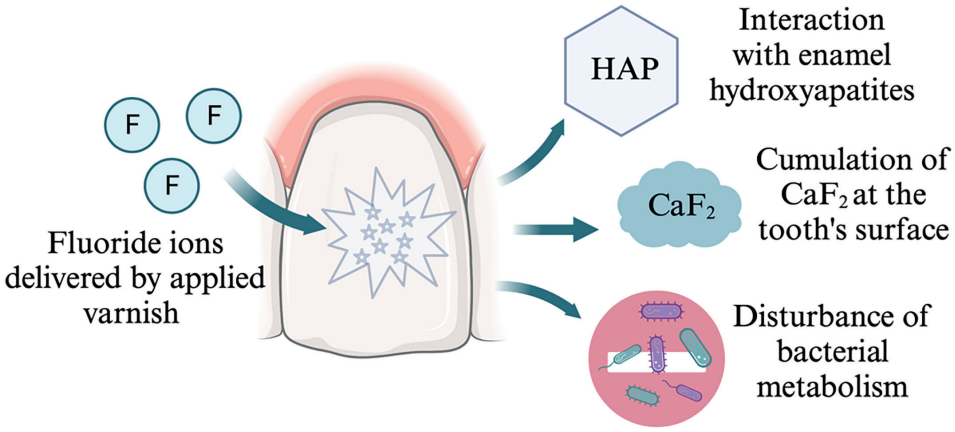

Fig. 2. The role of fluoride delivered by application of varnish at the tooth's surface

calcium and phosphate ions from saliva or from dissolved hydroxyapatite, forming small calcium fluoride crystals with embedded phosphates.[14,37,39,40,42–44,74,76–78] Since pure CaF_2 crystal is cubic rather than spherical, the resulting spherical deposits are described as calcium fluoride-like compounds, which on the one hand block acid diffusion, and on the other hand form a fluoride reservoir that is stable and insoluble in neutral pH environments. Phosphate ions increase its solubility compared to pure CaF_2.[24,79] With a decrease in pH, the membrane coating is dissolved and the release of fluoride ions and their incorporation into the enamel structure occurs. A decrease in fluoride concentration below 100 ppm and an increase in pH results in saturation of the enamel relative to the surrounding fluid and inhibition of the process described above.[12,29,37,40,42,44,53,74,76,80] Following the application of the varnish, more fluoride is retained on the demineralized rather than the healthy surface, contributing to the activation of repair processes in the enamel.[3,81]

The formation of CaF_2[24,25,34,40,50,67,82,83] is influenced by fluoride ions at sufficiently high concentrations, as well as calcium and phosphate ions presence in the oral cavity.[23,38,40,41,56] According to Attin et al.,[84] the supply of these ions in second-generation varnishes results in the deposition of larger amounts of calcium fluoride on the enamel surface, which in turn enhances the therapeutic effect.[23,38,48,49,60,78,85–92]

Clinical use of fluoride varnishes: indications, scheme, contraindications, and method of application

Varnish is defined as a professionally, topically applied fluoride compound. Its topical application is meant to slow down the release of active substances, including oxidative agents or chlorhexidine.[93] Varnishes have properties that facilitate their adhesion to the tooth surface. In comparison to sealants, fluoride varnishes are claimed to be easier to apply. In contrast to the multi-step technique of sealant application, varnishes can be successfully applied without etching or overdrying the surface of the tooth.[94]

Application and spreading of fluoride varnishes on tooth surfaces is usually performed with small brushes or cotton pellets. In the process of application, the clinician applies around 0.30–0.75 mL of varnish per single tooth. Additionally, the clinician may soak the dental floss in a varnish and apply it between teeth to cover hard to reach interdental spaces. Prolonged adhesion to the enamel enables the varnish to constantly release the fluoride, lowering long-time sensitivity and vulnerability of the tooth toward bacterial activity and generally caries.[29] The whole process should take about 1–4 min, which is dependent on number of teeth that need to be covered. The indications after successful application of fluoride varnish are as follows: Patient should avoid eating for around 2 h and in order to allow varnish to remain longer on the tooth surface, tooth brushing should be avoided on the same day, after which it should be continued. Fluoride varnishes have no age restrictions. For moderate risk, they should be applied twice a year, while for high risk up to 4 times. Disposable doses of fluoride varnish containing 5% NaF (22,600 ppm of fluoride) are 0.10 mL for infants and 0.25 mL for children over 1 year old. Contraindications to the use of fluoride varnish include bronchial asthma, stomatitis and necrotizing ulcerative gingivitis.

The use of fluoride varnishes in dentistry is now recommended by international dental organizations and societies, e.g., European Academy of Paediatric Dentistry (EAPD), American Dental Association (ADA), American Academy of Pediatric Dentistry (AAPD), IAPD International Association of Paediatric Dentistry (IAPD), World Dental Federation (FDI), or World Health Organization (WHO).[95–101]

According to ADA experts, in adults at increased risk of developing caries, including root caries, professional prophylaxis should include the application of 2.26% fluoride varnish 2–4 times a year or the use of acidified fluorophosphate gel (1.23%) 2–4 times a year.[102] Fluoride varnishes possess many advantages which make it one of the best ways of topical application. In general, they are considered safe and acceptable.[103] Their prolonged,

slow release of fluoride allows teeth to be exposed to higher doses without the risk of overdose. The application is fast and simple, without any need of additional drying. Prior teeth prophylaxis is not mandatory before application. Rapid setting time makes it an ideal material even for younger children or patients with gagging reflux.[14] Indications of topical fluoride varnish applications are mainly dental prophylaxis treatment of incipient caries or root caries. Hypersensitivity of teeth or roots is another indicator where fluoride varnish can act as a pain relief. Varnishes are indicated for use in patients with bad hygiene that has no prognosis of getting better, including handicapped and senile patients or children. They are particularly recommended for children using orthodontic appliances, adults using prosthetic restorations and patients with decreased salivary secretion.[104]

