Alginate/chitosan core-shell beads with bioactive functionalities

Introduction

Bacterial infection still remains one of the most serious complications to human health. The widespread use of antibiotics has created evolutionary adaptation by bacteria, leading ultimately to the so-called multidrug resistance (MDR). The consequence of the increasing resistance of bacterial strains to biocidal agents is an increasing risk of chronic infections and difficulties in their treatment (*i.e.* wound infections, osteomyelitis, septic arthritis, endocarditis, *etc.*). Antibiotic therapies in many cases of bacterial chronic infections are very limited. Imperceptibly, multidrug resistance of bacteria has become a global problem, mainly because of the ease of spread of pathogens. Currently, the hospitalized patients in every region of the world are experiencing effects of the increased antimicrobial resistance. According to the ECDE/EMEA reports (the European Centre for Disease Prevention and Control/the European Medicines Agency), the most common bacterial infections are caused by antibiotic-resistant strains such as: methacillin resistance *Staphylococcus aureus* (MRSA), vancomycin intermediate resistance and vancomycin resistance *Staphylococcus aureus* (VISA, VRSA), penicillin resistance *Streptococcus pneumonia* (PRSP), carbapenem resistance non-fermentative Gram-(-) bacteria *e.g. Pseudomonas aeruginosa* [1]. Noteworthy, the most serious chronic infections, characterized by persistent inflammation and tissue damage, are caused by biofilm-growing bacteria. For example, chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients is caused by biofilm-growing mucoid strains. Whereas, in patients with diabetes, elevated blood glucose level directly affects the growth of the population of all bacterial and fungal pathogens, in particular *Pseudomonas aeruginosa*, or yeast-like fungi (*Candida albicans*).

In Poland 6% of the population has diabetes, it means about 2.5 million people between the 20th and 79th years of age (of which only half knows that he is ill). Moreover, 400-500 thousand people is at risk of diabetic foot, which often leads to the related complications and results in amputations. According to the Polish Diabetes Association, Poland is among the countries, which have the highest number of limbs amputation – 14,000 amputations per year (on average, every second patient with diabetic foot). This means that because of the complications of diabetic foot, on average, 38 amputations are made every day, of which at least 30 can be prevented [2]. The scale of the problem is therefore high. Only early intervention with appropriate treatment of difficult to heal wounds can prevent such a patient from amputation. Looking for the most effective way to fight down the bacteria that cause chronic wounds in diabetes still remains a huge challenge for nowadays scientists. Results of this project are mainly dedicated to diabetic foot infections treatment within long-term collaboration in this field with group of professor Piotr B. Heczko (Department of Microbiology of Jagiellonian University Medical College, Cracow).

Bacterial biofilm

After initial invasion, bacteria attach to living and non-living surfaces, such as prosthetics, medical devices or wounds and form a biofilm composed of polysaccharides, proteins, and other components. This complex structure facilitates bacteria colonization, subsequent acquisition of ecological niches as well as allows to survive in adverse environmental conditions. Thus, biofilm growth is bacteria defensible reply on changes in conditions of their living environment (pH, temperature, nutrition accessibility, applied antimicrobial therapies, *etc.*) and is associated with an increased level of bacteria mutations as well as with other complicated quorum-sensing-regulated mechanisms (*i.e.* a system of stimulus-response signals). In hosts, biofilm formation may trigger inflammation and drug resistance, resulting in persistent infections. Indeed, about half of bacterial infections are related to medical devices and implants for different biomedical applications. Moreover, the biofilm matrix provides a barrier to phagocytosis compared to free-living planktonic cells. Additionally, conventional resistance mechanisms such as chromosomal β-lactamase system, efflux pumps system and mutations in antibiotic target molecules of bacteria also contribute to high biofilm resistance. Finally, since biofilm is a consortium of bacteria embedded in a self-produced complex polymer matrix, it is a specific barrier for antimicrobial agents penetration and their activity [3-6].

