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Design and characterization of antimicrobial usnic acid loaded-core/shell magnetic nanoparticles

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ABSTRACT

The application of magnetic nanoparticles (MNPs) in medicine is considered much promising especially because they can be handled and directed to specific body sites by external magnetic fields. MNPs have been investigated in magnetic resonance imaging, hyperthermia and drug targeting. In this study, properly functionalized core/shell MNPs with antimicrobial properties were developed to be used for the prevention and treatment of medical device-related infections. Particularly, surface-engineered manganese iron oxide MNPs, produced by a microemulsion method, were coated with two different polymers and loaded with usnic acid (UA), a dibenzofuran natural extract possessing antimicrobial activity. Between the two polymer coatings, the one based on an intrinsically antimicrobial cationic polyacrylamide (pAcDED) resulted to be able to provide MNPs with proper magnetic properties and basic groups for UA loading. Thanks to the establishment of acid–base interactions, pAcDED-coated MNPs were able to load and release significant drug amounts resulting in good antimicrobial properties versus *Staphylococcus epidermidis* (MIC = 0.1 mg/mL). The use of pAcDED having intrinsic antimicrobial activity as MNP coating in combination with UA likely contributed to obtain an enhanced antimicrobial effect. The developed drug-loaded MNPs could be injected in the patient soon after device implantation to prevent biofilm formation, or, later, in presence of signs of infection to treat the biofilm grown on the device surfaces.

1. Introduction

In the last two decades, superparamagnetic iron oxide nanoparticles (SPION) have been the subject of intense research since they are considered promising tools in different applicative fields, such as the environment [1], the electronics [2] and the biomedical field [3]. In this latter sector, SPION can be alternatively used in diagnostics and therapy [4,5]. Indeed, their intrinsic magnetic properties enable them to be investigated both as contrast agents in magnetic resonance imaging [6,7] and as therapeutic nano-systems in hyperthermia treatments [8], and drug targeting.

As far as drug targeting is concerned, in principle, SPION can be transported by means of external magnetic fields and fixed to a target body area to achieve a site-specific therapy with improved efficacy and minimized systemic side effects. SPION can also permit the use of drugs having limited therapeutic applications due to toxicity issue or unfavorable physicochemical properties. Drawbacks limiting the use of SPION are possible magnetically induced aggregation, surface oxidation, and lack of functional groups. Therefore, SPION surface modification is mandatory and usually involves SPION coating by polymers, surfactants or inorganic compounds [9]. The resulting core/shell structure can provide good dispersion in water, protection against oxidation and efficient drug loading [4].

Several studies [10–14] have investigated the use of SPION in cancer treatment for the delivery of drugs in targeted therapeutic regimes. Recently, SPION have been also proposed for the treatment of microbial infections related to implantable medical devices [15–20]. These infections are localized to the site of device implantation and are supported by the formation on the device surfaces of sessile bacterial communities, known as biofilms, highly resistant to antibiotics [21]. The localization of drug-loaded SPION at the infected area surrounding the implanted device is expected to be able to potentiate drug activity against biofilm. Indeed, SPION are small enough to penetrate the biofilm matrix as well as they possess a high surface to volume ratio ensuring the loading and delivery of significant drug amounts.

The impact of SPION alone as antimicrobials is under investigation [22–24]. Specifically, SPION seem to possess inherent bactericidal

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activity against *Staphylococcus aureus* [22], *Staphylococcus epidermidis* [23] and *Escherichia coli* [24], this activity being related to their ability to interact and penetrate bacterial cell membrane as well as to produce free radicals via the Fenton reaction [16].

SPION have been also loaded with different classes of antimicrobial agents, including antibiotics and antifungal drugs [1,19,25]. To improve antibiotic loading, hydrophilic and functional polymers have been used for SPION coating [19]. At this regard, cationic macromolecules seem to be the most interesting for the development of antimicrobial drug-loaded SPION since they not only possess intrinsic antimicrobial activity but are also able to bind to the negatively charged bacterial cell membrane with a potential positive effect on drug uptake by microorganisms.