Among the contraindications, there are confirmed statuses like bronchial asthma, stomatitis or necrotizing ulcerative gingivitis. Each applied dose of a varnish contains up to 0.2 g of ethanol, and therefore usage during pregnancy or lactation is not recommended. Application of fluoride varnish may occasionally cause temporary discoloration of teeth after contact with fluoride, lasting around 24 h after the outer layer of varnish is removed with brushing.[29] There is a possibility of allergy to fluoride varnish, which can cause a burning sensation in the mouth, eventually causing dermatitis or stomatitis if the varnish had contact with either skin tissue or oral mucosa. A lot of varnishes, e.g., Duraphat® (Colgate Oral Care, Sydney, Australia), contain rosin. Children who were hospitalized during previous 12 months due to severe asthma or allergies or who are allergic to patches may be at risk of an allergic reaction to rosin. In such cases, usage of varnish without rosin should be considered (approved for caries prevention in the UK) or alternative age-appropriate fluoride preparations should be suggested (e.g., fluoride mouthwash or toothpaste with a higher fluoride concentration). Likewise, fluoride varnishes containing the CPP-ACP complex are not recommended for patients allergic to milk. Swallowing the topical fluoride varnish may result in elevation of plasma fluoride level, yet such increase is lower than when compared to fluoride gels.[105]

Discussion

The effectiveness of the cariostatic action of fluoride ions can be attributed, on one hand, to their multidirectional effect on the carious process.[106–110] On the other hand, it affects the hard tissues of the tooth from mineralization during development to external protection in the oral environment. Fluoride released from fluoride varnishes increases its concentration in both saliva and plaque, interacts with enamel hydroxyapatites, interferes with bacterial metabolism, and accumulates on the tooth surface in the form of CaF_2. These deposits, according to Øgaard et al.,[24] could be observed in vitro for up to 4 months after application, while in vivo, due to the processes involved in chewing, swallowing, speech, and individual hygiene procedures, the time period of CaF_2 deposit removal was definitely shorter when compared to in vitro observations, reaching up to a maximum of 3 days.[111] According to Tenuta et. al.,[77] it is the amount of calcium fluoride formed that will have a direct impact on the long-term maintenance of higher fluoride levels in the mouth and the effective long-term anti-carious effect. Other researchers[40,67,82,83] emphasize the importance of calcium and phosphate ions present in the mouth as a factor that has a significant impact on the amount of fluoride captured and bound.[24,25,34,50]

Despite a very large number of studies conducted both in vitro and in vivo, there is still no clear opinion regarding the choice of the best fluoride varnish. Numerous experiments have shown that all varnishes of both the first and second generation applied to tooth surfaces release fluoride ions and cause them to accumulate in the enamel and plaque.[23,25,46] In in vitro studies, fluoride release was greatest during the first few hours after application, then decreased over time, with some investigators observing this on the 1st day after application[46,69,112] and others within 3 weeks.[25,50] The highest increase in fluoride was usually measured either after the 1st[46,112] or the 2nd hour after application.[25,113,114] Cumulative fluoride release, even for varnishes containing the same amount of fluoride, was not uniform and was likely due to differences in formulation, consistency and viscosity of the formulations.[22–28,115] Shen and Autio-Gold[115] obtained lower cumulative fluoride release in artificial saliva from Duraphat® (Colgate Oral Care) compared to Duraflor® (AMD Medicom Inc., Montreal, Canada) and CavityShield® (3M ESPE Dental Products, St. Paul, USA) (all 3 varnishes with 5% NaF), but for all, the decrease in ion emission between 7 h and 213 h of the experiment was similar.

Under in vivo conditions, the decrease in fluoride release was faster due to the effects of saliva on fluoride leaching and retention, as well as due to cheek and tongue muscle work, chewing, diet acidity, or hygiene treatments, but the pattern overlapped with in vitro studies. Most fluoride was released during the first few hours after application; in some studies,[46,112] it was the 2nd hour of the study, and in others the first 4 h.[36,58] A study by Piesiak-Pańczyszyn and Kaczmarek[46] showed a significant increase in salivary fluoride release during the first 2 h after application, which decreased with time, with the highest increase in the 1st hour – more than 15 times the initial value, in the 2nd hour slightly less, representing 9 times the initial level, and decreasing in the 168th hour of the experiment to a value oscillating near the initial value and corresponding to the cariostatic level. Comparing first- and second-generation varnishes with each other, it turns out that opinions on their effectivness and the factors affecting them are divided.[45] Based on data from the literature, one can find

both works that, evaluating the amount of fluoride ions released, as well as the composition and mechanical properties of varnish-treated enamel surfaces, showed the superiority of second-generation varnishes over the first one,[23,38,45,46,48,49,60,78,85–92,112] as well as those that did not show these differences[28,76,78] or even studies reporting the higher effectiveness of non-enriched varnishes.[23,42,53]

In a number of comparative studies, the best performing varnish was the second-generation one containing the CPP-ACP complex, for which the highest cumulative fluoride release and the largest increases in released fluoride over time were recorded.[23,46,85,112] In addition, it is also noteworthy that this release proceeded most dynamically in the initial phase, causing the release of more than 90% of the total available amount of fluoride applied in the varnish in a fairly short time (up to 24 h).[46,112] This was confirmed in a laboratory study performed by the ADA in 2015.[116] This study considered 7 varnishes, from which only 2: – MI Varnish (GC Corporation, Tokyo, Japan) (with CPP-ACP complex) and Prevident® varnish (Colgate Oral Pharmaceuticals, Inc., Canton, USA) (with 5% NaF and xylitol) – released almost 100% of the available fluoride within the first 6 h after application, while the other 5 reached levels between 29% and 53%. In contrast, Milburn et al.[50] showed in vitro that CXP-containing Embrace™ varnish (Pulpdent, Watertown, USA) released as much as 10-fold more fluoride in the first 4 h after application than Duraphat® (Colgate Oral Care), Enamel Pro Varnish (Premier Dental Products, Plymouth Meeting, USA) and Vanish (3M ESPE Dental Products). There are other papers that show higher amounts of fluoride ions released from varnishes with amorphous calcium phosphate (ACP).[25,26,117] In a study by Jablonowski et al.,[25] highest cumulative release of fluoride and highest rate of release were recorded for the Enamel Pro varnish with the addition of ACP for timeframe up to 3 weeks after application. Limit of detection in the referenced work oscillated around 4th week of the experiment for Enamel Pro Varnish (Premier Dental Products) compared to Duraphat® (Colgate Oral Care) and Clinpro XT (3 M ESPE Dental Products) varnishes, which reached limit of detection after approx. 6–7 weeks. It translates to a conclusion that varnish containing ACP in relatively short time released all available fluoride, causing its high concentration in the initial phase, which is considered critical.

