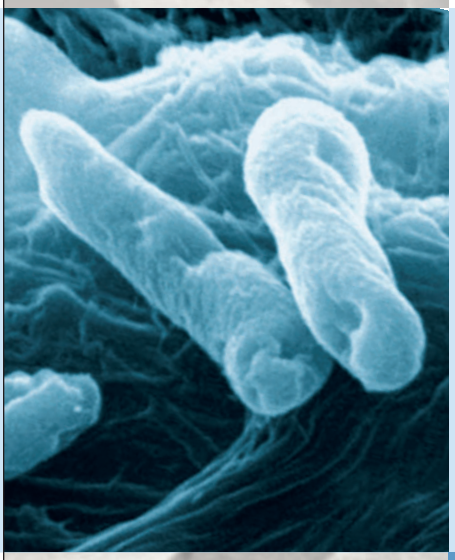
NIMBUS®
Technology

A Safe Harbor Against Bacterial Resistance



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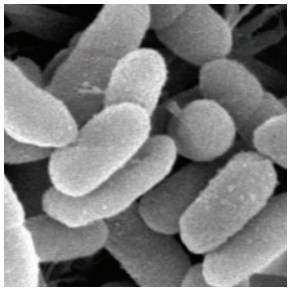
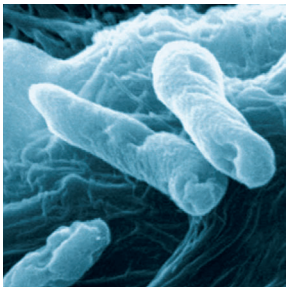




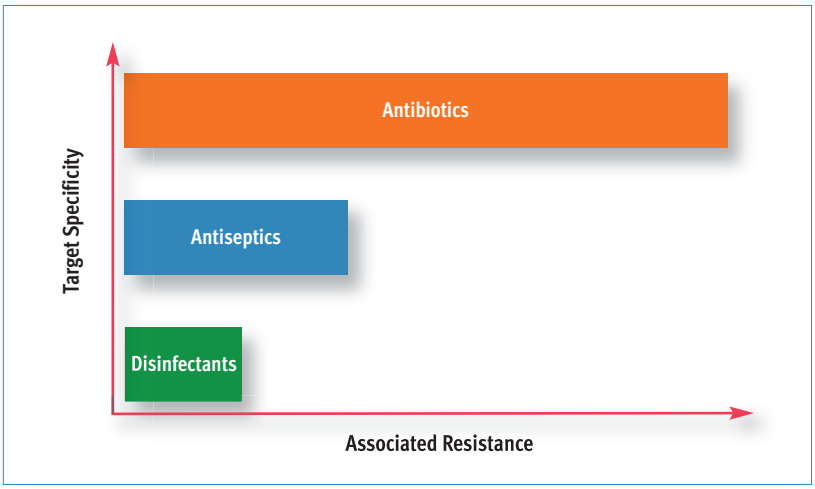
Summary

The emergence of bacteria resistant to antibiotics has been widely documented and publicized. This has in turn prompted concerns about the possibility of bacteria developing resistance to certain classes of microbicides. The microbicidal agent used in Quick-Med Technologies' NIMBUS® product line is a high molecular weight polymeric quaternary ammonium compound (a polycationic microbicide). Antibiotics rely on gaining entry to the cell interior for their biochemical mechanism of kill. NIMBUS technology is based on large molecules with many hundreds of charged sites per molecule, and employs a physical mechanism of kill based on cell wall disruption. Each individual NIMBUS molecule is very large and potent, and acts on the outside of the cell, as the large molecular size prevents entry of the microbicide into the cell. Testing was conducted on dressings utilizing NIMBUS polymeric cationic microbicidal technology, confirming that over the course of 10 iterations no bacterial resistance was generated.

General discussion

The body of research currently available suggests that the acquisition of resistance by bacteria depends on how a particular agent acts as an antimicrobial. Generation of resistance is accomplished more easily if the mechanism of microbicidal activity is through a very specific target, such as a metabolic process inside a cell. This requires the entry of the agent (for example, an antibiotic) into the cell. If the mechanism of microbicidal activity is general (as for cationic biocides), or acting against an innate structural component of the cell body, then it is more difficult for bacteria to develop resistance to that agent. Figure 1 shows a generalization based on types of microbicides, comparing target specificity to the likelihood of bacteria acquiring resistance.

