L9 - 10: Overcoming drug resistance & intracellular infections

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Overcoming drug resistance & intracellular infections

Intended learning outcomes

- To be aware of persistence in the population and recurrence in individuals of common and serious infections surviving inside cells
- To understand why infections inside cells are more difficult to treat and clear
- To understand why nanoparticulate antibiotics are very effective at treating chronic and serious infections, and also reduce the risk of antibiotic resistance
- To understand why some nanomaterials have broad spectrum antimicrobial activity for reducing the risk and spread of infections, including multi-drug resistance

Intracellular pathogen problem -1

Pathogens exploit niches in the host to survive. Many (*eg S. aureus, Mycobacterium tuberculosis, Salmonella enterica*) take shelter and persist in mammalian cells, making the infection latent or recurrent.

- Intracellular location protects from host defences and from antimicrobial therapy. Over two thirds of prescribed antibiotics are ineffective against intracellular infections.
- Develop 'small colony variants' (SCVs) – sub-populations with a slow growth rate, which are more resistant to antibiotics.
- Some antibiotic families (*eg* β–lactams & aminoglycosides) are hydrophilic with restricted penetration into host cells.
- Other antibiotics diffuse well into cells (*eg* fluoroquinolones & macrolides) but have low retention inside cells.
- Sub-cellular distribution is not uniform and varies with different antibiotics.

Intracellular pathogen problem -2

- Low cellular concentrations of antibiotics are often sub-therapeutic, resulting in low effectiveness against intracellular pathogens and the emergence of antibiotic resistance.
- High doses of antibiotics are often given to treat intracellular infections and to overcome drug resistance.
- High doses generate many side effects and toxicity (*eg* aminoglycoside ear & kidney toxicity).
- Only ~20 antimicrobial compounds in late stage clinical trials.
- Antibiotics forgotten because of their toxicity are being reconsidered (*eg* colistin/polymycin E).
- Given few new options, nanomedicines are considered to deliver new and existing antibiotics to intracellular locations with effective doses and pharmacokinetics, to reduce toxicities and drug resistance risks.

Examples: as well as persistent viral and parasitic, many common fungal & bacterial infections are intracellular

Example viral	Disease	Target cells
Herpes simplex	Type 1 oral, Type 2 genital	Epithelial
Hepatitis C	Liver cirrhosis, carcinoma	Hepatocytes
Resp syncytial virus	Pediatric respiratory	Epithelial
HIV	AIDS	T cells & others
Example protozoan		
Plasmodium	Malaria	Hepatocytes, erythrocytes
Leishmania	Cutaneous	Macrophages, neutrophils
Example fungal		
Candida albicans	Thrush, other cutaneous & mucosal forms	Epithelial
Aspergillus fumigatus	Pulmonary aspergillosis	Epithelial

Example bacterial	Diseases	Target cells
Mycobacterium tuberculosis	Tuberculosis	Macrophages, hepatocytes
Mycobacterium leprae	Leprosy	Macrophages, epithelial cells
Pseudomonas aeruginosa	Pneumonia, endocarditis, meningitis,	Macrophages, epithelial cells
Listeria monocytogenes	Listeriosis: diarrhoea, meningitis & septicemia in some	Macrophages, hepatocytes, enterocytes
Staphylococcus aureus	Pneumonia, mastitis, phlebitis, endocarditis, urinary, osteomyelitis	Macrophages, neutrophils
Salmonella spp	Salmonellosis (diarrhoea), typhoid fever	Macrophages, enterocytes
Escherichia coli	Diahorrea, urinary tract infections, meningitis in neonates	Epithelial cells, macrophages
Yesinia pestis	Bubonic Plague	Macrophages

