Biomedicine & Pharmacotherapy 169 (2023) 115875

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Review

Nano and microparticle drug delivery systems for the treatment of *Brucella* infections

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ARTICLE INFO

Keywords:
Brucella
Drug delivery systems
Nanoparticles
Nanotechnology
Survival
Therapeutics

ABSTRACT

Nano-based drug delivery systems are increasingly used for diagnosis, prevention and treatment of several diseases, thanks to several beneficial properties, including the ability to target specific cells or organs, allowing to reduce treatment costs and side effects frequently associated with chemotherapeutic medications, thereby improving treatment compliance of patients. In the field of communicable diseases, especially those caused by intracellular bacteria, the delivery of antibiotics targeting specific cells is of critical importance to maximize their treatment efficacy. *Brucella melitensis*, an intracellular obligate bacterium surviving and replicating inside macrophages is hard to be eradicated, mainly because of the low ability of antibiotics to enter these phagocityc cells . Although different antibiotics regimens including gentamicin, doxycycline and rifampicin are in fact used against the Brucellosis, no efficient treatment has been attained yet, due to the intracellular life of the respective pathogen. Nano-medicines responding to environmental stimuli allow to maximize drug delivery targeting macropages, thereby boosting treatment efficacy. Several drug delivery nano-technologies, including solid lipid nanoparticles, liposomes, chitosan, niosomes, and their combinations with chitosan sodium alginate can be employed in combination of antibiotics to successfully eradicate Brucellosis infection from patients.

1. Introducction

Brucellosis, caused by bacteria of the genus Brucella, is a chronic communicable disease targeting a variety of species, including humans, sheep, and cattle [1,2]. The bacterium is a Gram-negative, non--capsulated, non-motile, facultative intra-cellular cocco-bacillus, which can survive and replicate inside specific phagocytic cells (mainly macrophages), although it can also spread to other cells and tissues [1–3].

Most *Brucella* species' genomes have been sequenced and their data are available from GOLD database. Brucella have two chromosomes: a large one coding mostly for metabolically-related genes and a small one coding for genes contributing to pathogenicity of the bacterium [4].

Brucellosis is one of the most common zoonoses, with a global annual incidence of approximately 500,000 cases [2]. There are 12 different species belonging to the genus Brucella, targeting different hosts and featured by variable zoonotic potentials [3]. *B. melitensis*, *B. suis* and *B. abortus* have the highest zoonotic capacity, being hosted in sheep, goats, pigs and cattle, respectively. Some novel Brucella species are still awaiting their final genus affiliation [5]. Whilst *B. canis* (hosted in dogs), *B. inopinata* (unknown host), *B. ceti* (hosted in dolphins) and

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https://doi.org/10.1016/j.biopha.2023.115875
Received 5 April 2023; Received in revised form 6 November 2023; Accepted 7 November 2023

B. pinnipedialis (hosted in pinnipeds) typically cause mild infections in humans, *B. neotomae* (hosted in desert woodrat), *B. microti* (hosted in common vole), *B. papionis* (hosted in baboon), *B. ovis* (hosted in sheep) and newly characterized *B. vulpis* (hosted in red fox) are not zoonotic.

Given the intra-cellular life of Brucella, its eradication by antibiotics is rather complicated and Brucellosis can become a chronic disease in several mammals, including humans, sheep and cattle, with disease relapses potentially progressing to severe conditions requiring extensive medical care. The therapeutic efficacy of antibiotics against Brucellosis is in fact limited, since water solubility can hamper their penetration inside phagocites infected by the bacterium. An inappropriate use of antibiotics, not selected among those drugs effective against intracellular pathogens has amplified the issue of drug resistance. Furthermore, long-term treatment of Brucellosis may cause patient dissatisfaction and poor compliance [3].

1.1. Molecular mechanism of Brucella infection

Despite recent advances in the understanding of the molecular bases of the disease, the pathogenic mechanism of Brucellosis has not yet been fully elucidated.

Both antibodies/complement-opsonized as well as non-opsonized *Brucella* are able to survive and multiply inside target cells [1]. Lipid rafts, PI3-kinase and TLR-4 facilitate the entry of non-opsonized *Brucella* into murine and human macrophages, without activating these phagocytic cells [1,6,7]. Some *Brucella* mutants - lacking the O-polysaccharide (O-antigen) of LPS - are not capable to suppress macrophage activation and consequently die [8,9]. Some surface cell receptors are involved in the maturation processes of *Brucella*-containing vacuoles (BCVs), whose fusogenic characteristics can be modified by O-polysaccharide-antigen in the early phases of their formation. Loss of O- polysaccharide antigens expose other molecules that act like Pathogen Associated Molecular Patterns (PAMPs), stimulating the cell signaling pathway and triggering cell death regardless the LPS structure. Lipid rafts are also responsible for the absorption of mutants *B. ovis* and *B. canis* inside macrophages [10,11].

Two cell elements (BvrR/BvrS), an histidine kinase sensor located in the cell membrane (BvrS) and a cytoplasmic regulator (BvrR), control the expression of several genes responsible for the acylation of LPS lipid A and proteins of the outer membrane of *Brucella* (Omp3a and Omp3b), all involved in its entrance into the host cell [12]. Embodiment of *Brucella* inside macrophages (lipid raft- dependent) can occur via two receptors:

- class A scavenger receptor [13]; or
- cellular prion protein PrPc [14], which binds with heat- shock protein Hsp60.

UgpB locus encodes surface protein 41 (SP41) [15], a glycerol-3-phosphate binding ABC transporter located in the outer membrane of both pathogenic and non-pathogenic *Brucella*.

The microtubule network and the actin cytoskeleton of the host phagocytes adjust the entry of *Brucella* into cell. Some proteins activated at entry location are vital for cell invasion, namely small GTPases Cdc42, Rac, Rho and also Cdc42 [1,7,16].

When *Brucella* enters the host macrophage, it remains in BCVs, influencing the endocytic and secretory pathway of the infected phagocyte to survive [17]. As can be seen in Fig. 1, the early endosome interacts with cholesterol and flotilin-1 enriched BCVs (Fig. 1), tethering early endosomal antigen EEA1 and GTP-binding protein Rab5. On one hand these proteins play a role in lipid raft signaling, on the other they are involved in endocytic pathway and phagosome maturation. At the

