UNIVERSITY OF SÃO PAULO

SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

Efeito de carreador lipídico nanoestruturado contendo quitosana em células livres e biofilme de *Escherichia coli*

Effect of nanostructured lipid carrier containing chitosan on free cells and biofilm of *Escherichia coli*

MICHAEL OLUWOLE OSUNGUNNA

Ribeirão Preto
2019

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Doctoral thesis presented to the Graduate Program of School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences.

Concentration Area: Pharmaceuticals and Cosmetics

Student: Michael Oluwole Osungunna

Supervisor: Prof. Dr. Carolina P. Aires Garbellini

The corrected version of the Doctoral Thesis was presented to the Graduate Program in Pharmaceutical Sciences on 02/10/2019. The original version is available at the School of Pharmaceutical Sciences of Ribeirão Preto/USP.

Ribeirão Preto
2019

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73p; 30cm.

PhD Thesis, submitted to the Faculty of Pharmaceutical Sciences of Ribeirão Preto/USP – Area of concentration: Pharmaceuticals and Cosmetics.

Supervisor: Carolina Patrícia Aires Garbellini

1. Chitosan. 2. Catheter. 3. Nanostructured lipid carrier. 4. Biofilms.

APPROVAL PAGE

Name: Michael Oluwole Osungunna

Título: Efeito de carreador lipídico nanoestruturado contendo quitosana em células livres e biofilme de *Escherichia coli*.

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DEDICATION

This work is dedicated to the living glory of the Most High God and my late brother, Oluyomi Osungunna who died on 20th November, 2018.

SPECIAL ACKNOWLEDGEMENT

I would like to specially acknowledge the efforts and support of Professor (Dr) Osvaldo de Freitas at ensuring that I did not lose my admission and for his fatherly roles always.

ACKNOWLEDGEMENT

I want to give glory to the Almighty God, the omnipotent, omnipresent and omniscience, the Alpha and Omega, the Author and Finisher of my faith for His grace and mercy in the last four years of this program and the successful completion of this work. Your name O' God is worthy of my praise.

I would like to thank the University of São Paulo and the Faculty of Pharmaceutical Sciences of Ribeirão Preto for the opportunity given me as well as the provision of materials and equipment required for this work

I would like to thank my indefatigable supervisor, Professor (Dr) Carolina Patrícia Aires Garbellini for sourcing me, believing in me and supporting me throughout the period of this program. Your expertise, thoroughness, persistence, constructive criticisms and academic reinvigoration are acknowledged and appreciated.

I would also like to acknowledge the efforts of Professor (Dr) Renata Fonseca Vianna Lopez at ensuring that I did not lose my admission and for her supports throughout the period of the program.

I would like to appreciate the efforts of Professor (Dr) Priscyla D. Marcato, Taís Nader Chrysostomo Massaro and Letícia Bueno Silva for the development and analysis of the formulation used in this study and for always being there when the need arises. Thank you.

My special thanks go to Professor (Dr) Andre Pitondo da Silva for his professional supports always and for the provision of bacterial strain used in this work.

I would like to acknowledge the supports of Professor (Dr) Luciane Carla Alberici and Carem Gledes Vargas Rechia, my PAE tutors, for making my participation in the program worthwhile.

To the Postgraduate School of Faculty of Pharmaceutical Science of Ribeirão Preto - USP and all the workers, especially Eleni and Rafael, I say thank you for your attention and services always.

I would also like to acknowledge the efforts, technical sagacity and motherly roles of Mrs Ana Cristina Morzelli Polizello. Your analytical and problem-solving mien knows no bound.

Also to Mrs. Ana Caleiro Seixas Azzolini and Mr. Alcides Silva Pereira, I say thank you for your technical and administrative assistance in the course of this program.

My special appreciation goes to Ana Carolina Santos Ré for her assistance and intellectual contribution to this work. I am also indebted to my other colleagues in the Laboratory; Maria Carolina Bonjovanni and Erika Kawakita, I say thank you for your supports and cooperation.

I would like to thank the management of Obafemi Awolowo University, Ile-Ife, Nigeria, for granting me a four-year study leave with pay to enable me pursue the program with ease.

I appreciate the efforts of the head of my department in Nigeria, Dr Oladimeji F. O. and for always being there when the need arose.

To my immediate past supervisor, Professor (Mrs) Onawunmi G. O., I say thank you for your support and efforts at releasing me to come for the program.

My acknowledgement would not be complete without appreciating my father, brother and boss, Professor Alebiowu, for his fatherly roles and being there for me always.

To other academic and non-academic members of staff of the Department of Pharmaceutics, Faculty of Pharmacy, O. A. U. Ile-Ife, Nigeria especially Akinwumi Florence and Salau Oladimeji, I say thank you.

To my dear wife, Comfort Oluyomi Osungunna, and my children, Christianah Adewumi, Deborah Adeola, Israel Adedeji and Dorcas Adetola, I say thank you for your cooperation, endurance and perseverance throughout the period of this program.

The effort of Dr (Mrs) Adewole of the Department of Sociology and Anthropology, Faculty of Social Sciences, O. A. U. Ile-Ife is acknowledged and appreciated.

To my other colleagues, especially Dr Ogunjimi Abayomi, Victor Oloruntoba Bankole and Jaiyesimi Olakunle, I say thank you all and God bless.

This work was supported by the Higher Education Personnel Improvement Coordination - Brazil (CAPES) - Financing Code 001.

RESUMO

OSUNGUNNA, M. O. **Efeito de carreador lipídico nanoestruturado contendo quitosana em células livres e biofilme de *Escherichia coli*.** 2019. 73p. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2019.

A infecção do trato urinário (ITU) é a infecção mais comum em nível hospitalar, sendo os cateteres urinários responsáveis por desenvolver o risco de bacteriúria, o que pode agravá-la. Esta infecção geralmente está relacionada a formação de biofilme na superfície interna e externa do cateter. A bactéria uropatogênica *Escherichia coli* continua sendo o microrganismo mais isolado em cateteres e seus biofilmes são estudados de forma a desenvolver estratégias de controle das ITUs. O presente estudo examinou o efeito de um sistema de liberação nanoestruturado contendo quitosana no crescimento de biofilmes uropatogênicos de *E. coli*. Este trabalho foi dividido em duas etapas, sendo a primeira uma comparação de biofilmes crescidos nas superfícies mais utilizadas nos modelos de biofilme in vitro e a segunda a avaliação da suscetibilidade de células livres e biofilme de *E. coli* exposto a um carreador lipídico nanoestruturado contendo quitosana (CLN-quitosana). Assim, biofilmes de *E. coli* foram formados em cateter, lâminas de vidro ou placas de cultura de células por 5 dias, sendo a composição do biofilme avaliada. Na segunda etapa do trabalho, a CLN-quitosana foi preparada usando o método de emulsão e sonicação, sendo caracterizada em relação ao tamanho de partícula, índice de polidispersividade e potencial zeta. Após a determinação das concentrações inibitórias mínimas (CIM) e bactericidas (CBM), os biofilmes de *E. coli* foram crescidos em cateter. Após 48, 72, 96 e 120 horas de crescimento, os biofilmes foram expostos a solução de NaCl a 0,9% (controle negativo), solução de clorexidina a 0,12% (controle positivo) e CLN-quitosana (concentração final de quitosana de 0,28%). Após 24 horas de tratamento, os biofilmes foram coletados para análise de viabilidade bacteriana. Os dados foram analisados estatisticamente pelo teste de Tukey-Kramer ou Tukey, com nível de significância de 5%. A viabilidade bacteriana foi maior no cateter em comparação com lâminas de vidro ou placas de cultura ($p < 0,05$) e a menor contagem bacteriana foi observada na lâmina de vidro ($p < 0,05$). Embora as concentrações de carboidratos tenham sido menores no biofilme formado no cateter ($p < 0,05$), não foram observadas diferenças estatisticamente significativas na quantificação de proteínas para os grupos cateter e placa de cultura ($p > 0,05$) ou entre as lâminas de vidro e a placa ($p > 0,05$). Em relação à segunda etapa do trabalho, a preparação de CLN-quitosana apresentou distribuição bimodal de tamanho de partícula com tamanho médio de 292,9 ± 2,5 nm, índice de polidispersividade de 0,24 ± 0,03 e potencial zeta positivo (+19,1 ± 0,2), indicando o revestimento de nanopartículas pela quitosana. A análise dos valores de CIM e CBM revelou que a formulação inibiu o crescimento bacteriano e exerceu ação bactericida em concentrações 100 vezes maior do que a necessária para o digluconato de clorexidina (controle positivo). Comparado com os grupos controle, a CLN-quitosana afetou a viabilidade bacteriana dos biofilmes em todas as idades avaliadas ($p<0,05$). Sendo assim, os resultados sugerem que o cateter é a superfície adequada para estudar biofilmes de *E. coli*. Tanto as células livres quanto os biofilmes foram afetados pelo CLN-quitosana. No futuro, o cateter urinário pode ser utilizado como modelo para estudar ITUs com populações mistas de bactérias e o efeito de CLN-quitosana ou de sua associação com outros antimicrobianos poderá ser avaliado.

Palavras-chave: Uropatógeno, Biofilme, Cateter, Quitosana, Carreador lipídico nanoestruturado.

ABSTRACT

OSUNGUNNA, M. O. **Effect of nanostructured lipid carrier containing chitosan on free cells and biofilm of *Escherichia coli*.** 2019. 73p. Thesis (Doctorate). Faculty of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2019.