MPS/RES host cells -1

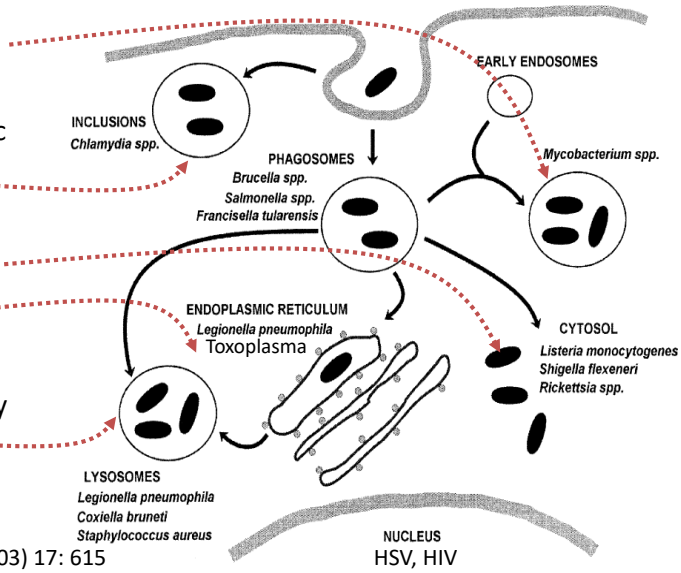
- Pathogens shelter / hide mainly in the mononuclear phagocyte system (MPS):
  - including the reticuloendothelial system (RES),
  - blood monocytes, tissue macrophages & dendritic cells.
- Remove foreign particles like pathogens by phagocytosis:
  - involving various endosomal signalling pathways,
  - normally resulting in lysis and digestion of pathogens to present their antigens to the adaptive immune system
  - intracellular pathogens subvert / hijack this process.

MPS/RES host cells -2

- 'Hijack' signalling pathways to avoid lytic mechanisms:
  - 'Trojan Horse' sanctuary in cell to survive & multiply
  - low activity of antimicrobials inside host cells
  - escape from the innate & adaptive immune systems.
- The macrophage reservoir allows pathogens to persist and to spread into other tissues (*eg* cross the BBB) and to cause recurrent infections.
- Knowing this intracellular niche and the cellular pharmacokinetics of the antibiotic are essential in formulating appropriate therapies.

Multiple pathways interplay for pathogens to survive and multiply in macrophages

1. Delay or block lysosome fusion
2. or non-phagocytic pathway with unlikely lysosome fusion
3. Disrupt & escape the endosome
4. Fuse with other organelles
5. Survive & multiply in mature phagolysosomes



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Diverse behaviour of antibiotics (between & within class)

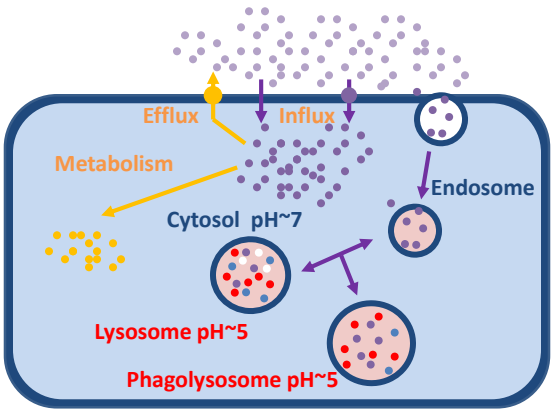
Activity may correlate weakly with extracellular concentration (C_e), or even intracellular concentration (C_i): balance between drug influx and efflux, accumulation (C_i/C_e, at equilibrium below), predominant localisation within cell, binding and metabolism, local physicochemical conditions, responsiveness of bacterium

Examples: Class	**Antibiotic**	**Influx**	**Efflux**	**C_i/C_e**	**Main localisation**
β-lactams	All	Fast	Variable	<1	Cytosol
Macrolides	Erythromycin	Fast	Fast	4-10	Lysosomes > cytosol
	Clarithromycin	Fast	Fast	10-20	
	Azithromycin	Fast	Slow	40-300	
Fluoroquinolones	All	Fast	Fast	4-10	Cytosol
Aminoglycosides	All	V slow	V slow	2-4	Lysosomes
Glycopeptides	Vancomycin	Slow	?	8	Lysosomes (kidneys)

Reasons for poor or variable activity against intracellular pathogens -1

- Maximal intracellular activities remain consistently lower than extracellular activities, irrespective of the level of drug accumulation and of the pharmacological class.
- Relative potencies of some antibiotics are markedly decreased against intracellular bacteria compared with those against extracellular bacteria (differences in expression).
- Gentamicin and oritavancin most affected, even though both drugs primarily concentrate in lysosomes and related vacuoles, where many intracellular bacteria hide.
- Lysosome vacuoles are acidic, which will markedly decrease the activities of some drugs (*eg* aminoglycosides).