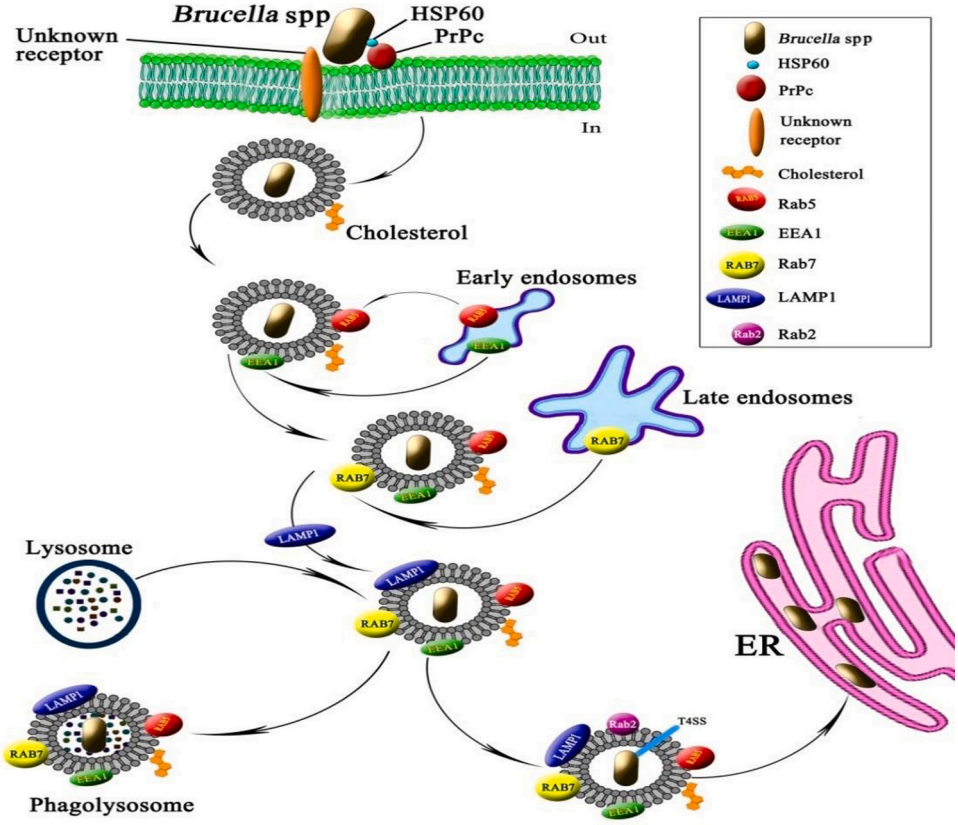

Fig. 1. Entrance of *Brucella* in the host cell and intracellular trafficking. Required molecules for maturation of Brucella Containing Vacuoles (BCV) can be noted in the upright quadrant. *Brucella* temporarily interacts with several components of the endocytic pathway. In phagolysosomes, virulent mutants lacking VirB T4SS degrade, whereas wild type Brucella can escape endoplasmic reticulum (ER) exit sites by fusing with late endosomes and lysosomes, therefore replicating in the phagosome.

beginning of their maturation process, BCVs lose their markers, gaining endosomal/lysosomal membrane protein LAMP1 (Fig. 1). Endocytic markers are essentially Rab7 (protein acquired by late endosome) and its effector, the Rab-interacting lysosomal protein (RILP), vital for BCV trafficking. BCVs mature by undergoing fusion processes with endosomes and lysosomes (Fig. 1).

Phagosome maturation involves acidification of BCVs, an important step for the survival of *Brucella* [18], and deviation from intracellular trafficking [19]. The acidic vacuolar pH of BCVs induces expression of *Brucella* genes,as the IV secretion system (T4SS) encoded by VirB Operon, stimulating the intracellular replication of bacteria [20]. VirB is involved in the transfer of bacterial proteins into infected cells, supporting the maturation of *Brucella*-containing vacuoles inside host cells [21–25].

Most BCVs become phago-lysosomal and the majority of *Brucella* (90%) are killed inside macrophages at the beginning of the infection. Ten percent *Brucella* manage to survive and proliferate inside macrophages by inhibiting phagosome - lysosomes fusion and VirB system [20, 26].

1.2. Treatment of Brucellosis

Appropriate treatment schemes for Brucellosis include doxycycline (100 mg every 12 h for 6 weeks) plus streptomycin (1 g/day for 2–3 weeks) [27,28]. Proper and timely use of antibiotics against brucellosis reduces the length of treatment, the risk of drug resistance and disease relapses.

Nevertheless, as already mentioned above, the intra-cellular life makes it difficult to eradicate *Brucella,* favouring chronic forms of the disease, featured by asthenia and even occasionally death [29].

WHO recommended to combine differefent antibiotics as a stratergy to eradicate Brucella infections; however, drug side effects and antibiotics resistance remain an issue [30].

2. Nano-technology

The Medical Standing Committee of the European Science foundation defined nano-medicine as "*the science and technology of diagnosing, treating, and preventing disease and traumatic injury, relieving pain, and preserving as well as improving human health, using a molecular tool and molecular knowledge of the human body*". The United States National Institutes of Health defined nano-medicine as "*an offshoot of nanotechnology, which refers to highly specific medical interventions at the molecular scale for curing diseases or repairing damaged tissues, such as bones, muscles, or nerves*" [31].

The conceptual principles of nanotechnology root back in 1959, when physicist Richard Feynman (Nobel Prize winner) delivered the lecture "*There's plenty of room at the bottom*" to the American physics society [32], albeit the term "*nano-technology*" was first used by Professor Noro Taniguchi only in 1974 [33].

Remarkable progress in the field of nanotechnology applied to pharmaceutics was achieved in the 1950 s and 1960 s, including development of miniaturized delivery systems for controlled release of medications [34], use of liposome for drug delivery [35] and albumin NPs [36]. Several biodegradable NPs were constructed as nano-based drug-polymer compounds combining poly (methyl cyanoacrylate) and poly (ethyl cyanoacrylate) in the 1970 s and developed pre-clinically in the 1980 s [35,37,38].

2.1. Applications of nanotechnology in medicine

One of the key factors for medical use of nanoparticles (NPs) is the ability to finely regulate their duration in the circulatory system by tailoring their surface charge with the specific objectives of their application. When NPs bear a positive charge, they are adapt at fostering their internalization inside cells, expediting the therapeutic action of the respective drug. Conversely, negative charges allow an extended sojourn of NPs within the bloodstream, optimizing their bioavailability. Furthermore, by enhancing NPs solubility in aqueous environments and refining their pharmacokinetic profile, the potency of the respective drugs conveyed is enhanced. The latter manipulation ensures precision delivery of drugs to their intended targets, minimizing unwanted side effects and lowering dosages required for their efficacy. Moreover, multiple drugs can be concurrently hosted by NPs, amplifying the respective therapeutic potential [39–41].

2.2. Therapeutic applications of nanoparticles

The dynamic integration of NPs into the realm of medicine has undeniably ignited a transformative era in the management of diseases once deemed insurmountable or associated with bleak prognostic outcomes. This remarkable advancement has transcended conventional boundaries, resulting in a therapeutic revolution extending beyond infectious diseases to encompass also an array of non-infectious conditions. Among these are disorders with metabolic or hormonal origins, enigmatic autoimmune afflictions, the formidable specter of cancer, and the pervasive turmoil of inflammations. In this revolutionary landscape, NPs have exhibited their prowess by orchestrating a multifaceted assault on diseases that have frequently eluded effective treatment interventions. Proficiency of NPs extends beyond conventional approaches as they exhibit the ability to neutralize resilient, multidrug-resistant pathogens. However, their application extends even further, as these innovative agents display a remarkable capacity to traverse the blood-brain barrier (BBB), thereby unlocking potential treatments for nervous system conditions once thought to be beyond reach. Surpassing conventional expectations, NPs have even embraced roles that venture into the realm of biological functionality. In an astonishing testament to their versatility, NPs have demonstrated their capacity to supplement oxygen delivery, effectively stepping into the roles traditionally held by red and white blood cells. This groundbreaking potential, as highlighted by Krishnan and George (2014), suggests a future where NPs could functionally emulate the vital roles played by these blood components, offering a glimpse into an era where medical solutions seamlessly intertwine with technological innovation [42].

2.3. Treatment of infectious and non-infectious diseases

The landscape of medical advancement has been reshaped by the emergence of ingenious smart drugs, capable of activation exclusively within their designated target organs and in response to specific contextual cues. This pioneering approach introduces an unprecedented degree of adaptability, precision, and personalization for the treatment of individual cases. Furthermore, these smart drugs bring a novel paradigm in dosage regulation, employing intricate slow-release mechanisms that can span days, and in some cases, even weeks. Such therapeutic innovation has paved the way for the effective eradication of viruses and other intracellular micro-organisms, marking a turning point in medical interventions. For instance, nanosized streptomycin and doxycycline have emerged as beacons of potential in combating Brucella melitensis infection, showcasing enhanced efficacy when juxtaposed with their traditional counterparts [43,44].