Urinary tract infection (UTI) is the most common hospital acquired pathological process and indwelling urinary catheters increase the risk of bacteriuria, which can progress to a serious condition. This infection usually follows formation of biofilm on both the internal and external catheter surface. The uropathogenic bacteria *Escherichia coli* is the most common infecting microorganism on catheter and its biofilms have been studied as a platform to select strategies to control UTIs. The present study examined whether a nano delivery system containing chitosan affected the growth of uropathogenic biofilms of *E. coli*. This work was divided in two stages, the first involved comparing adhesion surfaces most used in in vitro biofilm models and the second evaluated the susceptibility of free cells and biofilm of *E. coli* to a nanostructured lipid carrier coated with chitosan (NLC-chitosan). Thus, *E. coli* biofilms were formed on catheter, glass slides or tissue culture plates for 5 days and the composition of biofilm was evaluated. In the second stage of the work, NLC-chitosan was prepared using the emulsion and sonication method, and further characterized with respect to particle size, polydispersity index, and zeta potential. After determining the minimum inhibitory (MIC) and bactericidal concentrations (MBC), *E. coli* biofilms were grown on catheter specimens. At the 48, 72, 96, and 120 hours of growth, biofilms were exposed to 0.9% NaCl solution (negative control), 0.12% chlorhexidine solution (positive control), or NLC-chitosan (final chitosan concentration of 0.28%). After 24 hours of treatment, the biofilms were collected to analyze their bacterial viability. Data were statistically analyzed by Tukey-Kramer or Tukey test with a level of significance of 5%. Bacterial colony viability was higher in catheter compared to glass slides or plates ($p<0.05$) and the lowest bacterial count was observed for glass slide ($p<0.05$). Although concentrations of carbohydrate were lower in biofilm formed on catheter ($p<0.05$), no differences were observed between catheter and plate ($p>0.05$) as well as glass slides and plate ($p>0.05$) for protein quantification. Regarding second work stage, NLC-chitosan preparation had bimodal particle size distribution with mean size of 292.9 ± 2.5 nm and polydispersity index of 0.24 ± 0.03, and positive zeta potential (+19.1 ± 0.2) indicating the nanoparticle coating by chitosan. Analysis of MIC and MBC values revealed that formulation inhibited bacterial growth and exerted bactericidal action at concentrations 100 times higher than those required for chlorhexidine digluconate (positive control). Compared with the control groups, NLC-chitosan affected bacterial colony viability of biofilms at all ages studied ($p<0.05$). The results suggest that catheter is a proper surface to study *E. coli* biofilm compared to either glass slides or polystyrene plates. In addition, both free cells and biofilms of *E. coli* were significantly affected by NLC-chitosan, which can be a feasible approach for studies using uropathogenic bacteria. In future, urinary catheter can be used as model to study simulated UTIs, using mixed populations of bacteria, and the effect of NLC-chitosan or its association with other antimicrobial agents evaluated.

Keywords: Uropathogen, Biofilm, Catheter, Chitosan, Nanostructured lipid carrier

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LIST OF ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
CHX	0.12% solution of Chlorhexidine digluconate
DNA	Deoxyribonucleic acid
DL	Drug loading
EE	Encapsulation Efficiency
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
EPS	Extracellular polymeric substance
LB	Luria Bertani
mRNA	Messenger Ribonucleic acid
mm	millimeter
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
NaCl	0.9% solution of sodium chloride
NLC	Nanostructured Lipid Carrier
PDI	Polydispersity Index
SLN	Solid Lipid Nanoparticles
TPP	Sodium Tripolyphosphate
UTI	Urinary Tract Infection
UPEC	Uropathogenic *Escherichia coli*

LIST OF SYMBOLS

µL	microliter
α	alpha
<	less than
>	greater than
mL	milliliter
w/v	weight per volume
^{o}C	degree Celsius

SUMMARY

INTRODUCTION

1 INTRODUCTION

The use of indwelling medical devices is one of the major causes of urinary tract infections (DOLAN, 2001). The risk of developing a catheter-associated infection increases by approximately 10% each day the catheter is in place (PERCIVAL et al., 2015), and its treatment is challenging because uropathogenic microorganisms adhere to and accumulate on the surfaces of this medical device, producing biofilms (WI; PATEL, 2018).

Biofilms are communities of microorganisms attached to biotic or abiotic surfaces (KUMAR et al., 2017). Several studies have tested catheter as surface to form uropathogenic biofilms but other surfaces such as glass and tissue culture plates also have been used (LEBEAUX et al., 2013). Probably, these surfaces could have significant impacts on biofilm formation and its composition. One of the major features of biofilms is the self-production of extracellular polymeric substances composed of biomolecules such as polysaccharides and proteins that help to protect the microorganisms from external threats, including antimicrobials (KUMAR et al., 2017). In this sense, the development of delivery systems using nanotechnology could be a feasible approach to inhibit biofilm formation or control its growth.

Nanostructured lipid carriers (NLCs) consist of an unstructured solid lipid matrix formed by a mixture of solid and liquid lipids and an aqueous phase containing a surfactant or a mixture of surfactants (BELOQUI et al., 2016), showing advantages such as easy production, low costs and high stability (BUGNICOURT; LADAVIÈRE, 2017). As the unexpected growth of lipid nanoparticles limits their stability, adsorption of chitosan chains to nanoparticles has been used as a promising strategy to improve the formulation stability and bioadhesion (BUGNICOURT; LADAVIÈRE, 2017).

Chitosan is a biodegradable polysaccharide extracted from crustacean shells that is not toxic to animals and humans (MUXIKA et al., 2017). Chitosan and its derivatives have many pharmaceutical applications due to their antimicrobial activity (ALI; AHMED, 2018; CASADIDIO et al., 2019). Considering that the antimicrobial action of “chitosan/lipid” associations on uropathogenic biofilms has not been established in the literature, the present study examined whether cationic NLC covered with chitosan (NLC-chitosan) exert antimicrobial effect against biofilms of *Escherichia coli*, which is one of the most representative bacterial species in catheter-associated urinary tract infection.

LITERATURE REVIEW

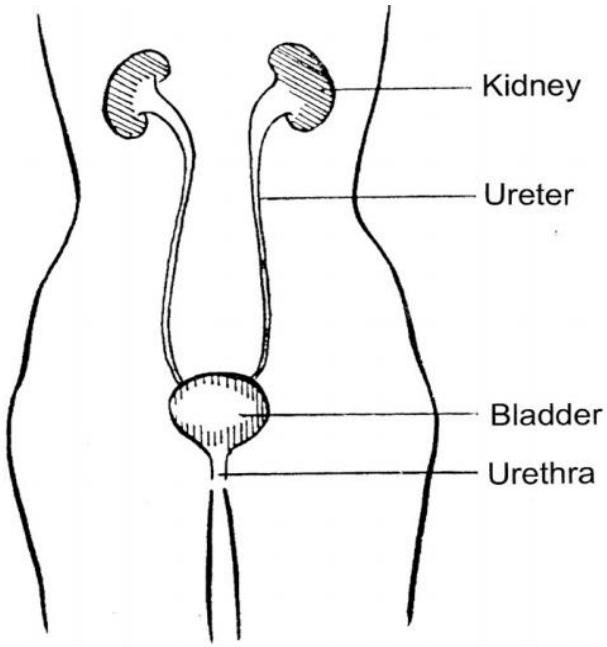
2 LITERATURE REVIEW

2.1. URINARY TRACT INFECTIONS (UTIs)

Urinary Tract Infections (UTIs), a major problem of catheterization, have been defined as the presence of significant levels of bacteria in urine, typically 10^4 – 10^6 colony forming units (CFU/mL) (HILBERT, 2011). UTIs affect about 150 million people each year worldwide (STAMM; NORRBY, 2001) and account for a high cause of morbidity in infant boys, older men, and females of all ages **(**FLORES-MIRELES et al., 2015).

UTIs may be with (symptomatic) or without (asymptomatic) symptoms. Symptomatic UTIs can be classified clinically as uncomplicated or complicated. Uncomplicated UTIs affect healthy individuals with no structural or neurological urinary tract abnormalities (NIELUBOWICZ; MOBLEY, 2010; HOOTON, 2012). These can be differentiated into lower UTIs (cystitis) and upper UTIs (pyelonephritis) (HANNAN et al., 2012; HOOTON, 2012) of which acute pyelonephritis is the most pressing. Figure 1 shows the urinary tract.

Figure 1: The urinary tract



Obtained from FENELEY, R. C. L.; HOPLEY, I. B.; WELLS, P. N. T. Urinary catheters: history, current status, adverse events and research agenda. **J Med Eng Technol**, London, v. 39, n. 8, p.459–470, 2015

Infection of one or both kidneys usually results from the ascent of microorganisms from the bladder through the ureter and is distinguished from other UTIs clinically, pathologically, and by characteristics of the causative microorganisms (WARREN, 2001). The patient with acute pyelonephritis classically presents the triad of fever, flank pain, and bacteriuria with or without diaphoresis, rigors, abdominal or groin pain, and nausea and vomiting.

Cystitis is often defined by the presence of 10^3 bacteria per milliliter or more in a midstream clean-catch urine sample from a patient with symptoms (FARO; FENNER, 1998; WARREN; ABRUTYN; HEBEL, 1999). Cystitis indicates that there is bladder involvement, and the symptoms include dysuria, urgency, frequency, suprapubic pain, incontinence, and malodorous urine. Cystitis may not cause fever and does not result in renal injury, unlike pyelonephritis (OYELAMI et al., 2005). Some of the risk factors associated with cystitis, include female gender, a prior UTI, sexual activity, vaginal infection, diabetes, obesity, and genetic susceptibility (HANNAN et al., 2012; FOXMAN, 2014).

Complicated UTIs, on the other hand, are associated with factors that compromise the urinary tract or host defense, among which is catheterization (LICHTENBERGER; HOOTON, 2008; LEVISON; KAYE, 2013). Reports indicate that 70% of UTIs are associated with urinary catheters (BURTON et al., 2011; WEBER et al., 2011). However, about 20% of patients will suffer a catheterization during their hospital stay, especially in intensive care units (SAINT; LIPSKY, 1999).

Nonetheless, the duration of the catheterization is a critical risk factor towards the development of catheter-associated urinary tract infections (CAUTI). Almost all long-term catheterized (>28 days) patients develop a CAUTI, whereas in short-term (<7 days) catheterized patients only 10–50% develop an infection (MORRIS; STICKLER; MCLEAN, 1999). CAUTIs account for up to 40% of hospital-acquired infections (SAINT et al., 2008) in the USA. In Brazil, however, crude mortality of patients with CAUTI was 30%, with extra mortality of 10.7% (SALOMAO et al., 2008).

Asymptomatic bacteriuria (ABU) on the other hand, has been defined as the colonization of urine by microorganisms in the absence of clinical symptoms (ROOS et al., 2006). ABU patients may carry a large number of bacteria, more than 10^5 CFU/mL, for months or years without developing symptoms. If left untreated in pregnant women, ABU can result in symptomatic UTI and cause renal injury despite being benign (OYELAMI et al., 2005).

Most patients with ABU do not need treatment, and in many cases the colonizing microorganism helps to prevent infection by other more virulent bacteria (HULL et al., 2000; DAROUICHE et al., 2001). The prevalence of ABU is determined by age, gender, sexual activity, and the presence of genitourinary abnormalities. Asymptomatic bacteriuria, at the rate of 3% to 5%, is commonly detected in women aged up to 60 years. It is more common in diabetic patients and the elderly (LIN, 2008).

Several species of bacteria have been implicated in the pathogenesis of UTIs but the most common infecting microorganism is *Escherichia coli* (HOOTON et al., 2010).

2.2 UROPATHOGENIC *Escherichia coli* (UPEC)

E. coli, a predominantly Gram-negative and facultative anaerobe in the bowel, is widely distributed in the intestine of humans and warm-blooded animals where they maintain the physiology of the healthy host (NEILL et al., 1994; CONWAY, 1995). *E. coli* is one of the many members of the *Enterobacteriaceae* family (EWING, 1986), which includes many genera, including known pathogens such as *Salmonella*, *Shigella*, and *Yersinia.* Although *E. coli* can be found as normal microbiota in the intestines of humans, it does not suggest that all *E. coli* are innocuous, as many strains of *E. coli* are pathogenic, causing many infectious diseases in humans as well as animals and birds.