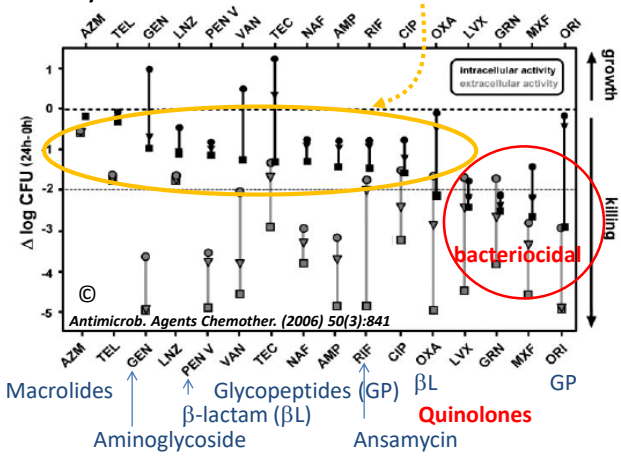
Reasons for poor or variable activity against intracellular pathogens -2



- Some antibiotics penetrate poorly, only taken up by pinocytosis
- Uptake accompanied by fast efflux
- Metabolism may inactivate drug
- Antibiotic and bacterial locations may differ
- pH & binding effects in different locations may reduce local antibiotic activity

Example: macrophage /*Staph aureus*

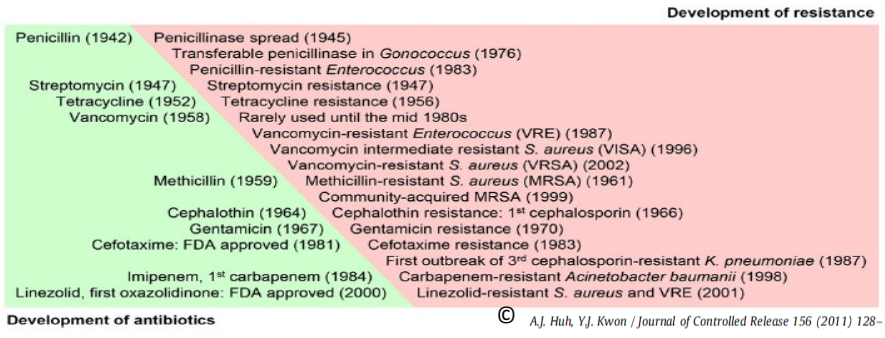
Many drugs recommended for treatment (*eg* β-lactams, linezolid & rifampicin) may **fail to demonstrate significant intracellular bacteriocidal effects**, whereas newer quinolones may be more effective



At an extracellular concentration corresponding to their C_{max} in humans, only the β-lactam, *oxacllin,* and *levofloxacin, garenoxacin & moxifloxacin* quinolones and, variably, the lipoglycopeptide *oritavancin* had truly intracellular bactericidal effects (2-log decrease or more)

Antibiotic resistance - inevitable

- Resistance to existing drugs, few new antibiotics developing.
- Problem may not be solved with new antibiotics.
- Antibiotics are small molecules with a single site of action, which makes them open to developing multiple antibiotic resistance mechanisms, and which always happens.
- Long history of resistance shortly after new antibiotic launch.



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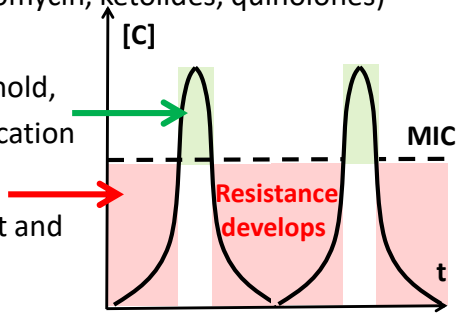
Antibiotic resistance and nanomaterials

- Antibiotic resistance develops particularly in the more 'concentration dependent' antibiotics/families (*eg* vancomycin, aminoglycosides, azalides/azithromycin, ketolides, quinolones)
  - antibiotics work when AUC or C_{max} / MIC above threshold, varying for drug / organism / location
  - when below threshold organisms are antibiotic tolerant and the likelihood of developing antibiotic resistance increases.



[MIC = minimum inhibitory concentration]

- Nanomaterials overcome drug resistance: (a) by bypassing resistance mechanisms and/or (b) by broad spectrum action against multiple conserved targets, which are less or not susceptible to antibiotic drug resistance mechanisms.