This heralds a new era where treatment modalities are no longer confined to the constraints of conventional pharmaceutical formulations. Among the groundbreaking developments, nano-formulations have surfaced as promising candidates under active development. Examples include PEG-AmB-LIP and poly lactide-co-glycolide (PLGA), harnessed to deliver Amphotericin B for the treatment of leishmaniasis and systemic mycotic infections. The nanoparticle-mediated administration of these formulations proved a staggering 20-fold increase in efficacy against intracellular Leishmania infections compared to their conventional counterparts. Similarly, isobutyl cyanoacrylate loaded with ampicillin has exhibited a remarkable 120-fold amplification in

therapeutic efficacy of latter penicillin against salmonellosis [45]. PEO-ß-PAA_1Na laden with streptomycin and doxycycline has also presented promising outcomes in the battle against brucellosis [46]. Notably, PLGA encapsulating rifampin, isoniazid, and pyrazinamide enabled complete eradication of Mycobacterium tuberculosis in lab animals with generalized TB infection [47]. Moreover, liposomes infused with etanidazole have shown potential in treating Trypanosoma cruzi infections in laboratory animals [48]. The global concern of antibiotic resistance has compelled a shift in strategy, and nanotechnology has emerged as a beacon of hope. NPs have unfurled a new arsenal of chemical and physical solutions for selective pathogens elimination with minimal host toxicity. NPs also display the ability to curtail bacterial attachment to target cells and impede biofilm formation [49].

A recent innovation comes in the form of silver ring-coated superparamagnetic iron oxide NPs, surpassing the limitations of conventional silver NPs. These biocompatible agents boast antimicrobial efficacy while preserving host cell health. The incorporation of external magnetic fields empowers precise targeting and penetration of NPs into biofilms for pathogen eradication [50,51].

In the battle against antibiotic-resistant bacterial strains, a multipronged approach has emerged. Nitric oxide (NO)-loaded NPs exhibit promising perspectives in defeating Metycillin-resistan-Staphylococcus-aureus (MRSA), while also manifesting anti-inflammatory properties that foster rapid wound healing with minimal scar tissue formation. Meanwhile, gold NPs dispersed on zeolites offer a radical and expeditious solution against antibiotic-resistant strains of E. coli and Salmonella typhi [52,53].

Likewise, the battle against S. aureus infections has witnessed a breakthrough with the deployment of antimicrobial peptides tethered to gold-coated NPs. Guided with precision, these NPs navigate to the bone marrow-derived mesenchymal stem cells within patients, effectively eradicating the pathogen [54,55].

ZnO and CuO NPs have shown potential against multidrug-resistant pathogens. Their mechanism of action hinges on the disruption of microbial membranes and generation of reactive oxygen species (ROS). This dynamic duo has displayed efficacy in tackling an array of pathogens, ranging from Metylicillin-sensistive-Staphylococcus-aureus (MSSA) and MRSA to MDR TB, E. coli, Strept. mutans, and Klebsiella pneumoniae [56,57]. A parallel avenue of innovation emerged through the application of nano-emulsions, oil droplets capable to kill pathogens by physically interacting and melting with their membrane [58].

Beyond the sphere of infectious diseases, NPs harbor immense promise in the realm of non-communicable and metabolic conditions. A trailblazing approach involves encapsulating secretory cells within NPs, ushering in new vistas for hormonal and enzymatic treatments of deficiency diseases. This ingenious approach employs nanopores within the NPs to house these secretory cells, allowing passage of their secreted materials while safeguarding cells themselves. Encapsulation of rat pancreatic beta cells within NPs allowed these cells to remain viable and continued insulin secretion for prolonged periods, shielded from immune attacks. This approach holds considerable potential in addressing the challenges of type 2 diabetes [59–61].

Another avenue of innovation in diabetes treatment includes an electro-active hydrogel membrane co-loaded with insulin and glucose oxidase enzyme. This membrane operates as a biosensor, detecting elevated glucose concentrations and dispensing insulin accordingly, for a dynamic release of the hormone aligned with glucose levels [62].

The transformative role of NPs extends also to mitigating the deleterious effects of ischemia. Within the body, superoxide dismutase (SOD) stands as a cornerstone of the antioxidant defense system against free superoxide anion radicals. Pioneering endeavors have succeeded in delivering SOD-loaded NPs to injury sites in laboratory animals, resulting in substantial reduction of tissue damage and fibrosis. This innovation holds potential in mitigating damage stemming from conditions such as renal ischemia or stroke, where tissue damage reduction by 50% has been achieved by SOD-loaded NPs [63–65].

Further applications of nanotechnology against non-communicable diseases involve conditions like Parkinson's disease, where NPs offer avenues for intervention [66]. Likewise, the potential of NPs extends to address the challenges posed by psoriasis [51].

In essence, the convergence of nanotechnology and medical science has unleashed a new wave of possibilities, reshaping the boundaries of what can be achieved in disease treatment and management.

2.4. Nano-particles

NPs are today a relatively novel yet promising approach for targeted delivery of biomaterials, including vaccines and drugs [38,67–69]. As drug carriers, NPs can offer an opportunity to treat intra-cellular infections and overcome the limitations of antibiotic entrance into host cells, optimizing their therapeutic effects [70]. The use of NPs enhances the uptake of poorly soluble antibiotics at the disease site, facilitating the accumulation of drugs inside cells, thereby reducing dose, number of administrations and length of treatment [71]. Lastly, NPs are also known to prevent the destruction of drugs inside the body, thus maximizing their bio-availability [46,70]. Several types of NPs have also been used to deliver antibiotics against *Brucella* [72–74].

Various types of nano-based products and technologies have been devised in pharmaceutics, allowing:

- inhibition of drug degradation, and/or
- drug absorption enhancement via facilitating diffusion through the epithelium, and/or
- modifications of pharmacokinetic characteristics and tissue distribution of drugs, and/or
- enhancement of intracellular penetration of drugs.

NPs as carriers of antibiotics have a number of advantages [75–78]:

- higher effectiveness
- less side effects
- possibility to overcome antibiotic resistance
- increasing half-life of the drug

Furthermore, some NPs (i.e. colloid metals) can also be useful for diagnostic purposes and improvement of imaging performance. New generation of biosensors called quantum dots, based on the optical properties of colloidal gold and fluorescent nanocrystals, are in fact ready for implementation in medical imaging [79].

Medication targeting is the selective release of a drug to a particular organ, tissue, or cell. Medications can be released from NPs in two ways:

- extracellularly, or
- intracellularly (via endocytosis).

Based on the biological life of their pharmaceutical carrier in the blood and the preferential accumulation of the drug-NPs complex at the location of interest, medications can be delivered passively or actively to the target organ [80]. In active drug delivery the surface of NPs needs to be recognized by specific receptors at the target site to be activated [81].

Widespread clinical application of NPs has prompted scientists to study the interactions with internal biological systems, especially under physiological conditions (pH=7.0–7.4). Before being introduced into human organisms, NPs must be carefully selected and modified by appropriate manufacturing processes, to enhance their stability in watery systems and the transport of drug active principles to the target sites [82,83].

NPs confer stability to drugs and allow to encapsulate both hydrophilic and hydrophobic molecules in the medication conveyed. From an engineering or biological perspective, the modest surface to volume ratio is a useful distinctive feature of NPs. However, approval of NPs requires careful examination of the risk-benefit ratio, since nano-toxicity

can be an issue [84]. For example, cationic NPs as gold and polystyrene can cause hemolysis and clotting, while anionic NPs are reportedly less harmful [85].