Pathogenic *E. coli* can be classified into intestinal pathogens causing diarrhea, and extra-intestinal *E. coli* causing a diversity of infections in both humans and animals including UTIs, meningitis and sepsis (KAPER; NATARO, 2004). The most common extra-intestinal *E. coli* infections are UTIs, which are caused by uropathogenic *E. coli* (UPEC).

About 80 – 90% of community-acquired UTIs are caused mainly by UPEC (FOXMAN, 2014; FLORES-MIRELES et al., 2015). UPEC express some virulence factors such as pathogenicity islands, fimbriae, aerobactin, serum resistance, and encapsulation, which contribute to their pathogenicity and colonization (KAPER; NATARO; MOBLEY, 2004). However, four main UPEC phylogroups (A, B1, B2, and D) have been identified by the occurrence and the expression of these virulence factors (BIEN; SOKOLOVA; BOZKO, 2012). Usually, many of these virulence factors

are found in varying percentages among various subgroups of UPEC (JOHNSON; STELL, 2000) and are required for UPEC to cause UTIs (HANNAN et al., 2012).

The various processes and stages involved in UPEC causing UTIs include (a) colonization of the periurethral, vaginal areas, and urethra by UPEC; (b) ascending into the bladder lumen and growth as planktonic cells in urine; (c) adherence to the surface and interaction with the bladder epithelium defense system; (d) biofilm formation; (e) invasion and replication by forming bladder intracellular bacterial communities (IBCs) where quiescent intracellular reservoirs are developed and reside in the underlying urothelium; (f) kidney colonization and host tissue damage with increased risk for bacteremia/sepsis (FLORES-MIRELES et al., 2015; SPAULDING; HULTGREN, 2016).

Replication of bacteria in the IBC can easily reach as many as 10^5 bacteria per cell; furthermore, bacteria in the IBC undergo morphological changes, flux out of the infected cell, and go on to infect neighboring cells (FLORES-MIRELES et al., 2015; SPAULDING; HULTGREN, 2016). The flushing of urine removes most of the invading bacteria, along with UPEC-filled exfoliated bladder epithelium cells (KAPER; NATARO; MOBLEY, 2004). Colonization of the bladder by UPEC is performed by using a variety of virulence factors, among which biofilm development is critical (ZALEWSKA-PIATEK et al., 2009).

2.3 BIOFILMS

Biofilm can be defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (DOLAN; COSTERTON, 2002).

The self-produced EPS, although varying in their compositions with respect to the biofilm-forming bacteria, perform the same roles which include dissemination of nutrients that are necessary for cell growth (CHENG et al., 2007), trapping of external nutrients that are required for cell sustenance (CHENG et al., 2007), and protection from external stress compared to planktonic bacteria (PANG et al., 2005). Components of EPS include extracellular polysaccharides, proteins and DNA. Additionally, some bacteria produce other substances which make their matrix unique. For example, cellulose has been reported to be a crucial component of the

extracellular matrix of *Salmonella typhimurium* and *Escherichia coli* (ZOGAJ et al., 2001; SOLANO et al., 2002).

Extracellular polysaccharides can be capsular or exopolysaccharides. The polysaccharides that remain associated with the cell following cellular harvest and centrifugation are capsular while those in the supernatant are exopolysaccharides (BRANDA et al., 2005). Diverse benefits that have been ascribed to the polysaccharide component of the biofilm matrix include adhesion, protection, and structure (LIMOLI; JONES; WOZNIAK, 2015).

On the other hand, biofilm matrix proteins play diverse roles in biofilm formation and dissolution. They are involved in attaching cells to surfaces, stabilizing the biofilm matrix via interactions with exopolysaccharide and nucleic acid components, developing three-dimensional biofilm architectures, and dissolving the biofilm matrix via enzymatic degradation of polysaccharides, proteins, and nucleic acids (FONG; YILDIZ, 2015). In addition, the role of extracellular DNA in the establishment of biofilm structure cannot be understated (WHITCHURCH et al., 2002).

Biofilm formation offers protection against antibiotics (GOLDBERG, 2002), disinfectants (PENG; TSAI; CHOU, 2002), and the dynamic environment (CHEN, 1998) with protection from environmental insults and assaults being foremost (DAVEY; O'TOOLE, 2000). Despite obvious similarity in some components of the biofilm matrix, it is noteworthy that structurally, developed biofilms are not homogeneous monolayers of microbial cells on a surface, but rather heterogeneous in both time and space (LEWANDOWSKI, 2000).

2.4 SURFACES FOR BIOFILM FORMATION

Biofilm development requires the presence of microbes and a surface for attachment. The absence of either of these two essential requirements means that a biofilm will not form (DUNNE, 2002). Attachment, the first stage in biofilm formation, of a bacterium to a surface is governed by two events: (i) that a bacterium accesses a surface and comes in close contact for attachment to occur and (ii) that the outer surface of a bacterium adheres to the surface (substratum) (FLETCHER; LOEB, 1978). The possibility of a bacterium accessing a potential surface depends on many factors such as culture concentration and bacterial motility, whereas attachment is dependent upon the chemical and physical interactions between the potential

surface, the bacterial surface, and the polymeric adhesive (FLETCHER; LOEB, 1978).

The extent of surface colonization by bacteria depends on the characteristics of the surface such as the surface smoothness/roughness as well as the hydrophobicity/hydrophilicity of the surface. It has previously been reported that the extent of microbial colonization increases as the roughness of the surface increases (CHARACKILIS et al., 1990). This increase can be attributed to a decrease in shear forces and an increase in surface area as surface roughness increases. However, subsequent studies with wild and fully adherent bacterial strains showed that smooth surfaces are colonized as easily as rough surfaces and that the physical characteristics of a surface influence bacterial adhesion to only a minor extent (COSTERTON et al., 1995).

Hydrophobicity of the surface is another property that determines the extent of microbial colonization. Surfaces differ in their degree of hydrophobicity hence their ability to support bacterial attachment. Surfaces have been classified into three types based on their degree of hydrophobicity, into hydrophobic, polymers of increased wettability, and hydrophilic surfaces (FLETCHER; LOEB, 1978). Hydrophobic materials include nonpolar polymers with no ionogenic functional groups and whose surface charge, if any, arises from low-level impurities or through adsorption/desorption of ions from a surrounding medium (HAYDON, 1964). Examples include Teflon, polyethylene, and polystyrene (PS). These materials have been reported to bear a large number of attached bacteria and are usually covered within 2 h with a monolayer of bacteria (FLETCHER, 1977). It is noteworthy that hydrophobicity of a surface can vary markedly depending on the molecules in the conditioning film (AN; DICKSON; DOYLE, 2000; BOLAND; LATOUR; SUTZENBERGER, 2000).

Polymers of increased wettability may contain significant quantities of polar groups. The polar groups are typically introduced by radiofrequency discharge, and some of the polar groups may bear negative charges such as carboxyl and phenolic groups (COOPER; PROBER, 1960; OWENS, 1975). Examples include Nylon 6.6, poly (ethylene terephthalate) (PET), and epoxy resin. PS and PET may be treated to the extent that their receding water contact angles approach zero through commercial processing, for example Falcon tissue culture products. Hydrophilic

surfaces such as glass are made of inorganic materials and have a zero water contact angle (FLETCHER; LOEB, 1978).

Notwithstanding the hydrophobicity of the surface for bacterial attachment, the hydrophobicity of the bacterial cell surface also plays a role in its adhesion to biotic and abiotic surfaces as well as its penetration of the host tissues (GOULTER et al., 2009; HEILMAN, 2011). The more hydrophobic cells adhere more strongly to hydrophobic surfaces, while hydrophilic cells strongly adhere to hydrophilic surfaces (KOCHKODAN et al., 2008; GIAOURIS et al., 2009). When two hydrophobic surfaces interact, they tend to become attached by removing the water molecules between them (HERRMANN et al., 1988).

Considering the heterogeneity of the microbial population, a mixed culture of hydrophilic and hydrophobic cells suggests that only part of them will participate in the adhesion, depending on the type of surface (KRASOWSKA; SIGLER, 2014). It is important to note however, that microorganisms can switch between hydrophobic and hydrophilic phenotypes in response to changes in environmental conditions (temperature, nutrient composition, etc.) and growth phases (BORECKA-MELKUSOVA; BUJDAKOVA, 2008; BUJDAKOVA et al., 2013). This observation may reinforce the earlier submission that the hydrophobicity of the surface plays a more prominent role in bacterial adherence than the hydrophobicity of the bacteria (PAVITHRA; DOBLE, 2008).

A multitude of studies exist where bacterial attachment (biofilm growth) to different surfaces has been studied with different bacterial species under different conditions. However, in evaluating surfaces for biofilm formation, two primary goals are possible. The first is to determine the suitability of the surface for research purposes with a view to selecting the best surface for the microorganism of choice. This became necessary because apart from providing suitable growth requirements, the natural habitat of the microorganism must be mimicked to minimize both phenotypic and genotypic variations as a result of a difference in real and experimental environments (BRANDA et al., 2005; RUMBAUGH; AHMAD, 2014).

The second goal in the attachment studies has been to invent colonization resistant surfaces. While surfaces or surface coatings that retard bacterial adhesion have been described, none have been developed that prevent it entirely (DUNNE, 2002). However, despite encouraging laboratory tests in the discovery of surfaces

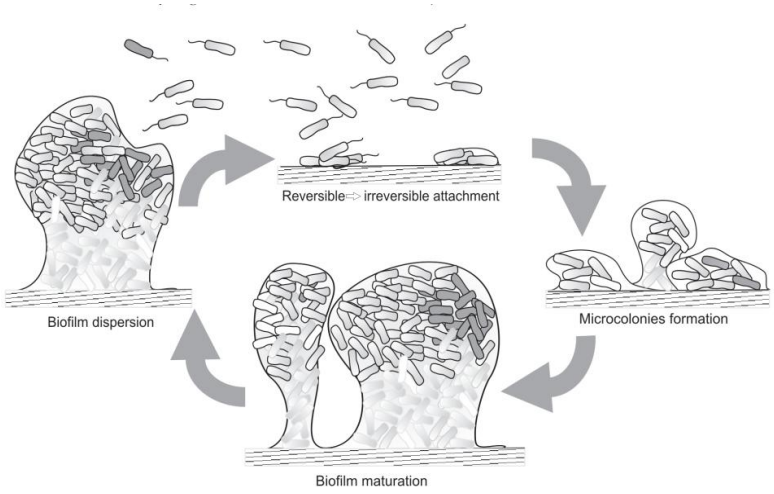
that are inherently resistant to bacterial colonization, practical tests have been disappointing (COSTERTON; LAPPIN-SCOTT, 1995).