Examples: drug resistance mechanisms
genetic - spread in population

- **Reduced uptake** by thicker & less drug-permeable cell envelope and/or increased drug efflux eg
  - tetracyclines, sulfonamides, quinolomes, aminoglycosides, chloramphenicol, macrolides, strepogrammins
  - Gram-negative *P. aeruginosa*, *E. coli via* inner membrane H+/drug anti-porters linked through periplasm to outer membrane pore.
- **Altered target** with lower drug binding affinity *eg*
  - beta-lactams, glycopeptides, sulfonamdes, quinolones, macrolides, aminoglycosides, tetracyclines, linezolid, rifampicin.
- **Drug inactivation enzymes** *eg*
  - β–lactams, aminoglycosides, chloramphenicol, tetracyclines, macrolides, quinolones, streptogrammin.
- **Competitive inhibitors** *eg* *p*-aminobenzoic acid against

Drug tolerance mechanisms
physiological – exist in population

- **Intracellular persistence** includes obligate (mycobacteria, chlamydia, rickettsia) and many facultative pathogens.
- **Low metabolism persisters** (few as 1 in 10^6 in population) are less affected (*ie* unaffected until metabolism switches on and drug still present).
- **Swarming** - cell elongation into filaments and formation of drug-tolerant rafts.
- **Biofilms** tolerate high concentrations of antibiotics through extracellular polymer matrix barrier to diffusion, binder of drugs, extracellular enzyme degradation of drugs, reduced nutrients and lower metabolic state (more persisters).

Overcoming drug resistance /tolerance with antibiotic nanomaterials

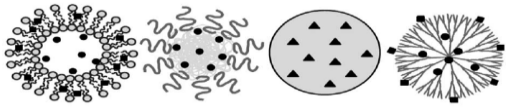
Antibiotic nanomaterials overcome drug resistance by:

- targeting to site of infection, including intracellular, allowing higher doses with lower toxicity
- increasing uptake and avoiding efflux resistance mechanisms
- increasing drug concentration around the infecting microorganisms for longer periods
- nanoantibiotics packaging multiple drugs into same particle to kill cells developing a single resistance mechanism

Nanomedicine antibiotics -1

No report yet of complete elimination of infections, but many pre-clinical studies in animal models and some clinical trials:

- improved penetration of mucus (*eg* inhaled, oral-gut)
- accumulation in inflamed / infected tissue
- sustained release in tissues
- less frequent dosing
- less toxic – encapsulation in nanoparticle until released
- higher doses may be used
- most nanocarriers considered (*eg* liposomes, solid lipid nanoparticles, polymer nanoparticles, dendrimers)



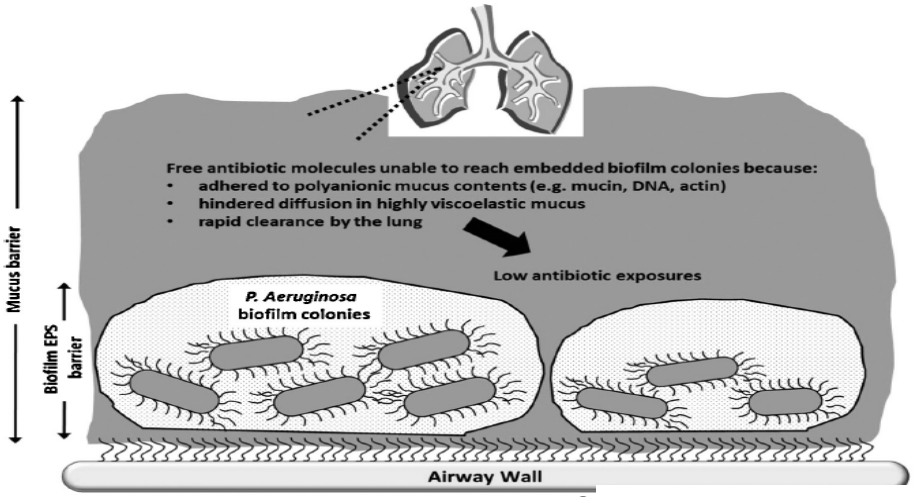
Nanoantibiotics -2

Nanparticulate (NP) antibiotics continued:

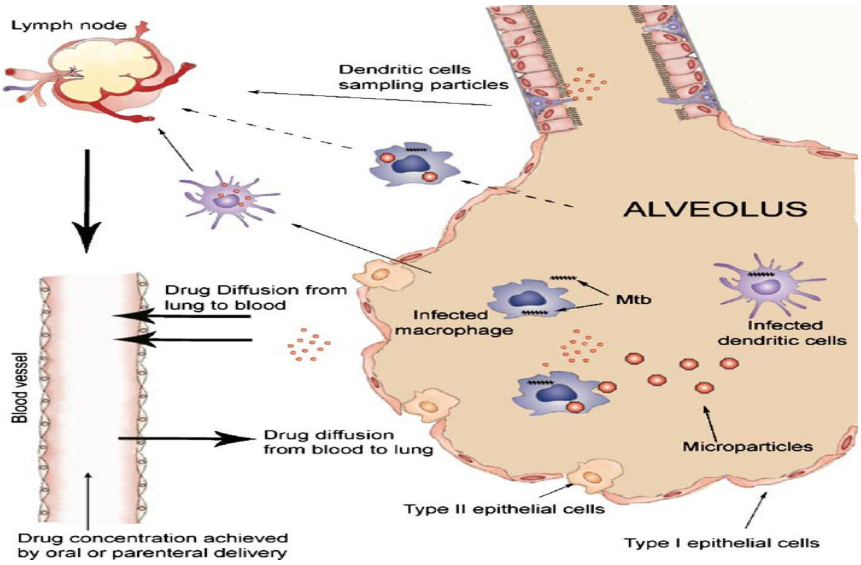
- greater activity against intracellular pathogens
- water-soluble drugs can cross membranes in NPs
- poorly water-soluble lipophilic drugs solubilised in NPs
- intracellular targeting, local sustained release
- intracellular release may be active (*eg* liposomes with phospholipase, pH-responsive peptides or polymers)
- avoidance of efflux pumps
- slower elimination
- multiple drugs in same NP to avoid single resistance mechanism

Chronic lung infections

Inhaled antibiotics have a short retention time (~2h), requiring frequent higher doses (*eg* ~25x, twice daily for 28 days).

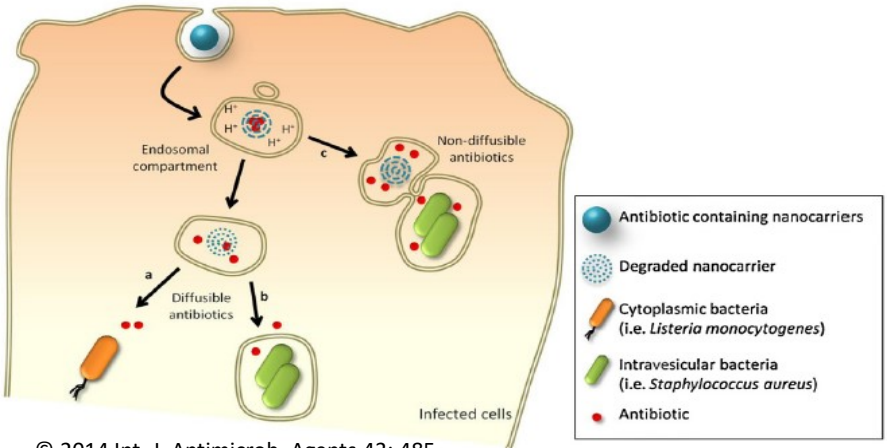


Inhaled *v.* Blood (IV/oral) NPs

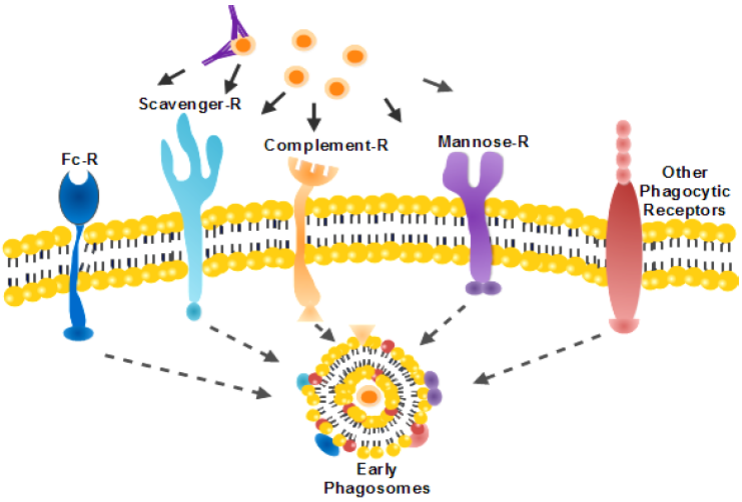


Passive targeting of macrophages

Clearance of nanoparticles by RES/MPS advantageous for treatment of intracellular infections



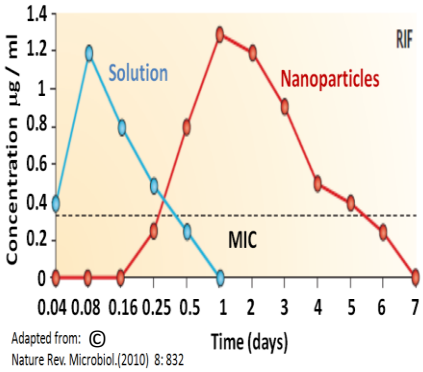
Selective targeting *via* macrophage receptors