2.5. Liposomes

Liposomes are minute spherical sacs with a self-assembled lipid coat layer of 25 nm-1 μm diameter, surrounding a central aqueous environment. The outer lipid membrane can be modified by natural elements as proteins, polysaccharides, glycolipids, antibodies, enzymes or synthetic molecules as PEG [86,87]. Hydrophilic compounds can still be incorporated in the lipid membrane of liposomes and their aqueous environment can also contain hydrophobic molecules.

Liposomes have been used as NPs for the delivery of different biological materials, including vaccines, drugs as well as antibiotics for the eradication of intracellular pathogens [88,89]. These NPs are stable in blood, but when entering the host cell are disrupted by lysosomes and their content is released in the cytosol [90]. Liposomes can have different formulations and different ligands can be attached, such as antibiotics for specific targeted delivery. For instance, conjugation of O-stearylamylopectin (O-SAP) to liposomes can promote its affinity to lung tissue. Since liposomes can target specific cells, antibiotics coveyed by them are more rapidly absorbed by cells, their therapeutic effectiveness is enhanced, the number of adminsitrations reduced and the respective dose contained, thereby reducing drug toxicity [87,91].

Liposome-formulated antibiotics have been successfully used to treat infections sustained by different pathogens including *Mycobacterium tuberculosis* [92–95] *Listeria monocytogenes* [96–98] *Salmonella* species [99,100] *Francisella tularensis* [101] *Staphylococcus aureus* [102], and *Brucella* [103–107], showing higher antibacterial activity than the respective free antibiotics.

2.6. Polymeric nanoparticles

Polymeric NPs (PNPs) are biodegradable and biocompatible compounds, easy to prepare, capable of targeting specific tissues and organs for the delivery of biological materials, including vaccines and drugs.

Two main classes of PNPs exist:

- **Synthetic PNPs**: PLA (poly(lactic acid)); PLG (poly(D,L-glycolide)); PLGA and PCA (poly-(cyanoacrylate)) are the main polymers employed for the fabrication of synthetic PNPs.
- **Natural PNPs:** Chitosan, alginate and gelatin are natural polymers used for the preparation of natural PNPs.

PNPs have been employed for the delivery of different antibiotics against various pathogens. For instance, two cationic antibiotics, doxycycline and streptomycin, have been incorporated into anionic PNPs via electrostatic interactions, creating nano-formulations more effective than the respective free drugs in reducing the load of *Brucella melitensis* in liver and spleen of infected mice [46]. Solubility, cytotoxicity and efficacy of different concentrations of co-trimoxazole or rifampicin loaded onto a monomethoxy poly (ethylene glycol)-oleate (mPEG-OA) NPs were compared in vitro with the respective free antbiotics against murine phagocytic cells infected by *B. melitensis*. Whilst the application of co-trimoxazole-loaded NPs did not reduce the number of bacteria inside murine macrophages, the antibiotic effectiveness of rifampicin increased [107].

In another experiment, 48 h pre-treament of macrophages with nanobiotics including rifampicin encapsulated in polyanhydride NPs managed to prevent intracellular infection by *B. melitensis* as opposed to free antibiotics [108]. Moreover, infected BALB/c mice treated by nano-formulation of rifampicin encapsulated in polyanhydride NPs showed a significantly reduction of *B. melitensis* in their liver and spleen compare to mice treated by free drugs [108]. The latter formulation may be effective against a range of intracellular bacterial pathogens, including mycobacteria and Burkeholderia species as well as for the treatment of antibiotic-resistant infections. Gentamicine-loaded chitosan NPs also proved effective to treat Brucella- infected J774A.1 murine macrophages in another experiment in vitro [109].

Although a formulation combining pH-responsive curcumin niosomes hydrogel with doxycycline-loaded sodium alginate NPs was not fully effective to eradicate Burusera infection in guinea pigs, the reduction of the number of residual micro-organisms in the treatment group was still significantly stronger and occurred within a shorter period of time compare to animals treated by free drugs [110]. The latter manufactured formulation may be useful for effective treatment of Brucellosis infections at the recommended therapeutic doses.

2.7. Solid lipid nanoparticles

NPs can be combined with lipid-based parenteral emulsions based on non-toxic and biodegradable lipid components, generating solid NPs (SLN) [111].

Solid lipids have some advantages in comparison with liposomes, emulsions and PNPs: preservation from deterioration, physical stability and more effective release of their cargo [112]. Disadvantages of SLN include limited loading capacity, drug dismissal during long-term storage and high water content. COVID-19 m-RNA vaccines as Spikevax (manufactured by Moderna) and Comirnaty (Pfizer Biontech) coat their fragile nucleic acids strands with pegylated lipid NPs [113]. In particular, doxycycline-loaded nano-antibiotics proved more effective than free doxyciclin against *Brucella melitensis* in rats [44].

In 2007, Lecaroz et al. introduced a pivotal approach by crafting micro- and NPs from distinct compositions of PLGA, namely PLGA 502 H and PLGA 75:25 H. Their experimentation on mice unveiled the successful delivery of PLGA 75:25 H micro particles to the liver and spleen, the target organs of *B. melitensis* infection. While both polymers share identical molecular weights, their varying lactic acid/glycolic acid composition ratios account for their different performance. PLGA 502 H NPs, in contrast to micro particles of PLGA 502 H and 75:25 H, displayed almost complete degradation within the first week post-administration. Pharmacokinetic evaluations following a single intravenous dose of gentamicin-loaded microparticles showcased elevated areas under the curve (AUCs) and increased mean retention times (MRTs) for both liver and spleen. These findings pointed toward successful phagocytic uptake within the target organs, accompanied by controlled antibiotic release. Despite resembling pharmacokinetic parameter values for liver, PLGA 75:25 H microparticles exhibited superior efficacy than PLGA 502 H in targeting the spleen and reducing splenic loads B. melitensis infection loads (114).

Moving forward, Seleem et al. embarked on a novel trajectory by constructing macromolecular nanoplexes via cooperative electrostatic interactions between cationic drugs and anionic polymers. This strategy facilitated the simultaneous binding of streptomycin and doxycycline into nanoplexes, subsequently proving to outperform the respective free drugs. Two doses of these nanoplexes displayed a significant reduction in *B.melitensis* loads within the spleens and livers of infected mice, improving the efficacy of the respective free drugs. Notably, nanoplexes exhibited superior performance in both spleen and liver, underscoring their potential for tackling Brucella infection (46).

In 2006, Lecaroz et al. spearheaded another approach centered around particulate carriers for targeted drug delivery to the mononuclear-phagocytic system, the very domain where Brucella resides. This approach hinges on the intracellular accumulation of the antibiotic after particle degradation. Moreover, the uptake of particles can trigger macrophage activation, augmenting the production of ROS intermediates, critical for host defense against intracellular pathogens. The focus was on polymeric NPs for gentamicin entrapment, using various PLGA polymers formulations. NPs < 350 nm size were used, and the choice of polymer influenced gentamicin encapsulation efficiency and in vitro release patterns. PLGA 502 H NPs emerged as the most

suitable treatment option due to their high entrapment and sustained release. Further evaluations demonstrated successful phagocytosis by murine monocyte cell lines and effective biodistribution to target organs in mice. Collectively, these findings underscored the potential of these nanocarriers as gentamicin delivery systems for treatment of brucellosis (115).