In the case of second-generation varnishes, fluoride release is often observed in 2 phases, which was decribed by Pichaiaukrit et al.[66] The phase of initial rapid release of large amounts of fluoride usually lasts until the first 4 h after application, depending on the varnish; between 12 h and 24 h, a steady, slower increase in fluoride commences, reaching a plateau.[34,46,50,112] In line with previous opinions[26,44] that a sufficiently high initial concentration of fluoride allows the formation of more calcium fluoride on the enamel surface and facilitates its binding to tooth tissues, and because the retention time of the tooth varnish is limited under in vivo conditions, such fluoride release characteristics can have a pronounced impact on the therapeutic efficacy of the applied agent. Some researchers also noted the highly significant effect of medium pH on the amount of fluoride ions released.[46] In the studies by Piesiak-Pańczyszyn and Kaczmarek[46] and by Piesiak-Pańczyszyn et al.,[112] the level of fluoride released for both first (Duraphat®; Colgate Oral Care) and second-generation varnishes: MI Varnish (GC Corporation) and Embrance Varnish (Pulpdent, Watertown, USA) was significantly lower in neutral environments than in pH = 4 and pH = 5 environments, as confirmed in post hoc tests. In addition, regression analysis showed that the acidity of the artificial saliva had a greater effect on fluoride ion release than time since application. On the other hand, Ten Cate et al.,[37] evaluating the enamel surface after application of 2% neutral NaF compared to acidified fluoride preparations, found the presence of significantly higher amounts of CaF_2 in the latter case. Similar conclusions were obtained in the Fluor Protector study.[36,37]

Fluoride varnishes, according to current knowledge, are the safest and most effective means in the treatment and prevention of tooth decay and hypersensitivity.[118] Despite the fact that they contain almost twice as much fluoride as the gels and foams used, and more than 15 times as much fluoride as everyday toothpastes, they do not entail health risk.[119] The most commonly used fluoride varnishes in dentistry are those with concentrations of 5% NaF, which is equivalent to 22,600 ppm of fluoride.[102,119,120] Indeed, it has been shown that the maximum fluoride concentration in serum after application of varnish with 5% NaF in young children is only 1/7 of the maximum values found after application of APF gel with 1.25% NaF.[121,122] This is due to the ability to apply the product very precisely to the teeth, as well as its adherence to the enamel surface, which prevents unintentional swallowing.[122] An analysis of the number of adverse events reported to the U.S. Food and Drug Administration (FDA) confirmed that the use of fluoride varnish should be considered safe in the systemic aspect.[123] On the other hand, reports of varnish cytotoxicity appearing in the literature draw attention. Due to the close contact of fluoride varnish with enamel, dentin or adjacent soft tissues including the gingival mucosa, it is necessary to rule out cytotoxicity prior clinical use. Evaluation of the possible cytotoxic effect on human tissues caused by fluoride varnishes is important for the health of oral cavity tissues.[14,124–126]

Biocompatibility of materials that are prepared to be used clinically in dentistry is evaluated using primary human cells, including fibroblast cells or odontoblasts cells; e.g., Sengun et al.[127] and Hoang-Dao et al.[128] confirmed evaluating cytotoxic effects on primary gingival fibroblasts, as well as pulp fibroblasts from dentin slice, due to the fact that both of them are directly exposed during the procedure of fluoride varnish application. In such evaluations, it is extremely important to determine the cytotoxicity

of a specific agent in vitro. Cytotoxicity can be properly measured only when there is direct contact between the specific material and gingival cells. Hoang-Dao et al.[128] confirmed that toxicity of given material is generally lower on dentin slice when compared to gingival fibroblasts. It can be explained by the buffering capacity of dentin.[129] There is also additional risk of swallowing fluoride varnishes, which should be avoided because of renal toxicity risk.[18] Moreover, it is very important to follow the clinician's recommendations regarding the dosage of fluoride varnishes. Prolonged inappropriate effects of fluoride varnishes on soft tissues in the oral cavity can cause, although rare and preventable, acute topical toxicity involving oral mucosa irritation.[130,131]

Conclusions

Despite many studies conducted both in vitro and in vivo, we still do not know all the mechanisms affecting the effect of fluoride on dental hard tissues, nor are we able to identify all the relevant variables that may intensify or limit this effect. In addition, in light of emerging information about the local negative effects of fluoride varnishes on soft tissues, it is necessary to continue experiments to maximize efficacy and safety.

ORCID iDs

Dagmara Piesiak-Pańczyszyn https://orcid.org/0000-0002-5218-197X
Wojciech Zakrzewski https://orcid.org/0000-0002-6242-1180
Aleksandra Piszko https://orcid.org/0000-0003-0386-216X
Paweł J. Piszko https://orcid.org/0000-0002-7577-8509
Maciej Dobrzyński https://orcid.org/0000-0003-2368-1534