Chifiriuc et al. [26] adsorbed four cephalosporins (cefepime, ceftriaxone, cefuroxime and cefoperazone) onto chitosan-coated SPION finding an improved activity against different strains of *E. coli*. Indeed, a decrease from 2 to 7.8 times in the minimum inhibitory concentration (MIC) was recorded when using the antibiotic-loaded SPION instead of each antibiotic alone. Lately, the same research group loaded aminoglycosides (kanamycin and neomycin) onto the same chitosan-coated SPION obtaining an enhancement in the antibacterial activity against *S. aureus* and *Pseudomonas aeruginosa* as well [27]. The authors suggested that the observed enhanced activity was related to the binding of chitosan to the bacterial cell membrane causing an alteration of cell wall integrity and a more efficient penetration of the released drug [28]. Recently, SPION coated with cationic polyacrylamides were shown to possess good bactericidal activity against *E. coli* in planktonic state [29,30].

Our group has lately developed an intrinsically antimicrobial cationic polyacrylamide [31] active against *S. epidermidis* and was able to efficiently complex usnic acid (UA), a dibenzofuran natural extract known for its antimicrobial [32–35] and antiproliferative activities [36]. The use of UA in therapeutic application is rather limited due to its low water solubility [37] and toxicity issues [38,39]. Therefore, this drug is an ideal candidate for a targeted therapy. Some studies have investigated the UA entrapping in micro- or nano-carriers underlying the applicative potential of these controlled releasing systems [40–42]. Usnic acid has been also recently adsorbed onto the surface of oleic acid coated-magnetite and tested in terms of its ability to prevent biofilm development in *S. aureus*, *E. coli*, *P. aeruginosa* and *Enterococcus faecalis* [43,44]. Results showed how magnetite/UA nanoparticles exhibited efficient antimicrobial activity against planktonic and adherent cells of Gram-positive strains. The same research group applied these UA-loaded magnetic particles to develop either thin coatings to be applied to titanium [45] or bioactive wound dressings [46] obtaining great antimicrobial activity versus *S. aureus* biofilms.

In this study, the synthesis of antimicrobial magnetic nanoparticles and investigation of their biocidal activity are reported. Particularly, core/shell magnetic nanoparticles (MNPs), possessing a manganese iron oxide core and a polymeric shell, have been developed and used for UA loading. Manganese iron oxides were chosen for their high magnetization [47] and safety for human use [48]. To obtain MNPs with different surface properties (hydrophilicity/hydrophobicity, functional groups), two polymer coatings were investigated: i) a hydrophilic antimicrobial cationic polyacrylamide bearing tertiary amino groups [31] and ii) a newly synthesized hydrophobic star-branched polycaprolactone. The first polymer provided intrinsic antimicrobial activity and basic groups for UA adsorption while the second one could interact with UA by hydrophobic interactions. Among the two polymer coatings, we expect the antimicrobial cationic polymer to be more advantageous since it could act synergistically with usnic acid potentiating the antimicrobial activity as well as preventing the development of drug resistant bacteria. Indeed, the cationic MNPs could bind to the negative bacterial cell membrane causing an alteration of cell wall integrity and promoting the penetration of the released drug into the cell membrane [28]. The developed core/shell MNPs were characterized in terms of size, morphology and magnetic properties. Finally, the influence of the two polymer coatings on drug release and antimicrobial activity in vitro vs. a strain of *S. epidermidis* was evaluated.