In 2011, Imbuluzqueta et al. devised a unique methodology by forming a hydrophobic complex called GEN–AOT through the ion pairing of gentamicin (GEN) with the anionic AOT surfactant. This complex was then harnessed to create a particulated material either by precipitation with a compressed antisolvent (PCA) technique or by encapsulation into PLGA NPs. The latter approach yielded NPs of sizes ranging from 250 to 330 nm, achieving sustained drug release over 70 days and 100% encapsulation efficacy for different NP formulations, with no indication of drug-polymer interactions. In vitro experiments targeting intracellular infection by Brucella melitensis demonstrated unaltered bactericidal activity of gentamicin after ion pairing, precipitation, or encapsulation, confirming the potential of these formulations for treating infections caused by gentamicin-sensitive intracellular bacteria (116). GEN-AOT formulations considerably diminished Brucella infection in experimentally infected THP-1 monocytes, achieving reductions of over 2-log10 units in vitro at clinically relevant concentrations (18 mg/liter) (116).

Notably, gentamicin-AOT-loaded NPs managed to considerably reduce Brucella infection also in vivo, effectively delivering the drug to both liver and spleen of infected mice, maintaining therapeutic antibiotic levels for up to 4 days in these two organs, thereby enhancing antibiotic efficacy. In a striking contrast, while 14 doses of free gentamicin had no significant impact on the infection's course, only 4 doses of gentamicin-AOT-loaded NPs managed to reduce splenic infection by 3.23 logs and eliminate Brucella in 50% of the infected mice, all without triggering adverse effects. These findings make a strong case for PLGA NPs containing chemically modified hydrophobic gentamicin as a promising avenue for human brucellosis treatment (117).

Hosseini and colleagues in 2019 embarked on a quest to evaluate the antibacterial effect of SLNs loaded with doxycycline against Brucella melitensis in vivo (Fig. 2). The synthesized doxycycline-encapsulated SLN (DOX-SLN) were tested against acute and chronic Brucellosis in Wistar rats. At pathological examinations spleens and livers of DOX-SLN-treated rats exhibited significantly decreased B. melitensis CFUs compared to those treated with free doxycycline or left untreated, endorsing DOX-SLN against chronic brucellosis to achieve bacterial eradication and relapse prevention (44).

Ghaderkhani and collaborators in 2019 delved into the antibacterial potential of Rifampicin-loaded SLNs against *B.abortus* 544. By employing a modified microemulsion/sonication method, Rifampicin-loaded SLNs were formulated, characterized, and evaluated. These NPs displayed an average size of approximately 319.7 nm, a PI of about 0.20, and a zeta potential of around 18.4 mV, all while exhibiting a spherical shape. The encapsulation efficacy of rifampicin reached 95.78% with a drug-loading of 34.2%. The drug release profile spanned 5 days. Notably, the antibacterial activity of Rifampicin-loaded SLN was significantly higher compared to free Rifampicin in bacterial and cell culture media, endorsing the potential of SLNs delivery system to enhance Rifampicin's antibacterial activity against *B.abortus* (118).

Razei et al., in 2019 assessed the efficacy of chitosan NPs in delivering gentamicin to Brucella-infected J774A.1 murine cells in vitro (Fig. 3). The chitosan NPs, synthesized by ionic gelation, exhibited a mean size of 100 nm and a positive charge of + 28 mV. Their loading capacity reached 22%, and about 70% of the drug was released from the NPs within the first 8 h. The antimicrobial activity of the formulations revealed a significantly lower minimum inhibitory concentration (MIC) of the gentamicin-loaded chitosan NPs (Gen-Cs) compared to free gentamicin. The MIC of NPs-loaded gentamycin was also reduced compared to the free drug. Analysis on J774A.1 murine cells infected by intracellular bacteria exhibited notable reduction in bacterial load for

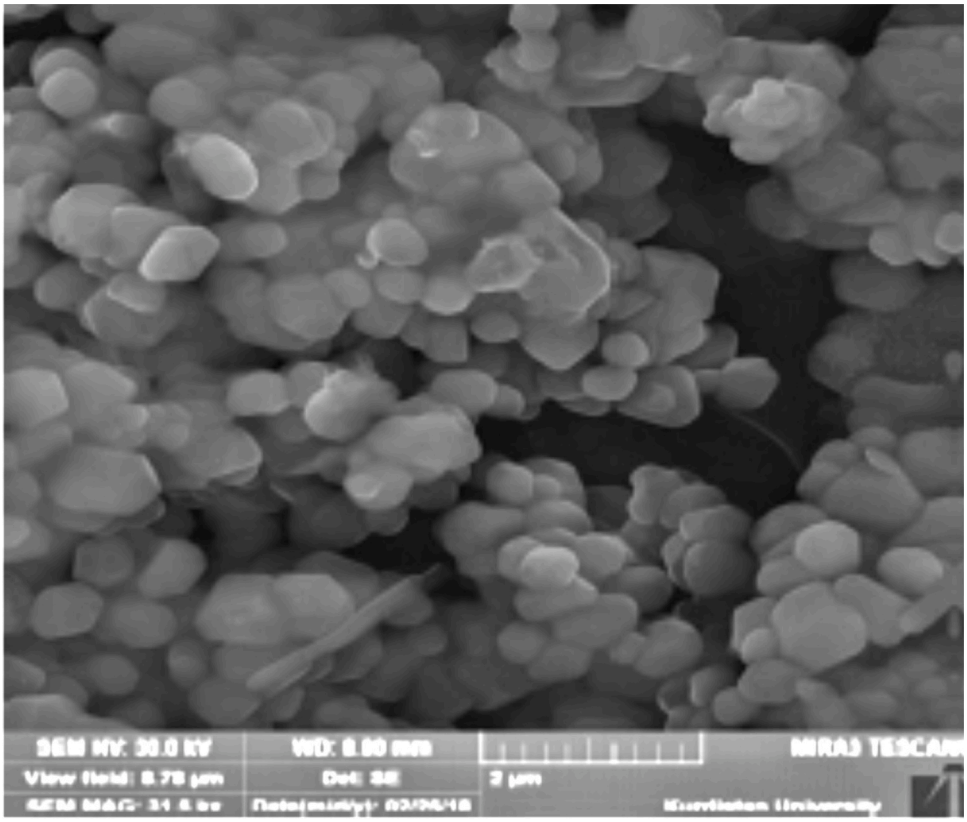

Fig. 2. Field emission scanning electronic microscope image of Drug Solid Nano-Particles (Drug-SLN) [44]; reused figure by official permission.

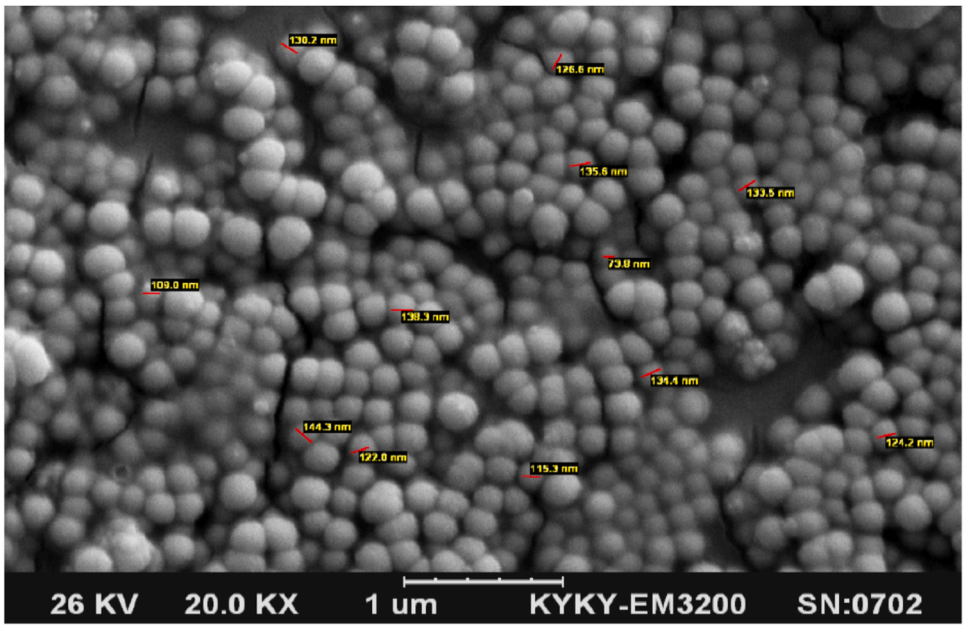

Fig. 3. Scanning electron microscopy of drug-loaded chitosan nanoparticles (NPs) [109]; reused figure by official permission.

both formulations, underscoring the potential of Gen-Cs as a promising avenue for optimal treatment of intracellular bacterial agents (109).