In practice, medical implants such as catheters are usually made from hydrophobic materials (silicon, stainless steel, Teflon, etc.). It therefore follows that hydrophobic, rather than hydrophilic microorganisms will attach easily to this surface (KRASOWSKA; SIGLER, 2014) and may explain the close association between *E. coli* and catheter-associated infections. However, effective and successful management of biofilm-associated infections requires that the mechanism of biofilm formation as well as control strategies be understood.

2.5 BIOFILM FORMATION AND CONTROL STRATEGIES

Biofilm formation requires the presence and interaction of microbes and surface (biotic or abiotic). The stages involved in biofilm formation are exemplified in Figure 2. The first stage in biofilm formation is an attachment to a surface, which can be reversible or irreversible. The reversible attachment, also known as docking phase, is governed by some physiochemical parameters that characterize the association between the bacterial cell surface and the conditioned surface of interest (AN et al., 2000). Docking depends on the net sum of attractive or repulsive forces generated between the two surfaces when the microorganism is brought into proximity to the surface, with the microorganism being propelled randomly or via chemotaxis and motility (DUNNE, 2002).

Figure 2: Stages involved in biofilm development



Obtained from MARKOWSKA, K; GRUDNIAK, A. M.; WOLSKA, K. I. Silver nanoparticles as an alternative strategy against bacterial bioflms. **Acta Biochim Pol**, Warszawa, v. 60, n. 4, p. 523-530, 2013.

These forces include electrostatic and hydrophobic interactions, steric hindrance, van der Waals forces, temperature, and hydrodynamic forces (CARPENTIER; CERF, 1993). Because most bacteria and inert surfaces are usually negatively charged, electrostatic attractions tend to favor repulsion (CARPENTIER; CERF, 1993). The situation, however, differs where the microorganism assumes an overall positive charge at a physiological pH thereby promoting docking to negatively charged materials e.g. Teflon. Hydrophobic interactions tend to have a more significant influence on the outcome of the docking phase (CARPENTIER; CERF, 1993).

Reversible attachment is usually followed by irreversible attachment, often called the locking step, and it is usually molecularly mediated using cell-produced adhesin. This stage is made possible because physical bacteria appendages (flagella, fimbriae and pili) have been able to overcome the physical repulsive forces of the electrical double layer (DE WEGER et al., 1987) thereby making contact with the bulk lattice of the conditioning layer, stimulating chemical reactions such as oxidation and hydration (GANESH; ANAND, 1998) thus consolidating the bacteria-surface bond. At this stage, the adhesion process becomes consolidated either by loosely bound microorganisms through the production of exopolysaccharides that complex with surface materials, or receptor-specific ligands located on pili, fimbriae, and fibrillae, or both mechanisms (DUNNE, 2002).

After this stage, the microorganism is attached firmly to the surface like a cocoon on a leaf because adhesion becomes irreversible in the absence of physical or chemical intervention (DUNNE, 2002). The attachment process can be affected by many interrelated factors such as the roughness and smoothness of the surface, nature and properties of the aqueous medium such as the pH, nutrient levels, ionic strength, and temperature as well as the characteristics of the cell surface such as hydrophobicity, presence of fimbriae/flagella, and production of EPS (DOLAN, 2001).

Following irreversible attachment of bacteria to the surface, the process of biofilm growth and maturation begins. This stage is characterized by an overall increase in density and complexity of biofilm due to active replication of surface-bound bacteria, interaction of extracellular components generated with organic and inorganic molecules in the immediate environment to create EPS, and domination over physical and chemical contribution to attachment by a biological process (DUNNE, 2002).

However, some factors such as the availability of nutrients in the immediate environment, the perfusion of those nutrients to cells within the biofilm, and waste removal do limit the growth potential of any bacterial biofilm. Nonetheless, an optimum hydrodynamic flow that favors growth and perfusion rather than erosion of the outermost layers does exist (CARPENTIER; CERF, 1993). Other factors that control biofilm maturation include internal pH, oxygen perfusion, carbon source, and osmolarity (O'TOOLE; KOLTER, 1998).

The increase in cell density and complexity continues until a critical mass is reached where a dynamic equilibrium begins to generate planktonic microorganisms from the outermost layer of growth, being the farthest from the surface. The microorganisms generated can escape the biofilm and colonize other surfaces (PETROVA; SAUER, 2016). Cells nearest the surface are characterized by reduced growth and metabolic activities due to a lack of nutrients or perfusion, decreased pH, pO_2, or an accumulation of toxic metabolic by-products (PROSSER et al., 1987).

Cell-cell signaling molecules, such as acylated homoserine lactones, which control population density-dependent gene expression may regulate the processes of initial development, maturation, and breakdown of a biofilm (DAVIES et al., 1998). Once fully matured, altered patterns of bacterial growth, physiological cooperation, and metabolic efficiency, all of which provide a form of functional communal coordination that imitates primordial eukaryotic tissue, can be generated by biofilm (COSTERTON et al., 1987). Several control strategies, preventive and curative, have been developed for biofilm control. While preventive strategies target the prevention of biofilm formation, curative measures target the formed mature biofilms.

Preventive measures include (i) stoppage of bacteria attachment; (ii) stoppage of biofilm growth; (iii) blockage of biofilm matrix synthesis; and (iv) disruption of cell-cell communication. Curative measures on the other hand, include (i) killing of mature biofilm; (ii) promotion of mature biofilm detachment; and (iii) binding and elimination of mature biofilm (FRANCOLINI; DONELLI, 2010).

However, to date, no known strategy can successfully prevent or control the formation of unwanted biofilms without causing adverse side effects (SIMOES; SIMOES VIERA, 2010). The success of biofilm control depends on the understanding of the mechanisms leading to biofilm formation (FRANCOLINI; DONELLI, 2010). However, both preventive and curative measures can be achieved through the use of antimicrobial agents, which can be conventional antibiotics or

natural source agents. However, the use of conventional antibiotics in biofilm control has been curtailed due to resistance of biofilm to some of the conventional antibiotics.

2.6 ANTIMICROBIAL SUSCEPTIBILITY IN BIOFILMS

The same strain of bacteria when grown as planktonic and biofilm will differ in their susceptibilities to the same antibacterial agent (ANDERL; FRANKLIN; STEWART, 2000). This is an indication that the standard mechanisms of resistance in planktonic bacterial cells such as efflux pumps, modifying enzymes, and target modifications (WALSH, 2000) are not responsible for antibacterial resistance in biofilms. In addition, antibiotic resistance of bacteria in biofilms is easily reproduced in vitro, showing that host factors are not required for this manifestation of biofilm defense (STEWART, 2002).

The existence of gene coding for multiple antibacterial resistance in biofilms has no vital role in mediating biofilm resistance to an antibacterial agent (BROOUN; LIU; LEWIS, 2000; MAIRA-LITRAN; ALLISON; GILBERT, 2000). This underscores the fact that resistance in biofilms is not always acquired by mutations or mobile genetic elements (ANWAR et al., 1989; WILLIAMS et al., 1997).

Antibiotic sensitivity is usually quickly restored when bacteria are dispersed from a biofilm to underscore an adaptive resistance mechanism rather than a genetic alteration (STEWART, 2002). However, conventional antibacterial resistance can develop in biofilms treated repeatedly or for a long time (BAGGE et al., 2000). It therefore follows that one should look beyond conventional mechanisms of resistance to understand biofilm resistance. Several hypotheses have been proposed for a better understanding of mechanisms of resistance in a biofilm.

The penetration limitation hypothesis suggests that only the surface layers of a biofilm are exposed to a lethal dose of the antibiotic due to a reaction-diffusion barrier that limits transport of the antibiotic into the biofilm. There are reservations to this hypothesis as some authors have reported retarded penetration of antibiotics (KUMON et al., 1994; SHIGETA et al., 1997) into the biofilm matrix, while others indicate rapid penetration (DAROUICHE et al., 1994; SUCI et al., 1994)**.**

The ability of antibiotics to penetrate biofilm has therefore been attributed to the biofilm type. For instance the penicillin class of antibiotics can penetrate biofilm formed by β-lactamase negative strains of bacteria better than biofilm formed by β-

lactamase positive strains of the same bacteria due to the ability of the later to deactivate penicillin (ANDERL; FRANKLIN; STEWART, 2000). Additionally, aminoglycoside antibiotics become adsorbed in the matrix of biofilm due to their interaction (being positively charged) with negatively charged polymers in the biofilm matrix, thereby retarding their penetration (KUMON et al., 1994; SHIGETA et al., 1997).

The physiological limitation hypothesis of reduced susceptibility of biofilms to antibiotics proposes that some microorganisms within the biofilm exist in a more recalcitrant phenotypic state. This hypothesis takes into consideration the effects of physiological factors such as growth rate, biofilm age, and starvation, on biofilm susceptibility to antibiotics. For instance, Aires, Batista and Pitondo-Silva (2017) reported a reduced susceptibility of *K. pneumoniae* biofilm to ceftriaxone, a cephalosporin antibiotic, with increasing age of the biofilm and in a similar way Singla, Harjai and Chhibber (2013) reported reduced susceptibility of *K. pneumoniae* to ciprofloxacin, amikacin and piperacillin antibiotics with an increase in age of the biofilm.

The physiological complexity of biofilm increases with age, hence reduced susceptibility. Notwithstanding this submission, there have been reports of newly formed biofilms being resistant despite being too thin to pose a hurdle to the penetration of either an antimicrobial agent or metabolic substrates (DAS et al., 1998; COCHRAN; MCFETERS; STEWART, 2000;). Another hypothesis to explain the reduced susceptibility of biofilms to antibiotics is the altered chemical microenvironment within the biofilm. Bacteria on the surface of biofilm have more access to oxygen than those in the interior. This may lead to the formation of two distinct environments within the biofilm with an aerobic environment at the surface and anaerobic in the interior (DE BEER et al., 1994). The same explanation holds for nutrients and such other growth conditions. The corollary to this submission is reduced growth rate and metabolism in the interior of biofilm compared to the outer surface of the biofilm.