Figure 1: Relative likelihood of bacteria being able to develop resistance vs. related to the target specificity



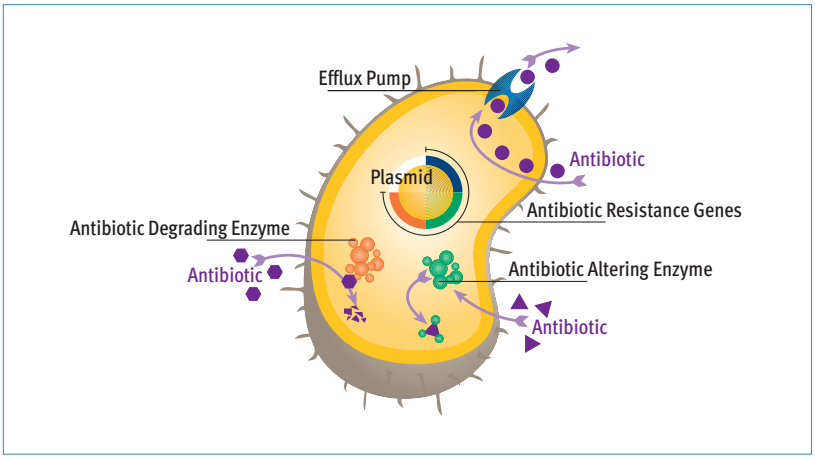
Antibiotics exert antibacterial efficacy by acting on a single cellular target which can be an intermediate in a metabolic pathway or a very specific structural component of the cell (Prescott, Harley and Klein's Microbiology, 5th Ed). Antibiotics are designed to be pharmacologically precise single-target drugs: this permits specific bacterial diseases to be precisely addressed, but is also the reason that bacterial resistance can be developed in a single one-step mutation. Antibiotic resistance is commonly traceable to a single mutation in a key bacterial gene (Poole, 2002).

Microbicides with antiseptic or disinfecting properties (silver, quaternary ammonium compounds, iodine, alcohols, hypochlorite bleach, cationic biocides etc.) act as multi-target agents: they are not limited to a highly specified single target in their mode of action (McDonnell and Russell, 1999). Such antimicrobial agents generate significantly less resistance because the resistance has to be expressed against a variety of structurally diverse targets. The likelihood of the development of biocide resistance in a single mutation step (similarly to antibiotic resistance) is negligible.

General mechanisms by which bacteria develop resistance to microbicidal agents

There are many instances where agents are known not to be effective against certain bacterial species — this is called natural or intrinsic resistance. Intrinsic resistances are well documented, and health care providers avoid using certain agents known to be ineffective against particular microbes.

Figure 2: Bacterial resistance mechanisms: antibiotic agents



Acquired resistance poses the highest risk in healthcare and community settings because in this instance, bacterial species show decreased susceptibility to previously effective treatments. The term "*resistant bacteria*" describes the fact that the organism is not inhibited by some previously effective standard measure that has now become unsuccessful.

Bacteria have shown a number of different mechanisms to develop resistance to microbicides and specifically antibiotics (Poole, 2002). Mechanisms include genetically encoded alterations in the metabolic pathway to circumvent antibiotic activity, or the production of enzymes that degrade or alter the antibiotic to render it ineffective [Figure 2].