The unique nature of a bacterial biofilm results in increased tolerance to antibiotics and disinfectants as well as resisting phagocytosis and other mechanisms of the body's defence system [4]. Therefore, **extensive efforts should be conducted toward finding antimicrobial agents with low toxicity to the host cell and a broad spectrum**

against a wide diversity of bacterial pathogens. It is equally important to develop effective delivery systems that integrate existing antibiotics and/or other biocidal agents with novel drug delivery systems to promote their antimicrobial activity.

Proposed antibacterial strategy

Noteworthy, most of bacterial infections can be prevented by early antibiotic or other antimicrobial agents prophylaxis or therapy. However, efficient antibacterial activity depends on successful biofilm penetration and cell wall reaching since action of the majority of antibiotic and other agents is localized in walls or inside the cells. Therefore, application of such strategies that increase biofilm susceptibility to antibiotics/agents is highly justifiable and reasonable. **A promising strategy, proposed in this project, is the use of enzymes that can dissolve the biofilm matrix (*e.g.* amylase, cellulase, lysozyme, alginate lyase, DNase, *etc.*) combining with subsequent antimicrobial agents application (*i.e.* antibiotic, functionalized metal nanoparticles, biofilm synthesis inhibitors, *etc.*).**

Currently used antimicrobial agents usually suffer from environmental toxicity, short-term antimicrobial activity or proteolytic instability and degradation. To overcome such disadvantages new antimicrobial agents are often physically incorporated into or chemically conjugated with polymers to enhance their antimicrobial efficacy and specificity, reduce cytotoxicity, prolong biostability and biocompatibility, and promote other biomimetic physicochemical properties. Two biopolymers – chitosan and alginate have nowadays attracted much attention due to their great potential in biomedical applications; *e.g.* they can be used as loading carriers to deliver different bioactive agents to target organisms, tissues, cells, and areas of the body. Both polymers are by far the most widely used polymer for immobilization and drug encapsulation technologies [7].

Chitosan is a cationic polymer from renewable resources (*i.e.* exoskeletons of marine crustaceans), obtained by N-acetylation of chitin. A major disadvantage is chitosan lack of solubility in near physiological pH, as the pK_a of the D-glucosamine residue is around 6.5 [8]. This property limits chitosan employment in enzyme immobilization since many enzymes are not stable at such low pH. This limitation could be overcome by surface modification of chitosan [9-11]. The other alternative is preparation of stable at physiological pH cross-linked beads and then enzyme immobilization. Noteworthy, chitosan can contribute into antimicrobial activity of the proposed hybrid capsules by its proposed mode of actions. Though demonstrated experimentally, the antibacterial activity of chitosan never has had its mechanism elucidated. Three possible modes of this activity have been considered. In one, electrostatic interactions between chitosan polycationic molecules and negatively charged cell membrane components are seen as responsible for the loosening of membrane structures and the consequent loss of cell components [12]. In the other one, chitosan is viewed as an agent penetrating through the cell wall inside the cells, where it interacts with DNA. This results in inhibition of both DNA transcription and mRNA and proteins synthesis. In the third mode by contrast, it is proposed that chitosan chelates metal ions and this leads to the production of toxins and to the arrest of cell cycle [8, 13, 14].

Alginates are naturally occurring polysaccharide copolymers, consisting of amino acid (1,4)-β-D-mannuronic and (1,3)-α-L-guluronic, covalently linked together by glycosidic linkages. This anionic polysaccharide is widely distributed in the cell walls of brown algae. Alginate has currently attracted much attention because of its unique properties such as: inexpensiveness, biocompatibility, a mild room temperature encapsulation technique, *etc.*. pK_a values of β-D-mannuronic acid and α-L-guluronic acid of alginate is 3.38 and 3.20, respectively [15]. Ionic complexation of alginate, having a negative charge, with chitosan possessing positive groups, ensure formation of gel or microcapsule system for various biomedical applications.