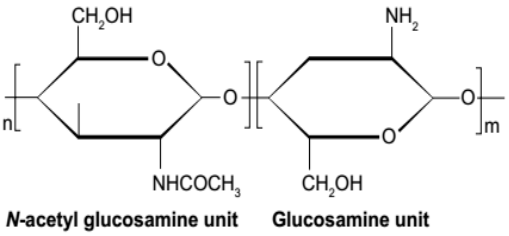
Because of the high metabolism on the surface of the biofilm, there may be an accumulation of waste products such that the pH of the surrounding surface changes compared to the pH in the biofilm interior (ZHANG; BISHOP, 1996). All these variations may lead to reduced activity of antibiotics on biofilm. For instance, aminoglycoside antibiotics are more effective in an aerobic environment than in an

anaerobic environment (TACK; SABATH, 1985). This implies that bacteria on the surface of biofilm will be killed at the expense of those in the interior. Penicillin acts on actively growing (actively metabolizing) cells because the target is cell-wall synthesis (TUOMANEN et al., 1986), such that bacteria on the biofilm surface will be killed by penicillin while less metabolizing in the interior will be spared. The hypothesis that bacteria in biofilm behave like spores remains a powerful and generic explanation of reduced susceptibility of biofilms to antibiotics (STEWART; COSTERTON, 2001).

2.7 CHITOSAN

Chitosan (Figure 3) is a partially deacetylated polymer of N-acetyl glucosamine that can be obtained through alkaline deacetylation of chitin (ZHAO et al., 2011). It is made up of a β-(1, 4)-linked-D-glucosamine residue with the amine groups randomly acetylated (SEVDA; MCCLUREB, 2004). It consists of alternating units of (1 → 4) linked N-acetyl glucosamine and glucosamine units. It is a white, hard, inelastic and nitrogenous polysaccharide (BADAWY; RABEA, 2011).

Figure 3: Repeating units of the chemical structure of chitosan



Obtained from Ahmed, T. A.; Aljaeid, B. M. Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. **Drug Des Devel Ther**, Auckland, v. 10, p. 483–507, 2016.

Chitosan can be of low molecular weight (< 50 kDa), medium molecular weight (50 – 150 kDa), or high molecular weight (> 150 kDa) (GOY; BRITTO; ASSIS, 2009). Chitosan, a weak base, is not soluble in water, but soluble in dilute aqueous acidic solutions below its pKa (~6.3), in which it can convert glucosamine units ($-NH^2$) into the soluble protonated form ($-NH^{+3}$) (GOY; BRITTO; ASSIS, 2009).

However, water-soluble chitosan derivatives can be obtained by quaternization of the nitrogen atoms of the amino when permanent positive charges in the polymer chains are introduced, leading to a particular cationic polyelectrolyte irrespective of

the pH of the aqueous medium. The solubility of chitosan depends on its biological origin, molecular weight and degree of acetylation (SHEPHERD et al., 1997). Chitosan is reputed for its biocompatibility, non-toxicity, low allergenicity, and biodegradability which make it suitable for various applications (KUMAR et al., 2004). Also, antimicrobial activity, among other activities of chitosan has been reported (GOY et al., 2016).

However, four mechanisms of antimicrobial action of chitosan have been proposed which include (i) binding of chitosan to the negatively charged bacterial cell wall causing disruption of the cell thus altering membrane permeability (CHIEN; YEN; MAU, 2015; SEVERINO et al., 2015); (ii) binding of chitosan with microbial DNA, leading to mRNA and protein synthesis inhibition at the entrance of the nuclei of the microorganisms of chitosan (SEBTI et al., 2005; YUAN et al., 2016); (iii) the chelation of metals, suppression of spore elements, and binding to essential nutrients to microbial growth (CHIEN; YEN; MAU, 2015; YUAN et al., 2016); and (iv) chitosan can form a polymer membrane on the bacterial surface thereby preventing nutrients from entering the cell (LIU et al., 2004; EL-TAHLAWY et al., 2005) or act as barrier to oxygen thereby inhibiting growth of aerobic bacteria (DEVLIEGHERE et al., 2004; YUAN et al., 2016).

Despite these proposed mechanisms of antimicrobial activity of chitosan, its actual nature of activity remains controversial with many authors submitting that chitosan is bacteriostatic while others favor bactericidal activity. However, recent studies have a propensity of characterizing chitosan as bacteriostatic rather than bactericidal (RAAFAT et al., 2008). Similarly, there is no agreement as regards whether Gram-positive bacteria are more susceptible to the activity of chitosan than are Gram-negative bacteria or vice versa. Some authors have shown chitosan to exhibit higher antibacterial activity against Gram-positive bacteria than Gram-negative, while some other studies have shown that Gram-negative bacteria are more susceptible than Gram-positive (CHUNG et al., 2004; WANG et al., 2004). Many works have demonstrated that there is no significant difference between antibacterial activity and bacterial species (ZHONG et al., 2008).

The antimicrobial activity of chitosan has been reported to be dependent on its molecular weight and the degree of acetylation. Although both parameters affect the activity independently, it has been suggested that molecular weight has a greater influence on chitosan activity than the degree of acetylation (SEKIGUCHI et al.,

1994). Antimicrobial activity of chitosan increases with a decrease in molecular weight (TIKHONOV et al., 2006; JING et al., 2007) as well as a decrease in the degree of acetylation (TSAI et al., 2004; ANDRES et al., 2007). Other factors affecting the antibacterial activity of chitosan include pH, temperature, and reactive time (KONG et al., 2010).

2.8 NANOSTRUCTURED LIPID CARRIERS (NLC)

Nanostructured lipid carriers (NLC) have been described as the improved second generation of solid lipid nanoparticles (SLN) formulated by mixing unsaturated, amorphous (spatially different lipids) or liquid lipids (oils) to the solid lipids (GELFUSO; CUNHA-FILHO; GRATIERI; 2016). They were developed to address decreased encapsulation efficiency (EE) and drug loading (DL), the two potential limitations of SLN (SWIDAN et al., 2016).

These identified limitations of SLN are caused by drug leakage in storage as a result of crystallization of the purified solid lipids (DAS; CHAUDHURY, 2011). The incorporation of spatially incompatible liquid lipid prevents the crystallization process (LASON; SIKORA; OGONOWSKI, 2013) and also induces a melting point depression in the solid lipid. Despite the induced melting point depression, the blends obtained remain solid at room and body temperature **(**MÜLLER; OLBRICH, 2000; LASON; SIKORA; OGONOWSKI, 2013).

Apart from the unique advantages of NLC, it also shares some advantages of the SLN (MEHNERT; MÄDER, 2001). Such shared advantages include sustained release, protection against drug degradation, high physical stability, and the facility to scale-up the production process, allied to the absence of organic solvents (MULLER; MADER; GOHLA, 2000; MEHNERT; MADER, 2001). NLC, a versatile tool with high potential of applications, can also be used to solubilize a number of molecules with different physico-chemical properties in a biocompatible and biodegradable matrix with well-established safety profiles (CORTESI et al., 2017).

NLC has been applied to the delivery of natural molecules with antimicrobial activity (CORTESI; et al., 2017), probably due to the fact that several natural extracts or pure natural compounds with proven antimicrobial activity suffer from some drawbacks represented by low water solubility, instability, as well as storage characteristics, thereby limiting their applications. Grape (*Vitis vinifera)* seed oil is not an exemption to these drawbacks.

Nonetheless, it is possible to formulate NLC in combination with chitosan. The advantages inherent in this combination include improved interaction and internalization in cells (SANDRI et al., 2010), decreased NLC internalization by macrophage (SERMENTO et al., 2011), enhanced oral absorption of drugs e.g. insulin (FONTE et al., 2011) oral administration of peptide drugs (GARCIA-FUENTES; TORRES; ALONSO, 2005) and sustained release (MARCATO et al., 2013). NLC-Chitosan combines the advantages of NLC with the biological properties of chitosan. Such biological properties include bioadhesiveness, biocompatibility, non-toxicity, low allergenicity and biodegradability (KUMAR et al., 2004). The bioadhesion property of chitosan can increase the retention of the drug at the application site.

Additionally, the antimicrobial activity of chitosan can also be explored with this combination (RIDOLFI et al., 2011). The addition of chitosan in the production of NLC can result in a more stable dispersion with a more polydispersed distribution and statistically larger particles (with a greater hydrodynamic radius) (RIDOLFI et al., 2011). Other advantages inherent in NLC-Chitosan combination include the reduction in pH of formulated NLC and low burst release of formulated NLC exemplified by a higher positive surface charge (GANESAN et al., 2016). Chemotherapeutic activity of formulated NLC can also be improved by combination with chitosan (KAMEL et al., 2017).

Despite the inherent advantages in NLC-Chitosan combination, information about their suitability for application in the control of catheter-associated biofilm-related infections is lacking, hence the need to investigate the NLC-Chitosan as a means of biofilm control strategy.

OBJECTIVES

3 OBJECTIVES

General objective:

The present study examined whether nano delivery system containing chitosan affected the growth of uropathogenic biofilms of *E. coli*.

Specific objectives:

i. To evaluate the effect of catheter, glass slide and tissue culture plate as adhesion surfaces on the viability of *E. coli* biofilm
ii. To quantify the carbohydrate and protein contents of *E. coli* biofilm matrix according to each adhesion surface
iii. To formulate and characterise NLC-Chitosan for its particle size, polydispersity index and zeta potential.
iv. To evaluate the formulated and characterised NLC-Chitosan for its antimicrobial activity against the free *Escherichia coli* cells.
v. To evaluate the formulated and characterised NLC-Chitosan for its antibiofilm activity against the preformed *E. coli* biofilm on catheter.