References

1. Lech T. Fatal cases of acute suicidal sodium and accidental zinc fluorosilicate poisoning: Review of acute intoxications due to fluoride compounds. *Forensic Sci Int*. 2011;206(1–3):e20–e24. doi:10.1016/j.forsciint.2010.06.027
2. Ozsvath DL. Fluoride and environmental health: A review. *Rev Environ Sci Biotechnol*. 2009;8(1):59–79. doi:10.1007/s11157-008-9136-9
3. Benson PE, Parkin N, Dyer F, Millett DT, Furness S, Germain P. Fluorides for the prevention of early tooth decay (demineralised white lesions) during fixed brace treatment. *Cochrane Database Syst Rev*. 2013;2013:CD003809. doi:10.1002/14651858.CD003809.pub3
4. Twetman S, Keller MK. Fluoride rinses, gels and foams: An update of controlled clinical trials. *Caries Res*. 2016;50(Suppl 1):38–44. doi:10.1159/000439180
5. Wong MCM, Clarkson J, Glenny AM, et al. Cochrane reviews on the benefits/risks of fluoride toothpastes. *J Dent Res*. 2011;90(5):573–579. doi:10.1177/0022034510393346
6. Marinho V. Cochrane fluoride reviews: An overview of the evidence on caries prevention with fluoride treatments. *Fac Dent J*. 2014;5(2):78–83. doi:10.1308/rcsfdj.2014.5.2.78
7. Walsh T, Worthington HV, Glenny AM, Marinho VC, Jeroncic A. Fluoride toothpastes of different concentrations for preventing dental caries. *Cochrane Database Syst Rev*. 2019;2019:CD007868. doi:10.1002/14651858.CD007868.pub3
8. Haugejorden O, Magne Birkeland J. Analysis of the ups and downs of caries experience among Norwegian children aged five years between 1997 and 2003. *Acta Odontol Scand*. 2005;63(2):115–122. doi:10.1080/00016350510019784
9. Olczak-Kowalczyk D, Mielczarek A, Jackowska T, et al. Fluoride agents in the prevention and treatment of dental caries and erosion in children, adolescents and adults: Recommendations of Polish Experts. Update of recommendations: Individual fluoride prevention in children and adolescents – recommendations of Polish Experts [in Polish]. *Nowa Stomatol*. 2022;27(2):35–59. doi:10.25121/NS.2022.27.2.35
10. Bonetti D, Clarkson JE. Fluoride varnish for caries prevention: Efficacy and implementation. *Caries Res*. 2016;50(Suppl 1):45–49. doi:10.1159/000444268
11. Fejerskov O, Nyvad B, Kidd EAM, eds. *Dental Caries: The Disease and Its Clinical Management*. 3rd ed. Chichester, UK: John Wiley & Sons Inc; 2015. ISBN:978-1-118-93582-8.
12. Olczak-Kowalczyk D, Borysewicz-Lewicka M, Adamowicz-Klepalska B, Jackowska T, Kaczmarek U, Kaczmarek U. Consensus statement of Polish experts on individual caries prevention with fluoride in children and adolescents. *Nowa Stomatol*. 2016;21(1):47–73. doi:10.5604/14266911.1199068
13. Heuser H, Schmidt H. Zahnkariesprophylaxe durch Tiefenimprägnierung des Zahnschmelzes mit Fluor-Lack. *Stoma*. 1968;21:91–100.
14. Chu CH, Lo ECM. A review of sodium fluoride varnish. *Gen Dent*. 2006;54(4):247–253. PMID:16903196.
15. Azarpazhooh A, Main PA. Fluoride varnish in the prevention of dental caries in children and adolescents: A systematic review. *J Can Dent Assoc*. 2008;74(1):73–79. PMID:18298889.
16. Carvalho DM, Salazar M, Oliveira BHD, Coutinho ESF. Fluoride varnishes and decrease in caries incidence in preschool children: A systematic review. *Rev Bras Epidemiol*. 2010;13(1):139–149. doi:10.1590/S1415-790X2010000100013
17. Marinho V. Cochrane reviews of randomized trials of fluoride therapies for preventing dental caries. *Eur Arch Paediatr Dent*. 2009;10(3):183–191. doi:10.1007/BF03262681
18. Marinho V, Worthington HV, Walsh T, Clarkson JE. Fluoride varnishes for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev*. 2013;2013:CD002279. doi:10.1002/14651858.CD002279.pub2
19. Strohmenger L, Brambilla E. The use of fluoride varnishes in the prevention of dental caries: A short review. *Oral Dis*. 2008;7(2):71–80. doi:10.1034/j.1601-0825.2001.70202.x
20. Autio-Gold JT, Courts F. Assessing the effect of fluoride varnish on early enamel carious lesions in the primary dentition. *J Am Dent Assoc*. 2001;132(9):1247–1253. doi:10.14219/jada.archive.2001.0367
21. Weintraub JA, Ramos-Gomez F, Jue B, et al. Fluoride varnish efficacy in preventing early childhood caries. *J Dent Res*. 2006;85(2):172–176. doi:10.1177/154405910608500211
22. Virupaxi SG, Roshan N, Poornima P, Nagaveni N, Neena I, Bharath K. Comparative evaluation of longevity of fluoride release from three different fluoride varnishes: An in vitro study. *J Clin Diagn Res*. 2016;10(8):ZC033–ZC036. doi:10.7860/JCDR/2016/19209.8242
23. Cochrane N, Shen P, Yuan Y, Reynolds E. Ion release from calcium and fluoride containing dental varnishes. *Aust Dent J*. 2014;59(1):100–105. doi:10.1111/adj.12144
24. Øgaard B. The cariostatic mechanism of fluoride. *Compend Contin Educ Dent*. 1999;20(1 Suppl):10–17; quiz 34. PMID:11908400
25. Jablonowski BL, Bartoloni JA, Hensley DM, Vandewalle KS. Fluoride release from newly marketed fluoride varnishes. *Quintessence Int*. 2012;43(3):221–228. PMID:22299122.
26. Ritwik P, Aubel J, Xu X, Fan Y, Hagan J. Evaluation of short term fluoride release from fluoride varnishes. *J Clin Pediatr Dent*. 2012;36(3):275–278. doi:10.17796/jcpd.36.3.q304488478w52334
27. Castillo JL, Milgrom P, Kharasch E, Izutsu K, Fey M. Evaluation of fluoride release from commercially available fluoride varnishes. *J Am Dent Assoc*. 2001;132(10):1389–1392. doi:10.14219/jada.archive.2001.0053
28. Shen P, Bagheri R, Walker G, et al. Effect of calcium phosphate addition to fluoride containing dental varnishes on enamel demineralization. *Aust Dent J*. 2015;61(3):357–365. doi:10.1111/adj.12385
29. Baik A, Alamoudi N, El-Housseiny A, Altuwirqi A. Fluoride varnishes for preventing occlusal dental caries: A review. *Dent J*. 2021;9(6):64. doi:10.3390/dj9060064
30. Wagner J, Kawamoto A. Fluoride varnish compositions including an organo phosphoric acid adhesion promoting agent. US patent US20130149391A1. July 10, 2014. https://patents.google.com/patent/US20130149391A1/en.