In the presented project polymeric core-shell beads constructed of alginate core loaded with active biocidal agent *e.g.* antibiotic, biofilm inhibitor or metal nanoparticles, *etc.* and with chitosan shell with immobilized enzyme increasing biofilm susceptibility to antimicrobial agents are proposed as a new effective biomaterials to fight down resistant pathogenic microorganisms producing biofilm. Noteworthy, since chitosan and alginate are well-known biopolymers, such hybrid biomaterials will show high biocompatibility, necessary in case of contact with healthy human cells. Firstly, outer chitosan shell with immobilized enzyme dissolves biofilm matrix. Then, after chitosan shell digestion by lysozyme (enzyme presents in human serum), cross-linked calcium-alginate inner core containing biocidal agent, disintegrating in physiological media by

phosphate ions, enables effective antimicrobial action. Chitosan was selected as the shell polymer since its antibacterial mode is well established and its surface can be easily functionalized [8, 9, 12, 13, 16-19].

Immobilized enzymes are generally more stable, therefore there are many potential applications, mainly in biotechnology and medicine. Enzyme immobilized polymeric capsules are common design systems providing a large surface for substrate and product diffusion. Nowadays, variety of enzyme immobilization techniques are available. Briefly, methods can be classified as physical (weak interaction) and chemical (covalent bonds). To the first group belongs enzyme adsorption, characterized by non-covalent bonding to insoluble support. The other techniques in this group are entrapment, encapsulation with liquid or solid matrix. The chemical immobilization techniques include covalent attachment to insoluble support and crosslinking [20].

To the best of our knowledge, there are any reports on a single antibacterial material containing biofilm dissolving enzymes and antibiotic for a diabetic foot treatment. However, there are increasing number of papers showing effectiveness of the separated application of enzymes having potency of biofilm matrix cleavage before antibiotic administration [21-23]. Noteworthy, in 2009 year Alipour *et al.* have shown that in case of mucin, sputum and biofilm growing *Pseudomonas aeruginosa* (PA) liposomal aminoglycosides have been found to be effective only when DNAse and alginate enzyme were applied [24]. Then, in 2012 year Aspe *et al.* have demonstrated important role of extracellular DNA in PA biofilms resistance to gentamicin. It was shown that the combination of alginate lyase and DNAase treatment of PA infections may be beneficial prior to administration of antibiotics [25]. Moreover, biodegradable polymer (chitosan) film loaded with antibiotic potentially advantageous as a clinically adjunctive treatment in musculoskeletal injuries to lessen or infection prevention has been presented by Smith *et al.* [26]. Likewise, Melake *et al.* have recently demonstrated bactericidal activity of tetracycline loaded chitosan microsphere towards PA biofilms. In the clinical field, such microspheres can be used as systems maintaining constant drug concentration for a prolonged period of treatment and maximize the therapeutic effect of antibiotics while minimizing antibiotic resistance. It was indicated that this approach can be a new strategy for the development of a specific drug delivery system increasing the efficacy of tetracycline in case of PA biofilm infections [27]. All these reports encourage for studying on biocompatible polymer systems including both biofilm digesting enzymes and antibiotics.

Summary

In conclusion, since bacteria resistance depends on their aggregation in multicellular community of polysaccharides, proteins, metabolites, DNA *etc.* (biofilm), an innovative strategy is to develop therapies that can disrupt the complex structure of the biofilm. The multicellularity of the biofilm defeat assures overcoming of the infection by the host defence system and/or the restore of the antibiotics efficacy. Proposed potential therapy includes usage of different types of hydrolytic enzymes (assuring cleavage of α- and β-glicosidic bounds), that can dissolve the polymeric matrix of the biofilm and subsequent application of a well-known bacteria-sensitive antibiotic.

Particularly in serious diabetic foot infections, the search for new therapeutic agents suitable for the fight down pathogenic microorganisms, is a very important challenge for modern science. **To the best of our knowledge, there are any papers presenting preparation and application of biopolymeric core-shell materials with bioactive functionalities such as biofilm dissolving enzymes and antibiotic for fighting down biofilm producing bacteria pathogens.** In future, prepared and studied in the presented project alginate/chitosan beads can be applied in diabetes wounds in form of aerosols, the most appropriate drug form in case of this serious chronic wounds.

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