MATERIALS AND METHODS

4 MATERIALS AND METHODS

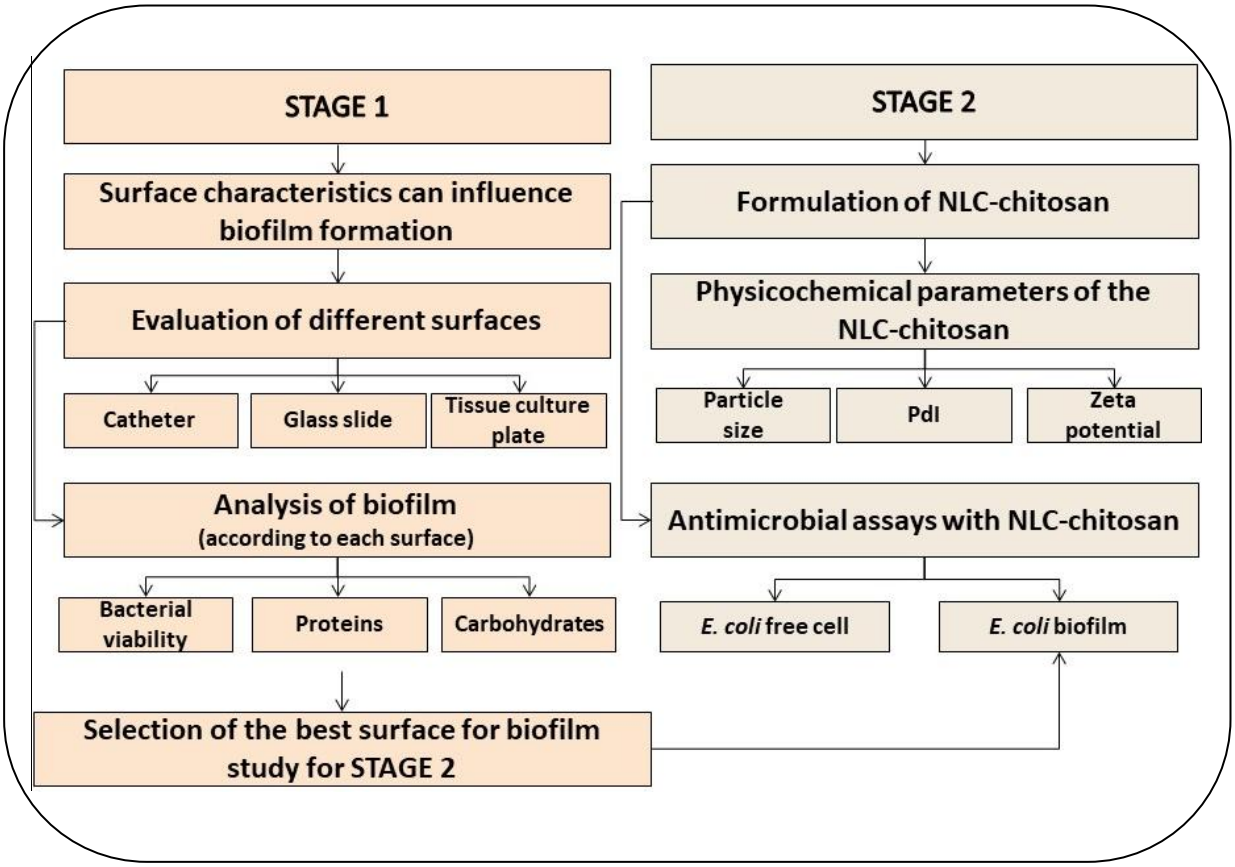
4.1 MATERIALS

Silicone-coated latex catheters 16 (5 mm outside diameter x 2 mm internal diameter x 10.0 mm height) were purchased from Rüsch Inc. (Georgia, USA). Microscopic glass slides (20 x 20 mm) were obtained from Knittelglaser (Varrentrappstr, Braunschweig, Germany). 12-well (25 mm diameter, 20 mm deep), U and flat bottom 96-well (8 mm diameter, 10 mm deep) tissue culture plates were obtained from BD Diagnostics (Sparks, MD, USA). Bovine serum albumin (BSA), chitosan (LMW, 50,000 - 90,000 Da; deacetylation degree of 75%), chlorhexidine digluconate, Tween® 80 (Polysorbate 80), tripolyphosphate (TPP) and resazurin were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and liquefied phenol from J.T. Baker (Pennsylvania, USA). Sodium chloride (NaCl), sodium hydroxide (NaOH), glucose, sulphuric acid and potassium chloride (KCl), were purchased from Merck (Darmstadt, HE, Germany). The Bio-Rad protein assay was purchased from Bio-Rad Laboratories (California, USA). Luria Bertani and Mueller Hinton culture media were obtained from Difco Laboratories (Detroit, MN, USA) and Brain Heart Infusion agar (BHI-agar) from Oxoid (Basingstoke, Hampshire, UK). Natural lipid of Illipe butter was purchased from LTP Lipid Technologies Provider AB (Karishamn, Sweden) and grape seed oil (*Vitis vinifera L*) was acquired from Mapric Produtos Farmacocosméticos (São Paulo, SP, Brazil). Ultrapure water from Milli-Q water system (Merck; Darmstadt, HE, Germany) was used to prepare the aqueous solutions. All other chemicals used in this study were of analytical grade.

4.2 EXPERIMENTAL DESIGN

This work was divided in two stages, the first involved comparing adhesion surfaces most used in *in vitro* biofilm models and the second evaluated the susceptibility of free cells and biofilm of *E. coli* to a nanostructured lipid carrier coated with chitosan (NLC-chitosan). Figure 4 shows the fluxogram of the experiment.

Figure 4: Fluxogram of the experimental stages



4.3 EVALUATION OF *Escherichia coli* ATCC 25922 BIOFILM GROWTH ON DIFFERENT SURFACES

Biofilm growth was done according to the modified method of Anghel and Grumezescu (2013), the main modification being the use of *E. coli* ATCC 25922 instead *S. aureus.* Briefly, *E. coli* overnight grown culture was diluted to 1:100 to achieve 0.5 McFarland (1.5×10^8 cfu/mL) and 1 mL of this suspension was aseptically distributed into 1.5 mL microtubes containing catheter disks suspended with a nylon thread. The same suspension was also used for biofilm growth on glass slides and 96-well plates. For glass slides, 5 mL of the bacterial inoculum was added into a 12-well culture plate containing the slides and for the plates, 200 µL of bacterial suspension was distributed into the wells of a 96-wells tissue culture plate. The microtubes containing the catheters, the glass slide and the plate, all with the bacterial suspension, were incubated at 37 ^{o}C. After 24 hours, all surfaces were washed three times in saline solution (0.9% NaCl) to remove non-adherent bacteria and re-suspended in fresh Luria Bertani broth with the incubation being continued for

4 more days. Each experiment was performed 3 times, being catheter in nonoplicate (n=27) and glass and plates in triplicate (n = 9).

On the day 5 of the experimental phase, the biofilm formed on each surface was washed three times in saline solution to remove non-adherent cells and excess of culture medium. Biofilms formed on glass and plates were removed by swabbing the whole surface of the exposed area. The swab was placed in sterile microcentrifuge tubes and suspended in 1 mL of saline solution. Catheter disks were transferred directly into sterile microcentrifuge tubes with saline solution. The biofilms of each surface were removed by sonication according to the method of Aires et al. (2008). A 100-µL aliquot of the sonicated biofilm suspension was diluted in 0.9% saline solution, and serial decimal dilutions were inoculated in duplicate on BHI-agar using the drop-count technique (HERIGSTAD; HAMILTON; HEERSINK, 2001). The plates were incubated at 37 ºC for 24 hours. The colony-forming units (CFU) were counted and the results expressed in CFU/mm^2 of surface area to make the results comparable.

4.4 BIOFILM MASS, PROTEIN AND CARBOHYDRATE QUANTIFICATION

The remaining biofilm suspension was lyophilized and the biological powders were pooled to estimate biomass that was formed according each surface. The protein content in the samples was estimated by the Bradford assay (BRADFORD, 1976) using BSA as standard. The total carbohydrate was determined by the phenol-sulphuric method (DUBOIS et al., 1956), using glucose as standard. The results were normalized to biofilm lyophilized biomass.

4.5 NLC-CHITOSAN: PREPARATION AND ANALYSIS OF PHYSICOCHEMICAL PROPERTIES

The NLC-chitosan was prepared by the emulsion and sonication method (PIVETTA et al., 2018, 2019). Briefly, the oil phase composed of natural lipids of Illipe butter and grape seed oil was heated to 70 ºC. The aqueous solution of 1.25% Tween 80 and 0.48% chitosan was heated to 70 ºC and added to the oil phase. The mixture was sonicated for 10 minutes in the ultrasonic processor VCX 750 (Sonics & Materials; Newtown, CT, USA), using a 13-mm probe, at an amplitude of 40%. The dispersion was cooled to 25 ºC prior to adding 0.1% tripolyphosphate (to improve the

stability of NLC-chitosan). Samples of NLC-chitosan formulations (n= 6) were diluted in 1 mM KCl solution and the size, polydispersity index (PdI), and zeta potential were determined by dynamic light scattering (Zetasizer Nano ZS; Malvern Instruments, Worcestershire, UK) immediately after preparation and after 30 and 180 days of storage under static conditions.

4.6 DETERMINATION OF THE MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATIONS

The minimum inhibitory concentration (MIC) of NLC-chitosan was determined using the microdilution method (CLSI, 2012). Briefly, *Escherichia coli* ATCC 25922 inoculum of 5 x 10^5 colony forming units per mL (CFU/mL) was added to a microplate containing dilutions of CHX solution (0.0012 - 0.6 mg/mL; positive control), NLC without chitosan (vehicle control), or NLC-chitosan, (0.0027 - 1.38 mg/mL of chitosan). After 24 hours at 37 ^{o}C, bacterial growth was assessed using 0.2 mg/mL resazurin solution and the microplates were incubated at 37°C for 1 hour. MIC was defined as the lowest concentration of a given treatment that inhibits bacterial growth.

To determine the minimum bactericidal concentration (MBC), 10 µL aliquots of *E. coli* inoculum treated with the sample at a concentration higher than MIC were cultured on BHI agar for 24 hours, at 37 °C. MBC was the lowest concentration that allowed no visible bacterial growth on agar. All the assays were performed in triplicate.

4.7 EVALUATION OF ANTIMICROBIAL ACTIVITY OF NLC-CHITOSAN ON *Escherichia coli* ATCC 25922 BIOFILM

Biofilm was formed as described by Galvan et al. (2016), with the following modifications: the use of Luria Bertani (LB) broth as culture medium and catheter disk as surface for biofilm growth. Briefly, aliquots of *E. coli* grown overnight were centrifuged (10,000 g, 5 minutes), and the bacterial pellets were suspended in 0.9% NaCl to a concentration of 1.5 × 10^8 cells/mL (0.5 McFarland, at 625 nm). The suspension was diluted in Luria Bertani broth to a final concentration of 3 × 10^7 cells/mL. Catheter specimens were suspended with a nylon thread in 1 mL of final bacterial suspension being aseptically distributed into 1.5 mL microtubes. The tubes were incubated for five days, at 37 °C, and culture media was replaced daily.

At 48, 72, 96, and 120 hours of biofilm growth, the biofilms were exposed to 0.9% NaCl solution (negative control), 0.12% CHX solution (positive control), or NLC-chitosan (at final chitosan concentration of 0.28%) for 24 hours. After exposure, the catheter specimens containing biofilms were washed three times with 0.9% NaCl to remove loosely adherent cells, and individually transferred to microcentrifuge tubes containing 1 mL of 0.9% NaCl. Next, the biofilms were sonicated using the sonic dismembrator model FB 505 (Fischer Scientific, Pitsburgh, PA, USA) for 15-second pulses at 20% amplitude to improve homogenization (AIRES et al., 2008). Biofilm suspensions were ten-fold serially diluted in 0.9% NaCl and each dilution was inoculated in BHI agar plates (HERIGSTAD; HAMILTON; HEERSINK, 2001). After incubation for 24 hours, at 37 °C, the number of colonies grown was counted and the results were expressed in CFU/mm^2 of the total surface area of the catheter. The experiments were performed in triplicate.