31. Kolter K, Schönherr M, Gebert S, Meyer-Böhm K, Maschke A. Pharmaceutical formulation for the production of rapidly disintegrating tablets. US patent US20100178349A1. September 10, 2019. https://patents.google.com/patent/US20100178349A1/en.
32. Wang W, Xie Q, Xu T, Wang Q, Malmstrom HS, Ren YF. Fluoride release and anti-erosive effects of dentifrices containing PVM/MA copolymers. *J Dent*. 2013;41(2):148–154. doi:10.1016/j.jdent.2012.10.013
33. Levy FM, Rios D, Buzalaf MAR, Magalhães AC. Efficacy of TiF4 and NaF varnish and solution: A randomized in situ study on enamel erosive–abrasive wear. *Clin Oral Invest*. 2014;18(4):1097–1102. doi:10.1007/s00784-013-1096-y
34. Comar LP, Souza BMD, Grizzo LT, Buzalaf MAR, Magalhães AC. Evaluation of fluoride release from experimental TiF4 and NaF varnishes in vitro. *J Appl Oral Sci*. 2014;22(2):138–143. doi:10.1590/1678-775720130574
35. Wagner J, Kawamoto A. Fluoride varnish compositions including an organo phosphoric acid adhesion promoting agent. US patent US8852561B2. July 10, 2014. https://patents.google.com/patent/US8852561B2/en.
36. Twetman S, Sköld-Larsson K, Modéer T. Fluoride concentration in whole saliva and separate gland secretions after topical treatment with three different fluoride varnishes. *Acta Odontol Scand*. 1999;57(5):263–266. doi:10.1080/000163599428670
37. Ten Cate J, Larsen M, Pearce E, Fejerskov O. Chemical interactions between the tooth and oral fluids. In: *Dental Caries: The Disease and Its Clinical Management*. Oxford, UK: Blackwell Munksgaard; 2003:49–69. ISBN:978-1-4051-0718-1.
38. Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC. New approaches to enhanced remineralization of tooth enamel. *J Dent Res*. 2010;89(11):1187–1197. doi:10.1177/0022034510376046
39. Reynolds E. Calcium phosphate-based remineralization systems: Scientific evidence? *Aust Dent J*. 2008;53(3):268–273. doi:10.1111/j.1834-7819.2008.00061.x
40. Vogel GL. Oral fluoride reservoirs and the prevention of dental caries. *Mongr Oral Sci*. 2011;22:146–157. doi:10.1159/000325166
41. Santos LDM, Reis JILD, Medeiros MPD, Ramos SM, Araújo JMD. In vitro evaluation of fluoride products in the development of carious lesions in deciduous teeth. *Braz Oral Res*. 2009;23(3):296–301. doi:10.1590/S1806-83242009000300012
42. Carvalho TS, Peters BG, Rios D, et al. Fluoride varnishes with calcium glycerophosphate: Fluoride release and effect on in vitro enamel demineralization. *Braz Oral Res*. 2015;29:S1806-83242015000100287. doi:10.1590/1807-3107BOR-2015.vol29.0092
43. Jafari K, Hekmatfar S, Fereydunzadeh M. In vitro comparison of antimicrobial activity of conventional fluoride varnishes containing xylitol and casein phosphopeptide-amorphous calcium phosphate. *J Int Soc Prev Community Dent*. 2018;8(4):309–313. doi:10.4103/jispcd.JISPCD_67_18
44. Carey CM. Focus on fluorides: Update on the use of fluoride for the prevention of dental caries. *J Evid Based Dent Pract*. 2014;14:95–102. doi:10.1016/j.jebdp.2014.02.004
45. Walczak M, Turska-Szybka A. The efficacy of fluoride varnishes containing different calcium phosphate compounds. *Fluoride*. 2017;50:151–160. https://www.proquest.com/docview/1942213151. Accessed September 13, 2023.
46. Piesiak-Pańczyszyn D, Kaczmarek U. Fluoride release from fluoride varnish under in vitro and in vivo conditions. *Dent Med Probl*. 2017;54(4):327–331. doi:10.17219/dmp/78887
47. Chhatwani S, Hoppe J, Naumova EA, et al. Fluoride ion release characteristics of fluoride-containing varnishes: An in vitro study. *Appl Sci*. 2021;11(4):1452. doi:10.3390/app11041452
48. Schemehorn BR, Orban JC, Wood GD, Fischer GM, Winston AE. Remineralization by fluoride enhanced with calcium and phosphate ingredients. *J Clin Dent*. 1999;10(1 Spec No):13–16. PMID:10686853.
49. Güçlü ZA, Alaçam A, Coleman NJ. A 12-week assessment of the treatment of white spot lesions with CPP-ACP paste and/or fluoride varnish. *Biomed Res Int*. 2016;2016:8357621. doi:10.1155/2016/8357621
50. Milburn JL, Henrichs LE, Banfield R, Stansell M, Vandewalle K. Substantive fluoride release from a new fluoride varnish containing CXP. *Dentistry*. 2015;5(12):1000350. doi:10.4172/2161-1122.1000350
51. Karlinsey RL, Mackey AC, Schwandt CS, Walker TJ. SEM evaluation of demineralized dentin treated with professional-strength NaF topical pastes. *Am J Dent*. 2011;24(6):357–362. PMID:22263333.