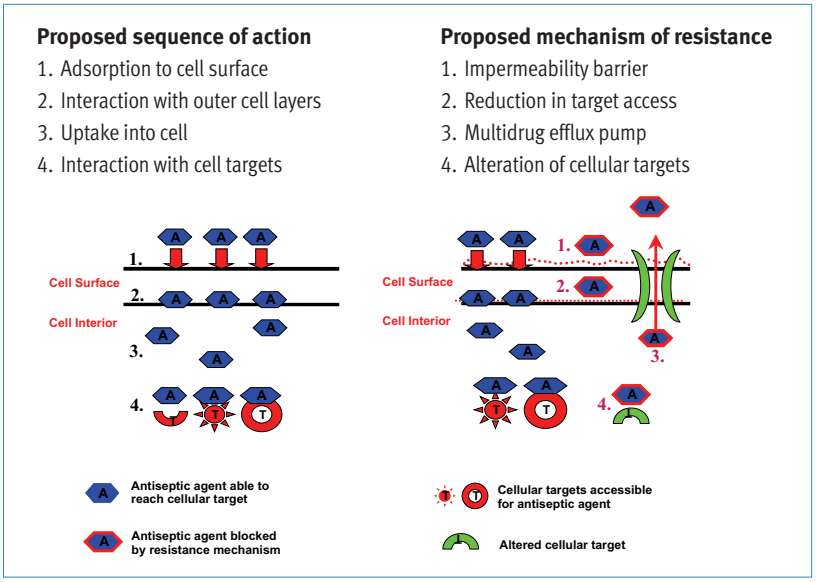
A resistance mechanism that is common for small molecules is the efflux pump: a mechanism by which the bacteria pump the biocidal agent out of the cell through the use of a transport protein. This enables the bacteria to withstand much higher concentrations of the agent (either antibiotic or antiseptic).

Bacterial response to antiseptics

The mechanism of bacterial resistance depends on the mode of action of the antimicrobial agent. Antiseptics generally act as multi-target drugs and generate significantly less resistance because the resistance has to be expressed against a variety of structurally diverse targets. The likelihood of the development of antiseptic resistance in a single mutation step (similarly to antibiotic resistance) is negligible.

The vast majority of antiseptics act on cell-surface components of the bacteria and/or the cytoplasmic membrane. Bacteria do not develop resistance to *antiseptic agents* as readily as to antibiotics, but some of the same defense mechanisms apply. The resistance mechanisms include stress response (adaptations to limit uptake of antimicrobial agent), the presence of efflux pumps, and target modification for small diffusible microbicides.

Figure 3: Mode of action and proposed mechanism of resistance generation to antiseptic agents



The modes of action of most antiseptics is to approach the cell wall, transfer into the cell interior and act on a cellular target (Maillard, 2002), as illustrated by the sequence in Figure 3. Bacteria develop resistance by implementing the mechanisms illustrated on the right hand side of Figure 3, including arresting the biocidal agent at the surface by presenting a barrier that is difficult to traverse, altering the cellular targets that are being acted on, and/or by using transport proteins to eject invading agents from the cell using an efflux pump mechanism.

The action of antiseptic microbicides illustrated in Figure 3 is attributed to high affinity binding to the negatively charged bacterial walls and membranes, and is directed against a wide target spectrum. This broad-action class of agents has significantly less potential for resistance development because resistance has to be expressed against a variety of structurally diverse targets (Gilbert and McBain, 2003).

Cationic agents

Cationic antiseptics include a range of agents that can be chemically distinguished through the number of positive charge sites resident on the molecule (or molecular repeat units for polymeric agents). Quaternary ammonium compounds (QACs) are commonly used cationic antimicrobials (Gilbert and Moore, 2005), of either monomeric (i.e. benzalkonium chloride – BAC) or polymeric types (i.e. NIMBUS). Quaternary ammonium compounds ('quats' or polyquats in the case of polymeric structures) have a fundamentally different mechanism of antimicrobial activity than small-molecule antiseptic agents such as silver or iodine that require entry into the cell in order to exert antimicrobial activity. Quats bind rapidly to the cellular envelope and displace otherwise stable calcium ions to chemically destabilize the cell wall structures. Cationic biocides cause the membrane to fragment, leading to generalized cellular leakage. The specific interaction and potency of the agents varies with number of charges, molecular configuration and, particularly for the monomeric compounds, with the length of alkyl chains bound to the quaternary moiety. Evidence of resistance to QAC biocides exist primarily for low molecular weight compounds such as benzalkonium chloride (Hegstad

et al., 2010). Known mechanisms of acquired biocide resistance are attributed to the changes in the cellular surface that suppress the absorption of biocide molecules into the cell, and plasmid mediated efflux that reduces the intracellular level of the agent (Russell, 2001; Poole, 2002).