4.8 STATISTICAL ANALYSIS

The data were analyzed with the aid of the SAS System (SAS Institute Inc. The SAS System, release 9.3. SAS Institute Inc., Cary:NC:USA, 2012). A generalized linear mixed model of analysis of variance was adjusted and the adherence of the residuals to the Gaussian distribution was evaluated by the Shapiro-Wilk test, by the coefficient of skewness, kurtosis and by graphical analysis. The "post-hoc" Tukey-Kramer (bacterial counting) or Tukey (carbohydrates and proteins quantification) test was used to compare means of significant effect. The significance level was fixed at 0.05 for all statistical tests.

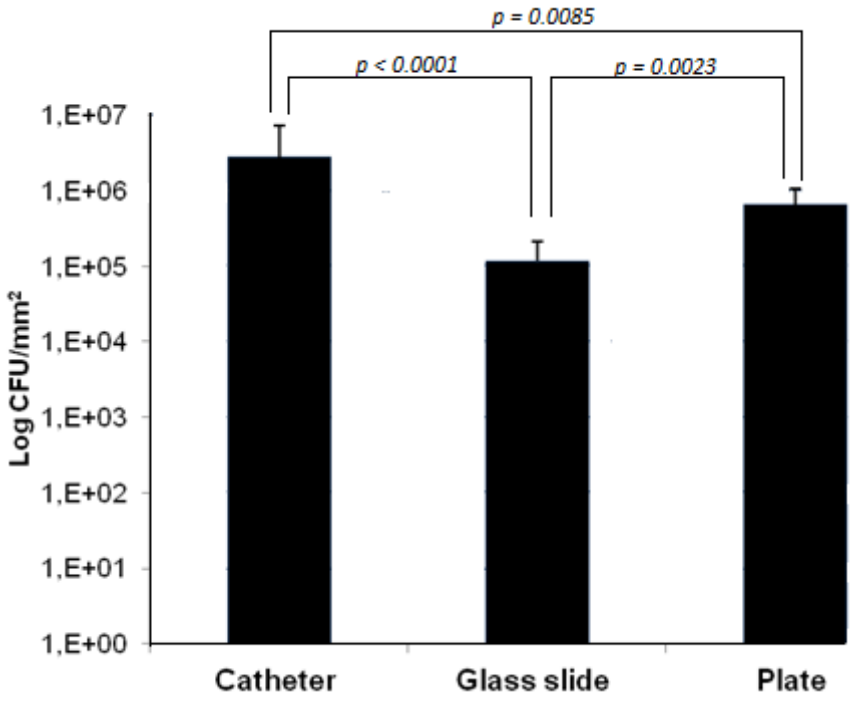
RESULTS

5 RESULTS

5.1 EVALUATION OF *Escherichia coli* ATCC 25922 BIOFILM GROWTH ON DIFFERENT SURFACES

The result (Figure 5) shows the counts of *E. coli* in the formed biofilms differ among the surfaces (catheter, glass slide and plate). Bacterial colony viability was higher in catheter surface compared to glass slides or plates ($p<0.05$). In addition, the lowest bacterial count was observed for glass slide surfaces ($p<0.05$).

Figure 5: Effect of different surfaces on viability of *Escherichia coli* ATCC 25922 biofilm.



The bars represent mean of three data points and SD (Mean ± SD). $P<0.05$ represents statistical differences between the groups (one-way ANOVA followed by Tukey-Kramer's pairwise multiple comparison).

5.2 BIOFILM MASS, PROTEIN AND CARBOHYDRATE QUANTIFICATION

Table 1 shows the total mass of *E. coli* biofilm that was recovered from each surface (catheter, glass slide and plate) as well as the quantification of proteins and total carbohydrates. Although the total biofilm biomass has similar weights, concentration of protein in *E. coli* biofilms was significantly different between catheter and glass slides ($p<0.05$). However, no differences were observed between catheter and plate ($p>0.05$) as well as glass slides and plate ($p>0.05$). For total carbohydrate,

catheter group shows lower concentration than in plate ($p<0.05$) and glass slide ($p<0.05$). However, no significant difference was observed among glass slide and plate groups ($p>0.05$).

Table 1: Total biomass of mature *E. coli* biofilm formed on different surfaces (catheter, glass slide and plate) and quantification of protein and total carbohydrate

	Total lyophilized biomass recovered	**Protein** (µg/mg biomass)	**Carbohydrate** (µg/mg biomass)
Catheter	56 mg ± 0.0	2.0 ± 0.7^{A}	0.2 ± 0.0^{A}
Glass slide	65 mg ± 0.0	11.7 ± 0.5^{B}	5.2 ± 1.2^{B}
Plate	63 mg ± 0.0	6.5 ± 3.4^{AB}	6.5 ± 0.7^{B}

Data are expressed as means ± standard deviations (n=2); values in a column (A, B) not sharing the same letter are significantly different from one other; ANOVA followed by the Tukey *post-hoc* test, $p<0.05$.

5.3 PHYSICOCHEMICAL PROPERTIES OF NLC-CHITOSAN

NLC-chitosan had bimodal particle size distribution (Figure 6). Analysis of particle size and PdI immediately after NLC-chitosan preparation and at 30 and 180 days of storage indicated that these parameters remained constant over time (Figure 7). The mean particle diameter and PdI values were 292.90 ± 2.50 nm and 0.24 ± 0.03 (n = 6), respectively. In addition, the positive value of zeta potential (+19.10 ± 0.20) indicated that the NLC nanoparticles were coated by chitosan.

Figure 6: Size distribution profile of nanostructured lipid carrier particles coated with chitosan (NLC-chitosan).

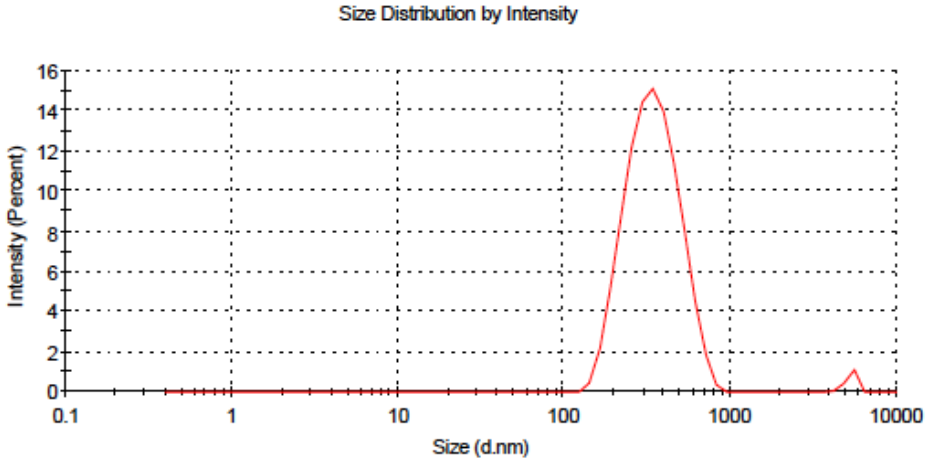
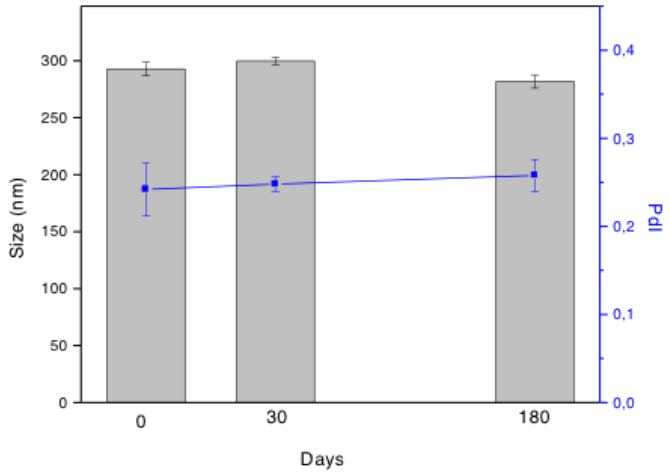


Figure 7: Particle size and polydispersity index (PdI) of nanostructured lipid carrier particles coated with chitosan (NLC-chitosan) immediately after preparation, and after 30 and 180 days of storage under static conditions.



5.4 DETERMINATION OF THE MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATIONS

Analysis of MIC and MBC values (Table 2) revealed that NLC-chitosan inhibited bacterial growth and exerted bactericidal action at concentrations 100 times higher than those required for CHX. Nanostructured lipid carriers without chitosan (NLC) did not interfere with growth of *E. coli* planktonic cells.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of NLC-Chitosan against planktonic cells of *E. coli* ATCC 25922

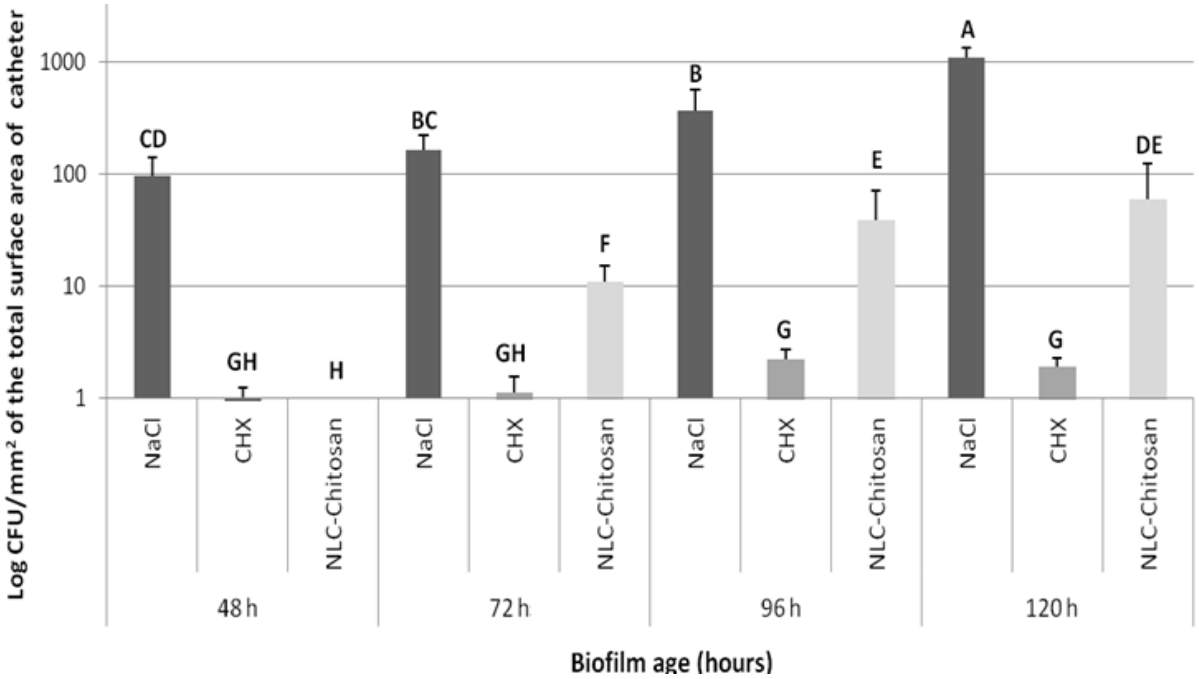
Treatment	MIC (mg/mL)	MBC (mg/mL)
NLC	n. d.	n. d.
NLC-chitosan	$3.45 \times 10^{-1} \pm 0.0$	$3.45 \times 10^{-1} \pm 0.0$
CHX	$2.34 \times 10^{-3} \pm 0.0$	$4.69 \times 10^{-3} \pm 0.0$

n. d.: not detected. CHX: 0.12% chlorhexidine solution (positive control); NLC: Nanostructured lipid carriers without chitosan (vehicle control); NLC-chitosan: Nanostructured lipid carriers coated with chitosan. Data are expressed as mean ± standard deviation