52. Elkassas D, Arafa A. Remineralizing efficacy of different calcium-phosphate and fluoride based delivery vehicles on artificial caries like enamel lesions. *J Dent*. 2014;42(4):466–474. doi:10.1016/j.jdent.2013.12.017
53. Mohd Said SNB, Ekambaram M, Yiu CKY. Effect of different fluoride varnishes on remineralization of artificial enamel carious lesions. *Int J Paed Dentistry*. 2016;27(3):163–173. doi:10.1111/ipd.12243
54. AlAmoudi SA, Pani SC, AlOmari M. The effect of the addition of tricalcium phosphate to 5% sodium fluoride varnishes on the microhardness of enamel of primary teeth. *Int J Dent*. 2013;2013:486358. doi:10.1155/2013/486358
55. Cross KJ, Huq NL, Palamara JE, Perich JW, Reynolds EC. Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. *J Biol Chem*. 2005;280(15):15362–15369. doi:10.1074/jbc.M413504200
56. Reynolds EC, Cai F, Cochrane NJ, et al. Fluoride and casein phosphopeptide-amorphous calcium phosphate. *J Dent Res*. 2008;87(4):344–348. doi:10.1177/154405910808700420
57. Farooq I, Moheet IA, Imran Z, Farooq U. A review of novel dental caries preventive material: Casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) complex. *Saudi J Dent Res*. 2013;4(2):47–51. doi:10.1016/j.ksujds.2013.03.004
58. Eakle WS, Featherstone JDB, Weintraub JA, Shain SG, Gansky SA. Salivary fluoride levels following application of fluoride varnish or fluoride rinse. *Community Dent Oral Epidemiol*. 2004;32(6):462–469. doi:10.1111/j.1600-0528.2004.00185.x
59. Patel PM. Comparison of the effect of fluoride varnish, chlorhexidine varnish and casein phosphopeptide-amorphous calcium phosphate (CPP–ACP) varnish on salivary streptococcus mutans level: A six month clinical study. *J Clin Diagn Res*. 2017;11(8):ZC53–ZC59. doi:10.7860/JCDR/2017/26541.10409
60. Tuloglu N, Bayrak S, Tunc ES, Ozer F. Effect of fluoride varnish with added casein phosphopeptide-amorphous calcium phosphate on the acid resistance of the primary enamel. *BMC Oral Health*. 2016;16(1):103. doi:10.1186/s12903-016-0299-4
61. Zhang Q, Zou J, Yang R, Zhou X. Remineralization effects of casein phosphopeptide-amorphous calcium phosphate crème on artificial early enamel lesions of primary teeth. *Int J Paediatr Dent*. 2011;21(5):374–381. doi:10.1111/j.1365-263X.2011.01135.x
62. Bakry AS, Abbassy MA. Increasing the efficiency of CPP-ACP to remineralize enamel white spot lesions. *J Dent*. 2018;76:52–57. doi:10.1016/j.jdent.2018.06.006
63. Świetlicka I, Kuc D, Świetlicki M, et al. Near-surface studies of the changes to the structure and mechanical properties of human enamel under the action of fluoride varnish containing CPP–ACP compound. *Biomolecules*. 2020;10(5):765. doi:10.3390/biom10050765
64. Yapp R, Powers J. Fluoride ion release from several fluoride varnishes. *Dental Advisor*. 2012;44:1–2. https://www.dentaladvisor.com/pdf-download/?pdf_url=wp-content/uploads/2015/02/fluoride-ion-release-from-several-fluoride-varnishes.pdf. Accessed September 23, 2023.
65. Premier Dental. Hygiene/Preventative equipment. Plymouth Meeting, USA: Premier Dental; 2023. http://premusa.com/dental/hygiene.asp. Accessed September 23, 2023.
66. Pichaiaukrit W, Thamrongananskul N, Siralertmukul K, Swasdison S. Fluoride varnish containing chitosan demonstrated sustained fluoride release. *Dent Mater J*. 2019;38(6):1036–1042. doi:10.4012/dmj.2018-112
67. Attin T, Lennon AM, Yakin M, et al. Deposition of fluoride on enamel surfaces released from varnishes is limited to vicinity of fluoridation site. *Clin Oral Invest*. 2007;11(1):83–88. doi:10.1007/s00784-006-0080-1
68. Colgate Professional. Update: Duraphat Product Labelling. New York, USA: Colgate-Palmolive Company; 2022. https://www.colgateprofessional.co.uk/products/duraphat-product-labelling-update. Accessed September 19, 2023.
69. Byeon SM, Lee MH, Bae TS. The effect of different fluoride application methods on the remineralization of initial carious lesions. *Restor Dent Endod*. 2016;41(2):121. doi:10.5395/rde.2016.41.2.121
70. Erdem AP, Sepet E, Kulekci G, Trosola SC, Guven Y. Effects of two fluoride varnishes and one fluoride/chlorhexidine varnish on *Streptococcus mutans* and *Streptococcus sobrinus* biofilm formation in vitro. *Int J Med Sci*. 2012;9(2):129–136. doi:10.7150/ijms.3637