Polycationic microbicides function very similarly to other cationic microbicides, but have some special features that emerge from their physical dimensions. The molecular weight of a polycationic quaternary microbicide such as poly-DADMAC, the active agent in NIMBUS, is orders of magnitude larger than antibiotics (see Table 1), or ordinary cationic microbicides. For comparison, Vitamin B12 represents one of the larger biological molecules that cells ingest, and is still orders of magnitude smaller than NIMBUS. Polycationic microbicide species act solely on the surface of the molecule, with no perceivable mechanism to permit internalization. Gilbert and Moore (2005) describe the mechanism of cell wall disruption induced by polymeric cationic biocides in excellent detail as shown graphically in Figure 4. The cationic polymer chains coordinate to the anionic segments of the phospholipid membrane, displacing stabilizing calcium ions. As increasing numbers of cell membrane molecules coordinate to the polymer, the integrity of the bacterial membrane is compromised, leading to gaps and holes as shown in the image. Polymeric cationic biocides have significant diffusion limitations due to their high molecular weight polymeric chains. Additionally, polymeric cationic biocides have a large multiplicity of charges with which to bind cell wall components, further limiting their potential for diffusion into the cell. The high local density of charges makes for a more effective biocide in that the perturbations induced within the cell wall matrix are much greater for each single molecule relative to monomeric cationic biocides such as BAC.

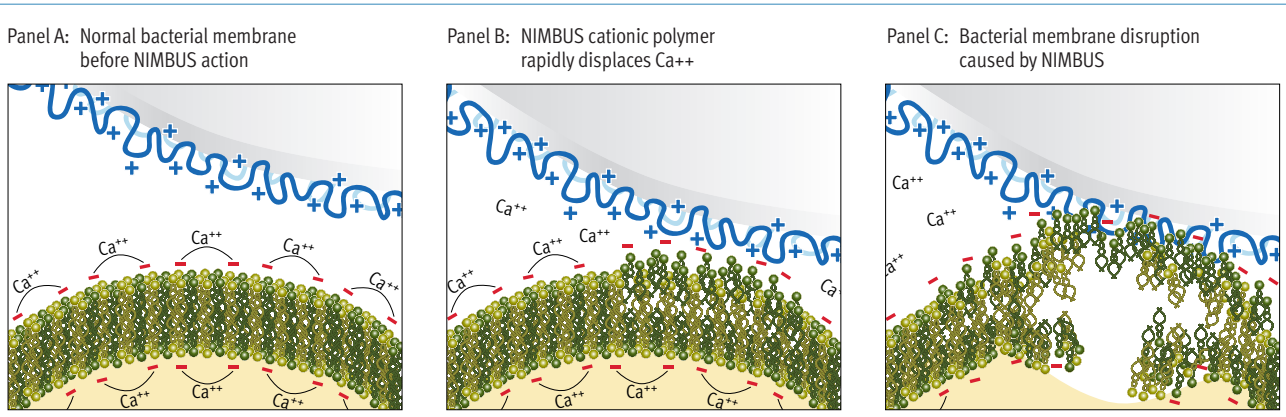
Table 1: Comparison of various molecules and their molecular weights

Molecule	Type of Molecule	Molecular Weight, g/mol
Penicillin	Antibiotic	373
Ciprofloxacin	Antibiotic	386
Vitamin B12	Vitamin	1355
Benzalkonium chloride	Cationic microbicide	354
Chlorhexidine gluconate	Cationic microbicide	898
PHMB	Polycationic microbicide	~2,000-4,000
Poly-DADMAC	Polycationic microbicide	~200,000-250,000

Polycationic biocide: NIMBUS

Quick-Med Technologies Inc. designed the NIMBUS antimicrobial polymeric technology, which utilizes a long chain (molecular weight >200,000 daltons) polyquaternary agent that is permanently bound to a solid substrate. Both the large size of the polymeric agent and the physical attachment to a surface preclude entry into cells, while the high charge density provided by hundreds of quaternary repeat units ensures high biocidal activity.