Example: inhaled rifampicin NPs

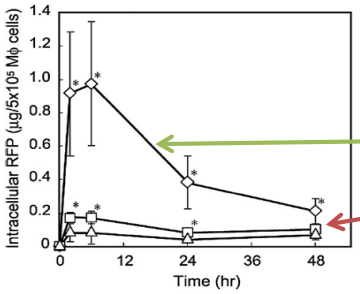
- Antibiotic solution degraded / excreted, requiring many doses.
- Slower sustained release from NPs in aerosol, drug > MIC for days
- NP penetration/retention in mucous layer, avoiding rapid clearance.
- NP uptake by endocytosis avoids barrier for free drug, results in higher intracellular antibiotic
- Higher intracellular drug for days above MIC more effective for slow-growing bacteria and minimises risk of antibiotic resistance development

Both free drug solution and NP-encapsulated drug in MDI aerosol



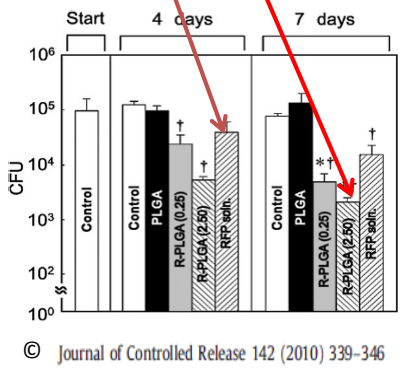
MIC – minimum inhibitory concentration

Example: inhaled rifampicin NPs targeted to lung macrophages harbouring mycobacteria



Inhaled rifampicin (RFP) – polymer (PLGA) nanoparticles (NPs) accumulated in alveolar macrophages, **with higher sustained levels of RFP within the macrophages**, **achieving greater killing of mycobacteria** than **RFP alone at higher levels**

Incubation time (h)	Samples[b]	Intracellular concentration (µg/mL)	Extracellular concentration (µg/mL)	Intra/Extra[c]
0	RFP solution	0	5.00	0
	R-PLGA (0.25)	0	0.25	0
	R-PLGA (2.50)	0	2.50	0
6	RFP solution	39.50	3.97	10
	R-PLGA (0.25)	82.05	0.04	2051
	R-PLGA (2.50)	461.38	0.40	1153
48	RFP solution	32.45	2.98	11
	R-PLGA (0.25)	48.02	0.07	686
	R-PLGA (2.50)	99.49	1.03	97



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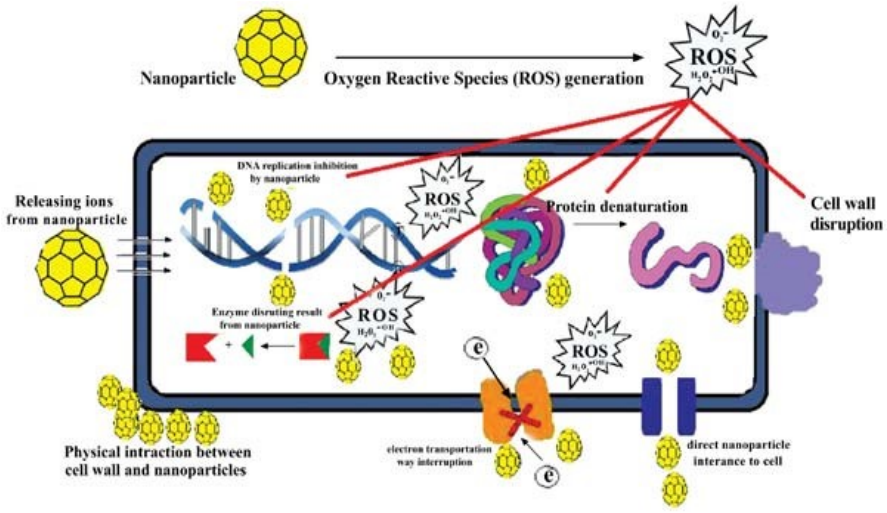
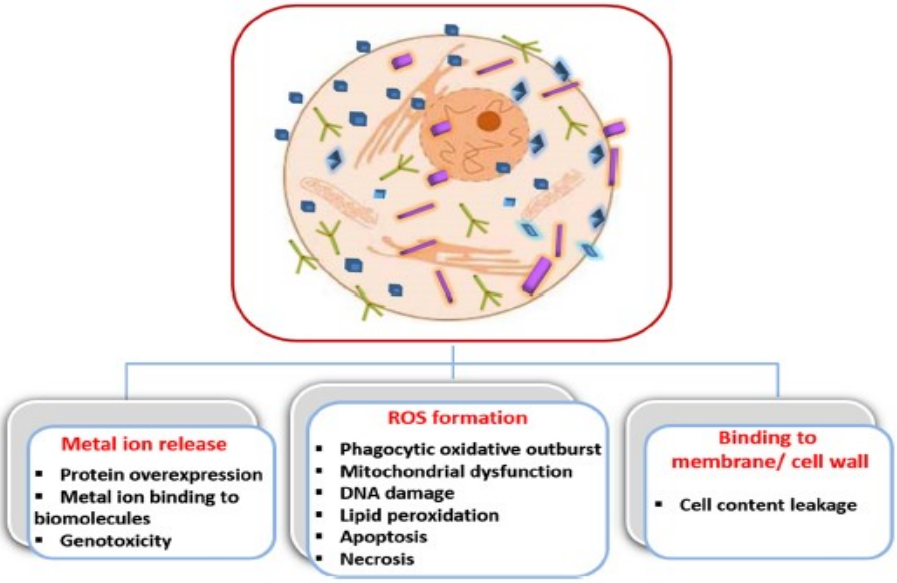
Antimicrobial nanomaterials & antibiotic resistance