Prior et al. ventured into uncharted territory in 2004 with biodegradable microspheres made of PLGA 50:50 and PLGA 50:50 H containing gentamicin sulphate. This innovation aimed to target Brucella abortus-infected J774 monocyte-macrophages. The treated infected cells received either 15 μg of free gentamicin or microencapsulated gentamicin. Efficacy tests revealed significantly decreased intracellular bacterial count by approximately 2 log10 in microencapsulated gentamicin within end-group uncapped PLGA 50:50 H microspheres compared to untreated infected cells. The addition of 2% poloxamer 188 further enhanced the bacterial reduction by 3.5 log10. Opsonization of particles with non-immune mouse serum had no impact on the microspheres' antibacterial effectiveness. End-group capped PLGA 50:50 microspheres exhibited less pronounced reductions in intracellular bacteria (~1 log10 reduction), yet again, the addition of poloxamer 188

Table 1
Some effective nano-antibiotics in the treatment of *Brucella* infections.

Nanoparticles	Drug	condition	activity	Reference
PLGA750H	Gentamicin	In vivo	The injection of three successive doses of the drug to nanocarriers reduced the amount of splenic *Brucella* infection compared to the free dose.	[114]
Nanoplex	Doxycycline + Streptomycin	In vitro & in vivo	The injection of two doses of the nanocarriers containing antibiotic considerably reduced the amount of *Brucella Melitensis* in the liver and the spleen of infected mice.	[46]
PLGA502H	Gentamicin	In vitro& in vivo	These nanocarriers were suitable for the delivery of Gentamicin to control Brucellosis.	[115]
PLGA	Gentamicin (AOT)*	In vitro	In this work, Gentamicin had been ion paired with the anionic AOT surfactant to obtain hydrophobic complex (GEN-AOT)* . Results demonstrated that the bactericidal activity of GEN had been$^{\text{stabled after ion pairing. Therefore these NPs had been used to treat}}$ the infections caused by GEN-sensitive inner cellular bacteria.	[116]
PLGA	Gentamicin (AOT)*	In vitro & in vivo	*Brucella melitensis*- infected macrophages showed remarkable improvement compared to the free dose of antibiotics. Combining nanoparticles with antibiotics revealed promising results on the advanced treatment of brucellosis.	[117]
SLN	Doxycycline	In vivo	The results showed that the nano drug showed much better therapeutic effects than the free drug.	[44]
SLN	Rifampicin	In vitro	They showed that the nano-antibiotic had more antibacterial effects than the rifampicin free drug.	[118]
Chitosan	Gentamicin	In vitro	the results of the experiment showed that this nano-formulation has a proper potential for treatment of *Brucella*-infected cells	[109]
PLGA	Gentamicin	In vitro	The results indicate that PLGA 50:50-microencapsulated gentamicin sulphate may be suitablefor efficient drug targeting and delivery to reduce intracellular *Brucella* infections.	[119]
PLGA+PLA	Gentamicin	In vitro	The results suggest that PLA:PLGA microspheres prepared by spray drying may be an appropriate delivery system for gentamicin sulphate to be used in the treatment of intracellular *Brucella* infections.	[169]
Noisome+ Chitosan–Sodium Alginate	Doxycylin + curcumin	In vitro & in vivo	reduced the viable *Brucella* count in a shorter time and sub-therapeutic doses.	[110]
Copolymer	doxycycline and rifampicin	In vitro & in vivo	results demonstrated that the use of nanotherapeutics was successful at increasing antimicrobial efficacy and improving in vivo activity through a combination of intracellular delivery, dose sparing, and extended release in treating chronic bacterial infections.	[108]

*GEN-AOT=gentamicin-bis(2-ethylhexyl) sulfosuccinate.

bolstered their intracellular antibacterial potency. Placebo PLGA 50:50 and PLGA 50:50 H microspheres displayed no bactericidal activity. These results point toward the potential of PLGA 50:50H-microencapsulated gentamicin sulphate to eradicate Brucella infections from phagocytes (119). (Table 1).

2.8. Nano drug delivery in treatment of brucella

Nanodrug delivery represents an innovative strategy that merges NPs with antimicrobial adjuncts in diverse forms, whether dispersed, absorbed, or affixed to NP surface. This approach holds the potential to modulate drug bioavailability and manage side effects, leading to increased therapeutic efficacy, reduced costs and less adverse reactions [120].

Nanomaterials, spanning the realms of organic, inorganic, or hybrid compositions, emerge at sizes smaller than a micron (<900 nm) and are tailored to various dimensions based on their intended applications. These materials come in forms like NPs, nanowires, and nanorods, each showcasing a spectrum of structural diversity, from fibrous networks to reticular structures, and hollow or solid spheres, characterized by both smooth and uneven surfaces. The advent of nanomaterials has ushered transformative progress across various domains, including medicine, by virtue of their exceptional properties [121,122].

The golden era of antibiotic discovery and introduction spanned from 1950 to 1970, marking a period during which no novel antibiotic class was unveiled for approximately 35 years [123,124]. In the last two decades, NPs and nano-drugs have revolutionized the treatment landscape for oncology and infectious diseases [124,125]. The implementation of nanocarriers for drug delivery not only ensures precise drug targeting within infected sites, but also allow to regulate dosing frequency and quantity, thereby mitigating the risk of treatment-related toxicity [126,127]. The primary role of mononuclear phagocytes and macrophages – the predominant habitat of intracellular bacteria [127,128] - is the engulfment and clearance of foreign particles and microorganisms through phagocytosis [127,129]. Nevertheless, pathogenic bacteria often possess mechanisms to evade macrophage's lethal actions, thereby increasing the risk of secondary infections and relapses, particularly when the host's immune response weakens. Recent studies have unveiled that infected macrophages serve as Trojan horses for bacteria, capable of disseminating them to different body regions [127,130–133].

In view of the above, if NPs possess dimensions that are deemed foreign by MPS and are thus taken up via phagocytosis, they can serve as carriers for drug delivery, releasing their therapeutic cargo within these cellular compartments to target bacteria concealed within macrophages [127,132] Investigations into the use of nanocarriers have been underway for over 45 years [127,133,134]. Essential requisites for drug nanocarriers encompass:

1) safety, non-toxicity, and biodegradability;
2) high drug loading capacity to achieve optimal drug concentrations at target sites;
3) controlled drug release to ensure sustained therapeutic effect yet minimizing side effects;
4) compatibility with the drug and its metabolism; and
5) successful drug delivery to the intended site.

An array of nanocarriers has been explored, including liposomes, micelles, dendrimers, nanotubes, and polymeric NPs like SLN [44,127,134].