5.5 EVALUATION OF ANTIMICROBIAL ACTIVITY OF NLC-CHITOSAN ON *Escherichia coli* ATCC 25922 BIOFILM

Bacterial colony viability increased with incubation time in the group treated with NaCl (negative control). The growth rates in 48- and 72-hour-old biofilms, and in 72- and 96-hour-old biofilms did not significantly differ from each other ($p>0.05$; Figure 8). Compared with NaCl, NLC-chitosan significantly decreased bacterial colony viability in biofilms of all ages studied (48, 72, 96, and 120 h) ($p<0.05$; Figure 8). NLC-chitosan suppressed bacterial growth as effectively as CHX (positive control) in 48-hour-old biofilms. CHX markedly reduced bacterial viability in biofilms of all ages studied, and more strongly than NLC-chitosan in biofilms older than 72 hours ($p<0.05$; Figure 8). The inhibitory potency of CHX did not change with the biofilm age.

Figure 8: Bacterial colony viability of *E. coli* biofilms treated with nanostructured lipid carrier containing chitosan (NLC-Chitosan)



CHX: 0.12% chlorhexidine (positive control). NaCl: 0.9% saline solution (negative control). NLC-Chitosan: 0.28% (Test sample). Data are expressed as mean ± standard deviation (n = 3). Columns not sharing the same letter (A-H) are significantly different from each other ($p<0.05$; ANOVA combined with Tukey's *post-hoc* test).

DISCUSSION

6 DISCUSSION

In this study, we evaluated whether the three adhesion surfaces most used in uropathogenic biofilm studies (catheter, glass slide and plate) could produce different responses regarding the bacterial colony viability of *E. coli* biofilms, as well as proteins and total carbohydrates quantification. The results of the bacterial count suggest that among of the three adhesion surfaces, *E. coli* colonized catheter surface better than plate and glass slide. Besides, the glass slides showed the lowest bacterial viability compared to other surfaces (Figure 5).

Considering that medical devices such as catheters are usually made from hydrophobic materials, this may explain, at least in part, why bacterial viability was higher in catheter than other surfaces. Culture plate is based on plastic materials such as polystyrene, which can present a high number of hydrophobic sites compared to glass slides. Although bacterial surfaces are complex, *E. coli* presents some hydrophobic residues (DOYLE, 2000), that may be involved in the hydrophobic interaction. Among the nonspecific interactions, hydrophobic bonds are usually the strongest non-covalent forces involved in bacterial attachment and has been defined as the attraction between apolar, or slightly polar, cells or other molecules, when immersed in an aqueous solution (DOYLE, 2000; GOMES et al., 2015).

Our results support the research of Gomes et al. (2015) where silicone catheter was the surface with the highest number of adhered *E. coli* cells until 24 hours of biofilm formation. However, in our study, the same tendency was observed with a mature biofilm formed until 5 days, which may suggest that hydrophobicity occurs regardless of the biofilm development stage.

Although *E. coli* presents higher bacterial colony viability on catheter surface, the results about biomass weight, protein and total carbohydrates contents show that biofilm of this surface is primarily composed of bacterial cells with a low presence of extracellular polymeric substances (EPS) (Table 1). On the other hand, *E. coli* biofilms grown on glass slide and plate have more protein and carbohydrates than those grown on catheter surface (Table 1). The EPS is essential for the biofilm architecture and development, probably on surfaces with lower chances of direct microorganism-surface interaction, the pronounced EPS production may help in the structuring of the biofilm on the surface. It has also been reported that polysaccharide polymers, which are a part of the EPS matrix, play an essential role in the attachment of biofilm to the hydrophilic surface rather than to hydrophobic surface (DOLAN,

2001). Although EPS presents as major constituents proteins and carbohydrates, future studies will be required to investigate in more detail the composition of these biofilms, such as specifying the type of carbohydrates and the content of other macromolecules.

However, biofilms of *E. coli* may not form easily on the hydrophilic glass surface but when they form adhered better, are better protected and are more stable, structure-wise, than biofilms on catheter surface. Probably, the mature biofilm formed on catheter surface is more susceptible to antimicrobial effects when compared with those obtained on the other materials. It is widely known that the extracellular matrix is essential for protection against antimicrobial agents (FLEMMING; WINGENDER, 2010; GOMES et al., 2015; FLEMMING et al., 2016). However, future studies are required to investigate if the amount of EPS may influence susceptibility to antimicrobial agents and whether the surface of adhesion can also be related.

Although the conditions presented in this study are far from those found in medical practice, the results suggest that distinct surfaces can affect *E. coli* biofilm formation and its EPS content, and that catheter could be a better surface to study *E. coli* biofilm compared to either glass slides or polystyrene plates even in advanced stages of biofilm development. Our findings can contribute to establishing an initial basis for the development of future studies involving strategies to prevent biofilm formation as well as the development of mature biofilms that probably present higher antimicrobials tolerance.

Considering that patients can use catheter for several days, the biofilm age poses a challenge to its removal with antimicrobials. Mature biofilms are up to 1,000-fold less susceptible to various antimicrobial agents than bacteria in planktonic culture or younger biofilms (STEWART; COSTERTON, 2001).

Rapid advances in nanoscale engineering may provide opportunities to develop new nanomaterials to prevent bacterial adherence and growth (PAULA; KOO, 2017). In this study, we examined the antimicrobial effect of NLC-chitosan against *E. coli* biofilms of different ages. The NLC-chitosan preparations were composed of a bimodal population with a first fraction (98%) of small particles (< 300 nm) and a second fraction of larger particles (Figure 6). Chitosan nanoparticles usually present bimodal population due to the rearrangement of particles after addition of tripolyphosphate and/or metal from the sonicator tip (RAMPINO et al., 2013).

The mean size of NLC-chitosan particles was greater than 100 nm. Despite the well-established protocols for the preparation of chitosan nanoparticles, it remains unclear how different processing factors affect the particle size (SREEKUMAR et al., 2018). High molecular weight chitosan can produce large nanoparticles with high PdI (RAMPINO et al., 2013; ZAKI et al., 2015). In the present study, the chitosan concentration and/or its molecular weight might have contributed to increase the nanoparticle size and its PdI; however, future studies are required to elucidate this hypothesis. Zeta potential is another essential parameter for the stability of nanosuspensions (MÜLLER et al., 2001). The positive zeta potential of NLC-chitosan indicated that the formulated NLC was coated with chitosan. In addition, the particle size and PdI remained unaltered for up to 180 days of storage (Figure 7). The presence of chitosan and Tween 80 on the particle surface probably confers steric and electrosteric stability, respectively to nanoparticles and improves their stability (MOORE et al., 2015), which could help to overcome the issues related to stability of NLC. Further studies are needed to analyze how chitosan coating affects the stability of NLC.

Data from the antimicrobial assays, i.e. the MIC and MBC values, demonstrated that NLC-chitosan inhibited the growth of and killed the planktonic *E. coli* cells (Table 2). Interestingly, NLC alone did not affect bacterial growth, indicating that the antimicrobial activity of NLC-chitosan resulted from the chitosan coating. NLC-chitosan effectively killed planktonic *E. coli* cells at a concentration about 100-fold higher than that required for CHX. The antimicrobial action of CHX against *E. coli* is well-known (KAMPF, 2019; SCHMUDDE et al., 2019), but its extensive use has raised concerns regarding development of acquired bacterial resistance to this potent antimicrobial (KAMPF, 2016), especially in the hospital environment (KAMPF, 2019). In this sense, the use of natural products such as chitosan may help to develop new antimicrobial tools.

The antimicrobial potential of NLC-chitosan became evident due to growth inhibition in biofilms formed for up to 120 hours (Figure 8). The biological activity of NLC-chitosan can be attributed to the presence of free protonated amino groups from chitosan (pKa of ~6.5) on the nanoparticle surface (ALI; AHMED, 2018), which may interact with components of the biofilm matrix and bacterial surface, such as the cell wall. NLC-chitosan exerted its maximum antimicrobial effect on 48-hour-old biofilms, which was comparable to that exerted by CHX, but became weaker as the biofilm

maturation time increased (Figure 8). Our findings corroborate a literature report that the susceptibility of *Klebsiella pneumoniae* mature biofilms to ceftriaxone is decreased when compared with young biofilms (AIRES; BATISTA; PITONDO-SILVA, 2017). It is well-established that extracellular polymeric matrix decreases antimicrobial penetration into deeper biofilm layers (FLEMMING; WINGENDER, 2010). At 48 hours, the extracellular polymeric matrix of *E. coli* biofilms was probably less structured than in older biofilms and allowed a better diffusion of nanoparticles through the biofilm matrix. Future studies are necessary to analyze the penetration of NLC-chitosan into different biofilm layers.

Despite the limitations of the biofilm model employed in this study, such as the use of only one bacterial species, it enabled evaluation of the antimicrobial potential of NLC-chitosan. As both lipophilic and hydrophilic compounds can be incorporated into NLC, NLC-chitosan is a useful carrier for other antimicrobial agents that can act in synergism with its intrinsic antimicrobial effect. Together, our findings indicated that the “chitosan/lipid” association exerted antimicrobial effects even in mature biofilms of *E. coli*, which is one of the most representative bacteria in catheter-associated urinary tract infection. The data reported here can support further studies to improve this nanoformulation, which seems to be a good strategy to fight against catheter-associated biofilms.

CONCLUSIONS

7 CONCLUSIONS

The results suggest that catheter is a proper surface to study *E. coli* biofilm compared to either glass slides or polystyrene plates. In addition, both free cells and biofilms of *E. coli* were significantly affected by NLC-chitosan, which can be a feasible approach for studies using uropathogenic bacteria. In future, urinary catheter can be used as model to study simulated UTIs, using mixed populations of bacteria, and the effect of NLC-chitosan or its association with other antimicrobial agents evaluated.

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