71. Premier Dental. Enamel Pro® Varnish. Plymouth Meeting, USA: Premier Dental; 2023. https://www.premierdentalco.com/product/hygienepreventative/fluoride/enamel-pro-varnish/. Accessed September 23, 2023.
72. Wefel JS. NovaMin®: Likely clinical success. *Adv Dent Res*. 2009;21(1): 40–43. doi:10.1177/0895937409335622
73. Simmer JP, Hardy NC, Chinoy AF, Bartlett JD, Hu JC. How fluoride protects dental enamel from demineralization. *J Int Soc Prev Community Dent*. 2020;10(2):134. doi:10.4103/jispcd.JISPCD_406_19
74. Kaczmarek U. Mechanizmy kariostatyczne fluoru. *Czas Stomatol*. 2005; 58(6):404–413. https://www.yumpu.com/xx/document/view/22985710/mechanizmy-kariostatyczne-fluoru. Accessed September 23, 2023.
75. Featherstone JDB. Prevention and reversal of dental caries: Role of low level fluoride. *Community Dent Oral Epidemiol*. 1999;27(1):31–40. doi:10.1111/j.1600-0528.1999.tb01989.x
76. Beltrán-Aguilar ED, Goldstein JW, Lockwood SA. Fluoride varnishes: A review of their clinical use, cariostatic mechanism, efficacy and safety. *J Am Dent Assoc*. 2000;131(5):589–596. doi:10.14219/jada.archiv.2000.0232
77. Tenuta LMA, Cerezetti RV, Del Bel Cury AA, Tabchoury CPM, Cury JA. Fluoride release from CaF_2 and enamel demineralization. *J Dent Res*. 2008;87(11):1032–1036. doi:10.1177/154405910808701105
78. Girish Babu K, Subramaniam P, Teleti S. Remineralization potential of varnish containing casein phosphopeptides-amorphous calcium phosphate with fluoride and varnish containing only fluoride: A comparative study. *Saudi J Oral Sci*. 2018;5(1):35–40. doi:10.4103/sjos.SJOralSci_44_17
79. Rosin-Grget K, Lincir I. Current concept on the anticaries fluoride mechanism of the action. *Coll Antropol*. 2001;25(2):703–712. PMID:11811302.
80. Mielczarek A, Michalik J, Kujawa M. The effect of selected fluoride products on microstructure of early caries lesions [in Polish]. *Nowa Stomatol*. 2013;3:120–124. http://www.nstomatologia.pl/wp-content/uploads/2014/10/ns_2013_120-124.pdf. Accessed September 14, 2023.
81. Holve S. An observational study of the association of fluoride varnish applied during well child visits and the prevention of early childhood caries in American Indian children. *Matern Child Health J*. 2007;12(S1):64–67. doi:10.1007/s10995-007-0294-0
82. Whitford GM, Wasdin JL, Schafer TE, Adair SM. Plaque fluoride concentrations are dependent on plaque calcium concentrations. *Caries Res*. 2002;36(4):256–265. doi:10.1159/000063931
83. Fejerskov E, Clarkson O. Fluoride in caries control. In: *Dental Caries: The Disease and Its Clinical Management*. Oxford, UK: Blackwell Munksgaard; 2003:49–69. ISBN:978-1-4051-0718-1.
84. Attin T, Hartmann O, Hilgers RD, Hellwig E. Fluoride retention of incipient enamel lesions after treatment with a calcium fluoride varnish in vivo. *Arch Oral Biol*. 1995;40(3):169–174. doi:10.1016/0003-9969(95)98804-8
85. Pithon MM, Baião FS, Sant'Anna LID, Tanaka OM, Cople-Maia L. Effectiveness of casein phosphopeptide-amorphous calcium phosphate-containing products in the prevention and treatment of white spot lesions in orthodontic patients: A systematic review. *J Investig Clin Dent*. 2019;10(2):e12391. doi:10.1111/jicd.12391
86. Majithia U, Venkataraghavan K, Choudhary P, Trivedi K, Shah S, Virda M. Comparative evaluation of application of different fluoride varnishes on artificial early enamel lesion: An in vitro study. *Indian J Dent Res*. 2016;27(5):521–527. doi:10.4103/0970-9290.195642
87. Somasundaram P, Mandke L, Vimala N. Protective potential of casein phosphopeptide amorphous calcium phosphate containing paste on enamel surfaces. *J Conserv Dent*. 2013;16(2):152–156. doi:10.4103/0972-0707.108199
88. Llena C, Leyda AM, Forner L. CPP-ACP and CPP-ACFP versus fluoride varnish in remineralisation of early caries lesions: A prospective study. *Eur J Paediatr Dent*. 2015;16(3):181–186. PMID:26418918.
89. Memarpour M, Fakhraei E, Dadaein S, Vossoughi M. Efficacy of fluoride varnish and casein phosphopeptide-amorphous calcium phosphate for remineralization of primary teeth: A randomized clinical trial. *Med Princ Pract*. 2015;24(3):231–237. doi:10.1159/000379750
90. Salman NR, ElTekeya M, Bakry N, Omar SS, El Tantawi M. Comparison of remineralization by fluoride varnishes with and without casein phosphopeptide amorphous calcium phosphate in primary teeth. *Acta Odontol Scand*. 2019;77(1):9–14. doi:10.1080/00016357.2018.1490967
91. Ekambaram M, Mohd Said S, Yiu C. A review of enamel remineralisation potential of calcium- and phosphate-based remineralisation systems. *Oral Health Prev Dent*. 2017;15(5):415–420. doi:10.3290/j.ohpd.a38779
92. Damyanova D, Angelova S, Targova-Dimitrova T. Clinical study remineralization effect of mineralization varnish. *IOSR J Dent Med Sci*. 2016;15(8):134–136. doi:10.9790/0853-150804134136
93. Centers for Disease Control and Prevention (CDC). Recommendations for using fluoride to prevent and control dental caries in the United States. Atlanta, USA: Centers for Disease Control and Prevention (CDC); 2001. https://stacks.cdc.gov/view/cdc/5160. Accessed September 8, 2023.
94. Chestnutt IG, Chadwick BL, Hutchings S, et al. Protocol for "Seal or Varnish?" (SoV) trial: A randomised controlled trial to measure the relative cost and effectiveness of pit and fissure sealants and fluoride varnish in preventing dental decay. *BMC Oral Health*. 2012; 12(1):51. doi:10.1186/1472-6831-12-51
95. Phantumvanit P, Makino Y, Ogawa H, et al. WHO global consultation on public health intervention against early childhood caries. *Comm Dent Oral Epid*. 2018;46(3):280–287. doi:10.1111/cdoe.12362
96. American Dental Association (ADA). *Fluoridation Facts*. Niagara Falls, USA: American Dental Association (ADA); 2018. https://ebooks.ada.org/fluoridationfacts/. Accessed September 14, 2023.
97. American Dental Association Council on Scientific Affairs. Professionally applied topical fluoride: Evidence-based clinical recommendations. *J Am Dent Assoc*. 2006;137(8):1151–1159. doi:10.14219/jada.archive.2006.0356
98. European Academy of Paediatric Dentistry (EAPD). Guidelines on the use of fluoride in children: An EAPD policy document. *Eur Arch Paediatr Dent*. 2009;10(3):129–135. doi:10.1007/BF03262673
99. Petersen PE, Ogawa H. Prevention of dental caries through the use of fluoride: The WHO approach. *Community Dent Health*. 2016;33(2): 66–68. doi:10.1922/CDH_Petersen03
100. World Health Organization (WHO). *WHO Expert Consultation on Public Health Intervention Against Early Childhood Caries: Report of a Meeting*. Bangkok, Thailand: World Health Organization (WHO); 2016. https://apps.who.int/iris/bitstream/handle/10665/255627/WHO-NMH-PND-17.1-eng.pdf?sequence=1. Accessed September 1, 2023.
101. FDI World Dental Federation. FDI policy statement on promoting oral health through water fluoridation. *Int Dent J*. 2014;64(6):293–294. doi:10.1111/idj.12150
102. Weyant RJ, Tracy SL, Anselmo T, et al. Topical fluoride for caries prevention. *J Am Dent Assoc*. 2013;144(11):1279–1291. doi:10.14219/jada.archive.2013.0057
103. Jafarzadeh D, Rezapour R, Abbasi T, et al. The effectiveness of fluoride varnish and fissure sealant in elementary school children: A systematic review and meta-analysis. *Iran J Public Health*. 2022; 51(2):266–277. doi:10.18502/ijph.v51i2.8680
104. Zero DT, Fu J, Espeland MA, Featherstone JDB. Comparison of fluoride concentrations in unstimulated whole saliva following the use of a fluoride dentifrice and a fluoride rinse. *J Dent Res*. 1988;67(10): 1257–1262. doi:10.1177/00220345880670100201
105. Ekstrand J, Koch G, Petersson LG. Plasma fluoride concentration and urinary fluoride excretion in children following application of the fluoride-containing varnish Duraphat®. *Caries Res*. 1980; 14(4):185–189. doi:10.1159/000260452
106. Kosior P, Kaczmarek U. Effect in vitro of environmental parameters on the release of fluoride ions from some materials used in dentistry [in Polish]. *Ann Acad Med Stetin*. 2004;50(Suppl 1):65–68. PMID:16892588.
107. Beiruti N, Frencken JE, Van't Hof MA, Van Palenstein Helderman WH. Caries-preventive effect of resin-based and glass ionomer sealants over time: A systematic review. *Community Dent Oral Epidemiol*. 2006;34(6):403–409. doi:10.1111/j.1600-0528.2006.00321.x
108. Ahovuo-Saloranta A, Forss H, Walsh T, et al. Sealants for preventing dental decay in the permanent teeth. *Cochrane Database Syst Rev*. 2013;2013:CD001830. doi:10.1002/14651858.CD001830.pub4
109. Kosior P, Kaczmarek U. Release of fluoride ions into saliva from some dental materials [in Polish]. *Ann Acad Med Stetin*. 2004;50(Suppl 1): 62–64. PMID:16892587.
110. Kosior P. Fluoride release from selected dental materials in experimental and clinical conditions [doctoral thesis]. Wrocław, Poland: Wroclaw Medical University; 2005.