Hence, through the innovative technology of nanodrug synthesis, treatments can be directed towards bacteria hidden inside cells and

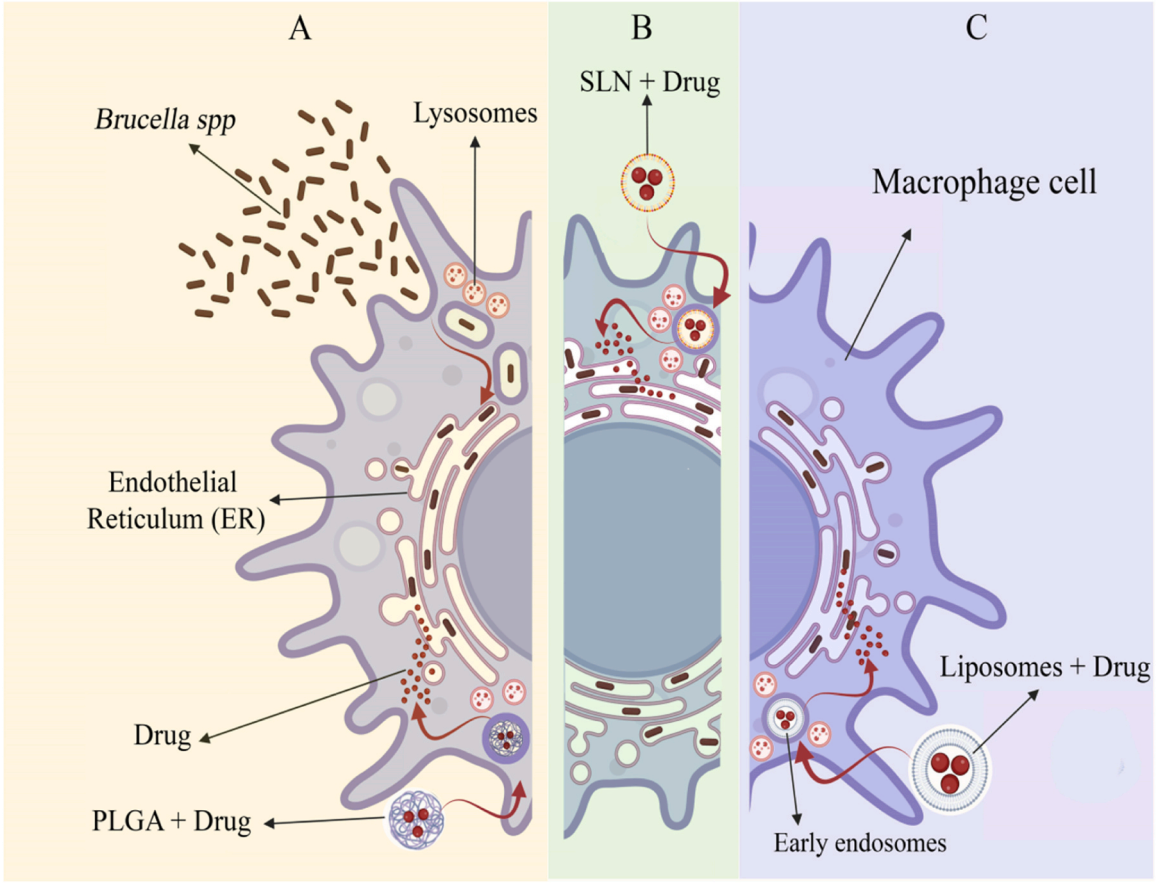

Fig. 4. The mechanism of entry of nanoparticles into the macrophage through polymer nanoparticles (A), solid lipid nanoparticles (B) and liposomes (C).

macrophages, charting a promising path toward disease eradication (Fig. 4).

2.9. Nanoparticles-mediated macrophage targeting

F-GNPs (surface-functionalized gelatin NPs), conjugated with mannose (m-GNPs) or PEG spacer (PEG), have been used as carriers to transport Amfotericin B (AmB) to macrophages for the treatment of visceral leishmaniasis. The spacer allows ligands to bind with macrophage receptors with little steric hindrance.

Forty-eight hours following incubation with Annexin-V-FITC/PI test, which measured cytotoxicity in treated macrophages, m-GNPs proved to be safe, increasing uptake and accumulation inside macrophage-rich tissues and improving anti-leishmanial activity (decreased IC50 by 5.4-fold compared to ordinary AmB) [135].

Direct coupling (M-PLGA) and PEG spacer conjugation (M PEG PPG) of PLGA to mannose were used to generate engineered PLGA NPs (M PNPs and M PEG PNPs) to deliver AmB to macrophages. Enhanced uptake, anti-leishmanial activity and increased accumulation in macrophage-rich tissues were all achieved with spacer-engineered PNPs [136–138]. Rifabutin-loaded mannosylated SLNs were devised for potential transport of rifabutin to alveolar macrophages [139], showing a six-fold increase in cellular uptake thanks to mannose coating [140].

2.10. Overview of interactions between phagocytic cells and nanoparticles

NPs recognized by surface receptors of phagocytic cells can be cleared from blood by the reticulo-endothelial system (RES), a set of phagocytic cells present in several organs [141]. When NPs are delivered, proteins from the surrounding environment immediately cover them to create a "*protein corona'* [142]. The composition of this protein layer's depends on the fluid (blood or cerebrospinal liquid) entering in contact with the NP [143] and the physical and chemical features of the NP (especially size and charge) [144]. Some of these proteins, called opsonins - as complement proteins and immunoglobulins - serve to identify and remove NPs from the body by phagocytic cells. By contrast, dysopsonin proteins antagonize the effect of opsonins, preventing NP uptake by phagocytes [145].

2.11. Liver macrophages have priority access to take up nanoparticles

In the liver, clearance of systemically administered NPs from bloodstream is predominantly accomplished by pinocitic activity of sinusoidal endothelial cells [146,147]. In particular, sinusoidal endothelial cells are able to remove soft and small (<0.5 μm) NPs [146,147] such as liposomes [148], polyion complex micellar NPs [149,150] and polymeric NPs [151]. By contrast, hard and larger (>0.5 μm) NPs cannot cross the pores within liver sinusoidal endothelial cells and are therefore primarily uptaken by Kupfer cells, specialized hepatic phagocytes [146,147,152].

After evading hepatic absorption, nanomaterials remaining in the central vein are re-absorbed by RES of other organs or the liver at a second blood passage.

The rate of NPs exiting the liver via hepatic veins is 1,000-fold reduced compare to the amount entering the liver via the portal triads. The anatomy and some characteristics of the liver explain why collection of NPs is privileged in hepatic macrophages [153]:

- large amount of blood flowing in the liver;
- Sluggish movement of NPs in hepatic capillaries;
- Largest phagocytic population in the body located in the liver;
- Higher access to blood-borne NPs by liver macrophages, with greater capacity to uptake and remove them from the blood.

2.12. Multiple stimuli-responsive drug-delivery systems (MSR-DDSs) responsive to exogenous and cellular/subcellular triggers

Exogenous stimuli could combine with endogenous (cellular/subcellular) stimuli to further facilitate the release of NPs inside macrophages or their effective endosomal escape (size shrinkage, charge reversal, ligands re- emergence) and achieve the highest therapeutic efficacy [154,155].

Protonation of endosomes / lysosomes can provide a positive charge to pH-responsive NPs, improving targeting of mitochondria, whose matrix has strong negative charge [156,157].

Cationic polymers linked to the surface of inorganic NPs can decrease their harmful effects. Despite high transfection efficiency, cationic polymers and liposomes are extremely hazardous.