- Broad spectrum against different microbial groups, similar to antiseptics and disinfectants
- Multiple killing mechanisms overcome resistance to any particular mechanism or specific drug target
- Reduces probability of resistance developing from any particular killing mechanism, and more difficult (*eg* membrane damage, DNA & protein disruption)
- Targets are essential, require multiple pathways and many genes, when many possible resistance mutations may kill or weaken the mutated microorganism
- Antimicrobial surfaces reducing/preventing drug-tolerant biofilm formation

Broad spectrum antimicrobial activity



Toxicity mechanisms



Antimicrobial nanomaterials summary

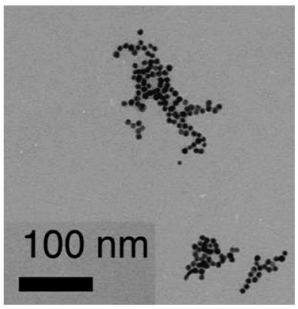
Nanomaterial	Mechanism	Applications
NO releasing	NO / ROS free radicals ROS – reactive oxygen species	Infected wounds *eg* diabetic foot
Ag NPs	Ag+ disrupts membranes, electron transport, DNA/RNA	Wound dressings, device coatings, potable water treatment
ZnO NPs	Membrane damage, ROS, H_2O_2, Zn^{++} enzyme inhibition	Creams, lotions etc, medical device coatings, mouthwash
TiO_2 NPs	Cell membrane damage, ROS	Toxic - disinfection
Au NPs	Weak, unless antibiotic or heating	Photothermal therapy, adjuvant after serious infection
Chitosan	Lipid membrane disruption, metal ion chelation	Toxic - disinfection, preservation
Graphene	High surface area	Wound dressing, device coatings, filters

Example: NO releasing NPs

- NO is lipophilic & crosses most biological barriers
- Reactive nitric oxide species (RNOS) formed by reaction of NO with superoxide (O_2^-):
  - peroxynitrite $OONO^-$
  - nitrogen dioxide NO_2
  - nitrogen trioxide N_2O_3
- Antimicrobial including against drug-resistant microorganisms when [NO] >1mM and above innate protection by enzymes (*eg* lactate dehydrogenase, flavohaemoglobins):
  - protein damage – RNOS react with cysteine, methionine, tyrosine, phenylalanine & tryptophan, Fe depletion in Fe-S and haem, thiol nitrosylation
  - DNA damage through strand breaks, deamination of C A & G nucelotide bases, increases in hydrogen peroxide & alkylation.

Example: hydrogel / glass composite NO NPs

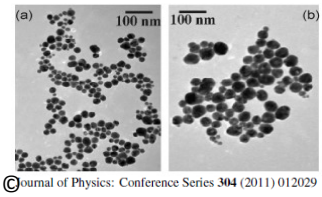
- Dose-dependent killing of antibiotic-susceptible and -resistant bacteria *eg* MRSA in wounds.
- Reduces bacterial burden, inflammation and increases speed of wound closure.
- Damages MRSA cell wall, causing osmotic damage and fluid to flow into the bacterial cell, followed by lysis of the bacterial cell
- Stimulates interferon production, inhibiting angiogenesis in abcesses and the systemic spread of bacteria into blood



Sugar glasses support redox reactions *eg* thermal reduction of nitrite, when the NO produced remains trapped in the glass until released by water.