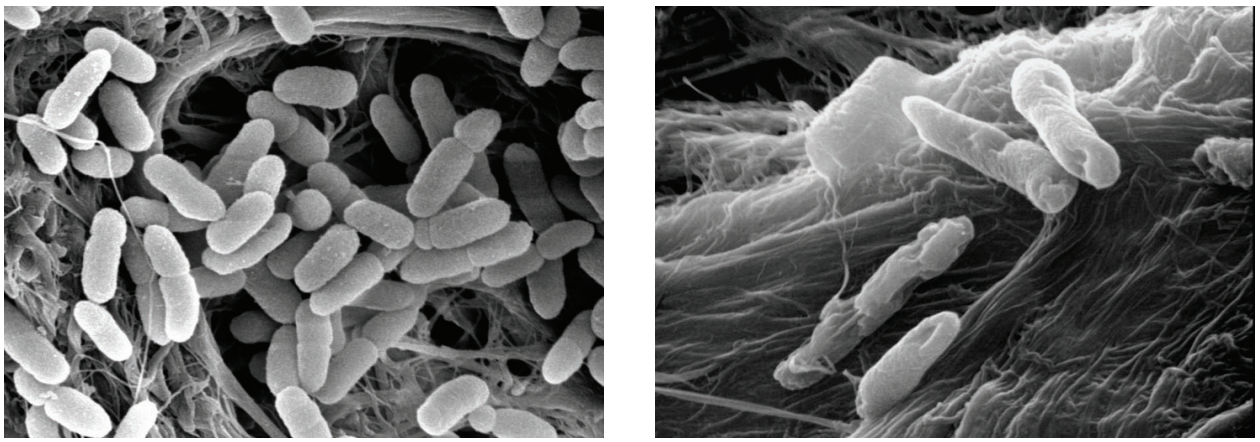
Figure 4: Conceptual Representation: action of polymeric cationic biocidal agent



Normal bacterial membranes (Panel A) are stabilized by Ca++ ions binding anionically charged phospholipids. NIMBUS quat-polymer rapidly displaces Ca++ (Panel B) leading to loss of fluidity (Panel C) and eventual phase separation of different lipids. Domains in the membrane then undergo a transition to additional smaller micelles.

NIMBUS does not require entry into the cell in order to exert antimicrobial activity but destabilizes the cell wall structures, inducing cellular collapse, as experimentally demonstrated by the high resolution SEM images in Figure 5. The chemistry of the cell wall is relatively immutable, so the generation of resistance to this mechanism is extremely unlikely.

Figure 5: SEM imaging of *E. coli* on untreated gauze wound dressing (left) and on NIMBUS treated wound dressing (right)

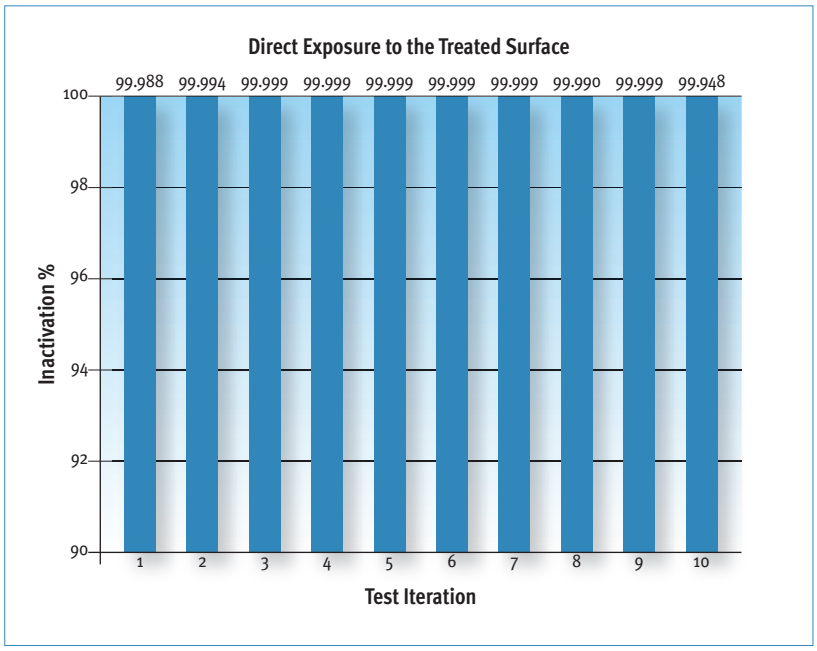


Test of bacterial resistance to NIMBUS

We evaluated changes in bacterial susceptibility to NIMBUS biocide after step-by-step adaptation training of *E. coli* culture to the active surface of the NIMBUS dressing. Sequential assessment of the minimum inhibitory concentration (MIC) was used as an additional confirmation experiment.