111. Eakle W, Kimberly G, Bastin G. *Dental Materials: Clinical Applications for Dental Assistants and Dental Hygienists*. Philadelphia, USA: Saunders; 2019. ISBN:978-0-323-60955-5.
112. Piesiak-Panczyszyn D, Watras A, Wiglusz RJ, Dobrzynski M. In vitro comparison of the fluoride ion release from the first- and second-generation fluoride varnishes. *Appl Sci*. 2023;13(12):7327. doi:10.3390/app13127327
113. Castillo JL, Milgrom P. Fluoride release from varnishes in two in vitro protocols. *J Am Dent Assoc*. 2004;135(12):1696–1699. doi:10.14219/jada.archive.2004.0121
114. Fernández CE, Tenuta LMA, Zárate P, Cury JA. Insoluble NaF in Duraphat® may prolong fluoride reactivity of varnish retained on dental surfaces. *Braz Dent J*. 2014;25(2):160–164. doi:10.1590/0103-6440201302405
115. Shen C, Autio-Gold J. Assessing fluoride concentration uniformity and fluoride release from three varnishes. *J Am Dent Assoc*. 2002;133(2):176–182. doi:10.14219/jada.archive.2002.0141
116. American Dental Association (ADA). Fluoride Varnish: A Clinical Perspective on the ADA Laboratory Evaluation. Niagara Falls, USA: American Dental Association (ADA); 2015. https://www.ada.org/en/es-MX/member-center/oral-health-topics/~/media/737FB2A4B88A4E9496F0F0A340525C29.ashx. Accessed September 23, 2023.
117. Gonzalez-Cabezas C, Flannagan S, Krell R, Uekihara M, Niquette C. Fluoride release from fluoride varnishes is affected by incubation conditions. American Association for Dental Research (AADR) 2012 Annual Meeting, March 21–24, 2012, Tampa, USA. Alexandria, USA: American Association for Dental Research (AADR).
118. Kirschneck C, Christl JJ, Reicheneder C, Proff P. Efficacy of fluoride varnish for preventing white spot lesions and gingivitis during orthodontic treatment with fixed appliances: A prospective randomized controlled trial. *Clin Oral Invest*. 2016;20(9):2371–2378. doi:10.1007/s00784-016-1730-6
119. Seshadri VRA, Varghese NS, Gurunathan D. Evaluation of the cytocompatibility of fluoride varnish and its effect on human gingival fibroblasts (hGFs): An in vitro study. *Cureus*. 2023;15(7):e41735. doi:10.7759/cureus.41735
120. Giacaman R, Fernández C, Muñoz-Sandoval C, Fuentes N. Longer retention time of fluoridated varnishes enhances enamel remineralisation in vitro. *Oral Health Prev Dent*. 2018;15(6):569–573. doi:10.3290/j.ohpd.a38778
121. Pendrys DG, Haugejorden O, Baårdsen A, Wang NJ, Gustavsen F. The risk of enamel fluorosis and caries among Norwegian children: Implications for Norway and the United States. *J Am Dent Assoc*. 2010;141(4):401–414. doi:10.14219/jada.archive.2010.0192
122. Browne D, Whelton H, O'Mullane D. Fluoride metabolism and fluorosis. *J Dent*. 2005;33(3):177–186. doi:10.1016/j.jdent.2004.10.003
123. Mascarenhas AK. Is fluoride varnish safe? Validating the safety of fluoride varnish. *J Am Dent Assoc*. 2021;152(5):364–368. doi:10.1016/j.adaj.2021.01.013
124. Eyüboğlu G, Yeşilyurt C, Ertürk M. Evaluation of cytotoxicity of dentin desensitizing products. *Oper Dent*. 2015;40(5):503–514. doi:10.2341/13-334-L
125. Parirokh M, Forghani FR, Paseban H, Asgary S, Askarifard S, Esmaeeli Mahani S. Cytotoxicity of two resin-based sealers and a fluoride varnish on human gingival fibroblasts. *Iran Endod J*. 2015;10(2):89–92. PMID:25834590. PMCID:PMC4372780.
126. Larpbunphol N, Chanamuangkon T, Thamrongananskul N. The fluoride release, abrasion resistance, and cytotoxicity to hGFs of a novel cyanoacrylate-based fluoride varnish compared with conventional fluoride varnish. *Dent Mater J*. 2022;41(5):757–766. doi:10.4012/dmj.2022-005
127. Sengun A, Buyukbas S, Hakki SS. Cytotoxic effects of dental desensitizers on human gingival fibroblasts. *J Biomed Mater Res*. 2006;78B(1):131–137. doi:10.1002/jbm.b.30464
128. Hoang-Dao BT, Hoang-Tu H, Tran-Hung L, Camps J, Koubi G, About I. Evaluation of a natural resin-based new material (Shellac F) as a potential desensitizing agent. *Dent Mater*. 2008;24(7):1001–1007. doi:10.1016/j.dental.2007.11.014
129. Camps J, Pashley DH. Buffering action of human dentin in vitro. *J Adhes Dent*. 2000;2(1):39–50. PMID:11317407.
130. Garcia RI, Gregorich SE, Ramos-Gomez F, et al. Absence of fluoride varnish-related adverse events in caries prevention trials in young children, United States. *Prev Chronic Dis*. 2017;14:160372. doi:10.5888/pcd14.160372
131. Quiñonez RB, Stearns SC, Talekar BS, Rozier RG, Downs SM. Simulating cost-effectiveness of fluoride varnish during well-child visits for MedicAid-enrolled children. *Arch Pediatr Adolesc Med*. 2006;160(2):164–170. doi:10.1001/archpedi.160.2.164