Example: silver NPs

- Antimicrobial activity of Ag NPs largely due to Ag^+ ions. Ag^+ NPs greater activity and toxicity than Ag metal. Ag NPs less toxic and need surface oxidation to produce Ag^+
- Smaller size and higher surface area shapes increase broad spectrum activity against fungi, bacteria, including antibiotic resistant.



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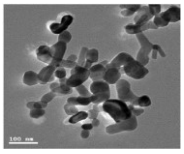
- Ag^+ binds to S & P groups of proteins and lipids in cell envelope.
- Ag^+ inhibits peptidoglycan and cellulose cell wall synthesis.
- Ag^+ binds to negatively-charged lipid → holes in membranes & lysis.
- Ag^+ enters microbial cells to inhibit cytochromes/electron transport, DNA replication, denatures ribosome.
- Ag^+ causes reactive oxygen species (ROS) with broad spectrum action.
- Resistance develops slowly - Ag^+ efflux pumps & lipids not binding Ag^+

Effect of metal NP characteristics

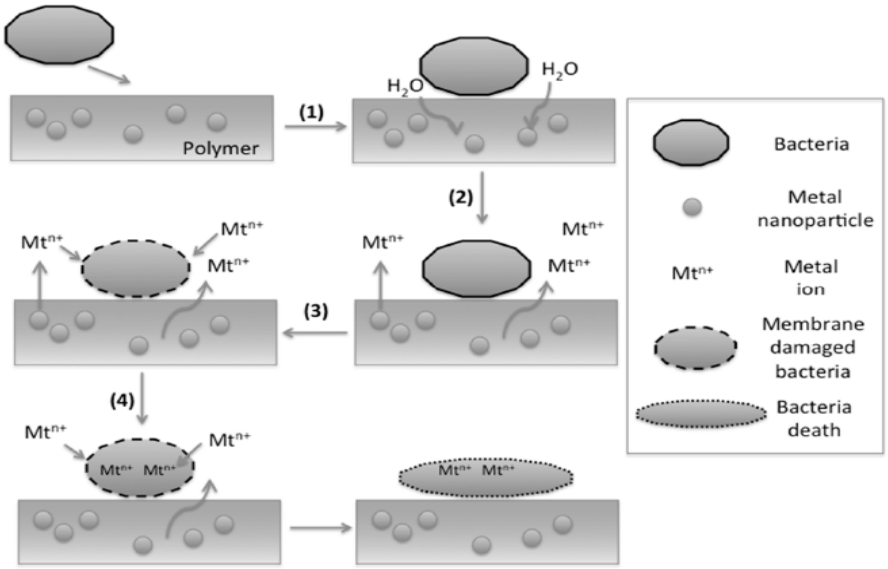
- Activity increases with decreasing particle size
  - related to higher dissolution of metal ions
- But other size/shape-dependent interactions
  - smaller / higher curvature may interact less with membrane surface
- Activity increases with larger lattice constant
- Crystallographic properties less understood and mixed conflicting effects often reported

Examples of other metal NPs



- **Zinc** oxide ZnO NPs - broad spectrum *via* binding to lipid membranes causing lysis, formation of ROS also damaging membranes by lipid peroxidation, resulting in oxidative stress in cell, release of Zn^{++} also inhibitory. © International Journal of Antimicrobial Agents 43 (2014) 95–104
- **Copper** oxide CuO NPs - also interact with amine & carboxyl groups.
- **Titanium** dioxide TiO_2 NPs– also photocatalytic ROS production.
- **Magnesium** halide/oxide MgX_2 / MgO NPs – also enzyme inhibition.
- **Bismuth** Bi NPs – when combined with X ray treatment reduce X ray dose required – Bi emits electrons producing free radicals when X ray irradiated, which damages DNA.
- **Gold** Au NPs low activity, but kill drug-resistant bacteria when antibiotic on NP surface, also photodynamic therapy with IR lasers.
- **Aluminium** oxide Al_2O_3 NPs – low activity and increase conjugation & plasmid transfer, and so spread of drug resistance.

Polymer / metal nanocomposites



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Example: graphene NPs

Possible uses in wound dressings, potable water filters *etc* and, given the very high surface area of graphene, possible use with adsorbed antimicrobials.

Single/few layer graphene nanosheets (esp graphene oxide) suggested (evidence otherwise) to agglomerate microoganisms and to lyse cells by edges penetrating & extracting lipids from membranes.