Cationic polymers of mesoporous silica NPs (MS-NPs) can bind medications, stabilizing cargo and decreasing drug leakage. Polymine-copolymerized lysine-modified MS-NPs have been used to convey Doxorubicin (DOX) and Bcl-2 siRNAs to breast cancer cells to induce apoptosis. Both DOX molecules and polymers were bound one another by electrostatic attraction, boosted by PEI- PLL's protonation of amine groups. By cleaving the disulfide bonds between polylysine-modified polyethylenimine (PEI-PLL) and MS-NPs, glutathione (GSH) enables to open the gatekeepers and increase the release of DOX. By adding 10 mM GSH, an acidic pH of 5.0 for tumor microenvironment (TME) /lysosomes and an environmental pH of 7.4 for increasing the release of DOX, the rate of apoptosis raised by 14.37% with DOX and Bcl-2 siRNA together, compared to DOX-loaded MS-NPs [158].

A conjugation of nano-carriers as gold NPs with a combination of doxycycline–streptomycin and hydroxychloroquine - an FDA-approved antimalarial medication for obligate intracellular Plasmodium parasite - may offer promising therapeutic prospects against brucellosis [159,160], since gold NP dosage can be selectively absorbed by macrophages of liver and spleen [159,160].

2.13. pH-sensitive nanomaterials

NPs may provide the encapsulated drug molecules with a stable habitat, preventing their eventual environmental degradation or inactivation, hence maximizing their bioavailability [161]. The ability of NPs to penetrate the endothelium and the capillary tubes can also be exploited to deliver medications targeting cells in inflammatory regions [162].

Some NPs degrade or deform under acidic environmental conditions. These pH sensitive NPs can be exploited to induce the release of encapsulated pharmaceuticals to specific sites by changes in pH [163–165].

2.14. Microparticles

Particle size and distribution influence several aspects NPs activity (Fig. 5), including drug loading, drug release, drug toxicity, biodistribution and stability, among other.

One of the main limitations of NP-enhanced delivery of drugs is their clearance by RES, which reduces their distribution and bio-availability. NPs exceeding 100 nm size can be trapped in blood and various organs such as the spleen, lungs, liver and kidneys. In order to improve intra/extra-cellular targeting of NPs as well as prolonging their circulation time, novel approaches of "host bioinvisibility" have been devised to inactivate the complement and mask the surface of nano-materials with self-identifying proteins, thereby reducing their immune recognition and uptake by phagocytic cells [141]. Furthermore, the size and diameter of NPs can be controlled during drug manufacturing process by adjusting the physical properties of NPs, especially the concentration of polymers or surfactants [166]. Furthermore, positively charged NPs penetrate cells more efficiently thanks to better uptake than neutrally and negatively charged NPs.

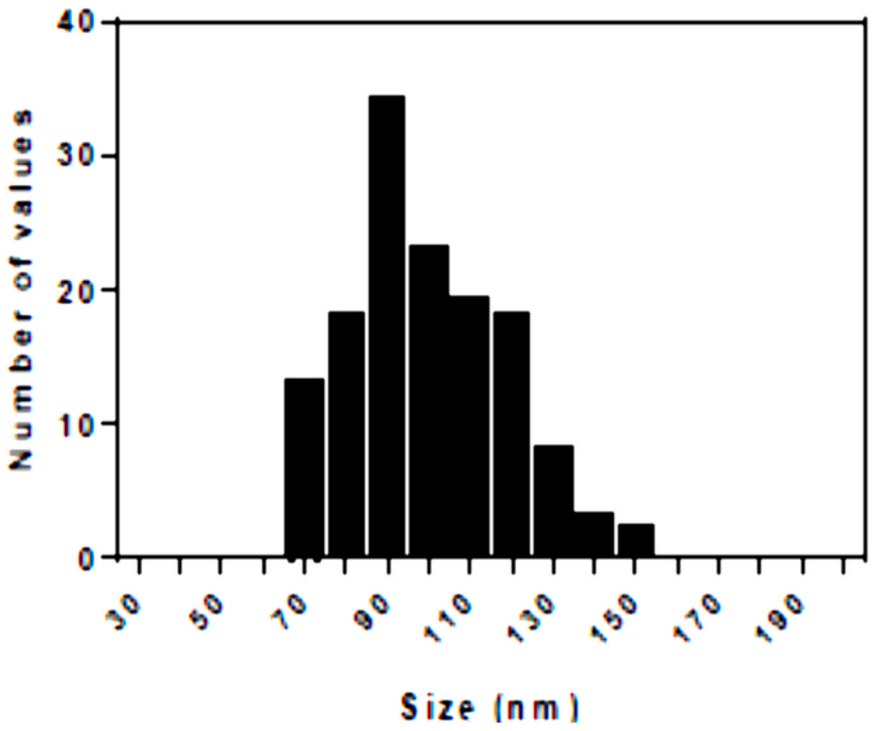

Fig. 5. Size distribution of 136 nanoparticles (NPs) [109]; reused figure by official permission.

Microspheres and microcapsules are common structures, larger than NPs, used for the structural and functional capacity of local drug delivery. Whilst NPs, smaller than 10 nm, can pass through the lymphatic duct, MPs' – whose average size is between 1 and 1000 micrometers - can act locally at a specific target site [167].

Depending on the formulation, MP can be used in different dosage forms, such as solids (capsules, tablets, bags), semi-solids (gels, creams) or liquids (solutions, suspended and even absorbed through the non-gastrointestinal tract) [168]. PLA and PLGA microspheres have been used for the delivery of gentamicin sulphate, a highly hydrophilic and cationic antibiotic effective against Brucella infections [169]. A research study evaluated the efficacy of gentamicin-loaded PLGA 50:50 H (PLGA 50:50 H) microspheres for the treatment of mice experimentally infected with *B.abortus* 2308, providing evidence of poor distribution in target organs due to issues associated with particle size and aggregation [170].

2.15. Density functional theory (DFT) for nanoparticles

Classical density functional theory (DFT) calculations are cost-effective statistical methods increasingly used in recent decades to calculate and predict properties of molecules with great precision and low processing costs, without any experimental input [171,172]. Quantum simulation systems allow to reliably calculate organic molecule geometric optimization, absoprtion spectra, lowest energy electronic transitions, define molecule's behavior as a nucleophile or electrophile charachterize the binding capacity of a drug and its release from carriers [173–175]. For instance, DFT enabled to establish that addition of surfactant AOT improves the interaction of Gentamycin with PLGA and the efficiency of targeted delivery of the antibiotic to treat of Brucellosis [30].

3. Conclusion

Brucellosis is one of the most widespread zoonosis in the world. Although different antibiotics regimens including gentamicin, doxycycline and rifampicin are available against the disease, no efficient treatment has been attained yet, due to the intracellular life of *Brucella* [176,177]. Thanks to their small sizes, nano-antibiotics can be easily uptaken by macrophages, the preferred habitat of Brucella, overcoming the difficulties to reach the intra-cellular environment of these bacteria [178,179]. Different nano- and micro-structures have been used to deliver antibiotics to target organs with promising results. Nano-medicines responding to environmental stimuli (temperature, pH, electric or magnetic field, light, ultrasound, salt concentration, among others) allow to maximize drug delivery inside macropages, thereby boosting treatment efficacy [180,181]. Since they can be delivered to specific target organs, nano-antibiotics would also reduce treatment costs and side effects frequently associated with chemotherapeutic medications, thereby improving treatment compliance of patients [182–186].

Ethics approval and consent to participate

Not applicable.

Funding

Not applicable.

CRediT authorship contribution statement

AR, MJ, AH, MH, SZ, HA, and MS designed and supervised the study, contributed to drafting the manuscript and wrote the manuscript. MKH, MA, and AHK performed the revision and editing the review. LC wrote the manuscript and revised it. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Not applicable.

Acknowledgments

We would like to acknowledge all colleague that intellectually help us to contact this scientific review study.

Consent for publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115875.

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