The selection vector was created by exposing serial passages of bacteria to the NIMBUS treated surfaces. Three or more isolated survivor colonies were selected and propagated into new inoculum. Exposure to the treated substrate was repeated for ten passages. The results of these experiments demonstrated that *E. coli* did not become resistant to NIMBUS after a prolonged and repeated exposure.

Figure 6: Determination of microbicidal activity of NIMBUS surface and testing of bacterial resistance



Conclusions

The acquired resistance mechanisms to microbicides are dependent on factors that involve entry into the bacterial cell. Whether it is followed by expulsion via an efflux pump, or by the alteration of an intracellular target site, the functionality of the agents is implicit to their size and their ability to cross the cell membrane to interfere with a specific metabolic pathway. However, very large polycationic microbicides, such as the active agent in NIMBUS, are able to counteract such mechanisms due to the massive size as a polymer, the external action on the bacterial cell, and the inability to enter the cell and become a part of a metabolic pathway. Without this intracellular interaction, bacteria cannot create a genetically-encoded resistance mechanism to interrupt or prevent the microbicidal action of high molecular weight polycationic microbicides.

Experimental evidence demonstrated that bacteria fail to develop resistance to NIMBUS over the course of many successive generations. The NIMBUS antimicrobial surface destroys bacteria by causing irreversible damage to bacterial membranes rather than by targeting a specific intracellular target, and therefore carries a low risk of resistance development. The design of this technology purposely minimizes opportunities for bacteria to generate resistance, thus permitting safe and effective prophylactic application.

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Glossary

Microbicide — a substance that is specifically destructive to microbes (bacteria, fungi, viruses, protozoal parasites). These substances are used as prevention rather than treatment of an infection.

Antibiotic — a substance produced by, or a semi-synthetic substance derived from, a microbe and able in dilute solution to inhibit or kill another microbe. Antibiotics are used as treatment rather than prevention of an infection. Antibiotics discriminate between prokaryotic and eukaryotic cells by using highly specialized mechanisms specific to the prokaryotic cell.

Antiseptic — a substance used externally on living tissue to suppress bacterial growth. Antiseptics are applied to living tissue/skin to reduce the possibility of infection and/or sepsis — in a preventative fashion rather than as a treatment. There are many antiseptic types that work by different mechanisms. The goal of antiseptic agents is generally to suppress the growth of bacterial cells while leaving intact the host mammalian cells.

Disinfectant — a substance used on non-living surfaces to destroy microorganisms. Disinfectants are generally utilized on inert surfaces rather than on skin and tissue, because they are agents whose activity level is extremely high. These agents are not selective and can be potentially harmful or toxic to living tissue at in-use concentrations.

Single-target site — refers to the mode of action of antibiotics. Antibiotics act on a single intracellular target in order to be effective against bacteria. The target is either a precise event in a metabolic pathway or a precise structural target within the cell.

Multi-target site — describes the microbicidal action of antiseptics as being less specific than that of antibiotics. These compounds physically denature and compromise components of the bacteria (such as proteins and cell wall components) by acting upon general cellular structures. This is described as multi-target because the compounds will not select specific proteins, or specific single points in the cell wall while carrying out these functions.

Cationic microbicides — microbicides that are positively charged. These typically act by coordinating to the negatively-charged bacterial cell membrane and disrupting it, leading to cell death. These may be either small molecules or large polymers.

Quaternary ammonium compounds (QACs) — positively-charged (cationic) disinfectants in which microbicidal activity is provided by a nitrogen atom with four chemical bonds ("quat"), with one or more alkyl groups of various lengths bonded to the nitrogen.

Polyquaternary ammonium compounds — a series of repeating quaternary ammonium compound units bonded together to form a high molecular weight "chain" that contains a multiplicity of positive charges.

Intrinsic resistance — the case of bacteria not being susceptible to a particular agent due to innate structural characteristics. For example, certain antibiotics are known to be ineffective against M. tuberculosis by their very nature — not due to any alteration of the bacteria after exposure to the antibiotic.

Acquired resistance — the case of bacteria developing reduced susceptibility to a compound that they were initially sensitive to. Acquired resistance poses the highest risk in both healthcare and community settings.