2. Materials and methods

2.1. Preparation of manganese iron oxide magnetic nanoparticles ($MnFe_2O_4$)

Manganese iron oxide nanoparticles were synthesized by coprecipitation of Fe^{3+} and Mn^{2+} from a water-in-toluene microemulsion system followed by thermal treatment as previously described [49]. Briefly, 12.5 mL of a 0.4 M sodium dodecyl benzene sulfonate (NaDBS, Aldrich) solution was added to 11.5 mL of a water solution containing 0.4 M $Fe(NO_3)_3$ (Fluka) and 0.2 M $Mn(NO_3)_2$ (Fluka). Later, excess toluene (1:20 water:toluene volume ratio) was added to the mixture and the resulting microemulsion was stabilized by stirring for 24 h. Finally, 20 mL of 1 M NaOH solution (in stoichiometric amount to obtain $MnFe_2O_4$) was added to the microemulsion under nitrogen flow to avoid Mn^{2+} oxidation. After 2 h stirring, the microsuspension was kept at 100 °C for 90 min under nitrogen flow (digestion process). Then, the precipitated powder was collected by centrifugation and repeatedly washed with a 1:1 water/ethanol solution. The obtained dark brown powder was first dried at 40 °C and then submitted to a thermal treatment of calcination in oven at 600 °C.

2.2. Synthesis of polymers and their characterization

To obtain magnetic nanoparticles with different surface properties, $MnFe_2O_4$ were coated with either a hydrophobic star-branched poly-ε-caprolactone or a hydrophilic basic polyacrylamide (Fig. 1).

The star-branched poly-ε-caprolactone was obtained by ring opening polymerization of ε-caprolactone (CL, Sigma) by using tetraethylenepentamine (TEPA, Fluka) as a polyfunctional initiator (5 reactive functionality) and $Sn(Oct)_2$ as a catalyst (0.1% with respect to monomer). The reaction was carried out at 110 °C for 24 h, employing a 50/1 [CL]/[TEPA] molar ratio. The polymer was named $sbPCL_{50}$, where 50 indicates the theoretical length of each arm.

The chemical structure of $sbPCL_{50}$ (Fig. 1) was confirmed by ^{1}H-NMR (300 MHz, d-DMSO): δ = 1.60–1.80 (m, –CH_2CH_2), δ = 2.60 (m, –CH_2CO), δ = 3.65 (t, –CH_2OH), δ = 4.20 (m, –CH_2OCO). ^{1}H-NMR analysis was also used to determine the number-average degree of polymerization of each arm of the polymer (DP_n), from the ratio of the integrated area between the peak at 3.65 related to the chain ends and the peak at 4.2 attributed to the methylene groups linked to the ester oxygen [50]. The number-average molecular weight (M_n) and polydispersity index (I) were measured by Gel Permeation Chromatography (GPC) using a 150-C Waters GPC apparatus equipped with a differential refractive index detector. Measurements were performed in HPLC grade tetrahydrofuran (THF) at 25 °C with a mobile phase flux of 1 mL·min^{-1}. The instrument was calibrated with narrow linear polystyrene (PS) standards with molecular weights ranging from 1.3×10^3 to 1.5×10^6 g·mol^{-1}. Two cross-linked polystyrene columns (Water Ultrastyragel) able to separate in the range 2×10^3–1×10^6 g·mol^{-1} (linear) and 200–3×10^4 g·mol^{-1} were used.

The basic cationic polyacrylamide was synthesized by radical polymerization of the monomer AcDED obtained by reaction between acryloyl chloride (Ac, Sigma) and N,N-diethylethylendiamine (DED, Sigma), as described elsewhere [31]. Briefly, for monomer synthesis DED (0.029 mol) was added to a solution of Ac (0.038 mol) in dimethylcarbonate (DMC, 75 mL) containing K_2HPO_4 (0.08 mol). The reaction was carried out for 4 h at room temperature. After reaction, the solution was filtered and AcDED recovered by solvent evaporation.

The AcDED polymerization was carried out at 25 °C for 24 h by using a 1 M water solution of AcDED and $K_2S_2O_8$ (2.8×10^{-4} mmol) plus $FeSO_4$ (2.4×10^{-4} mmol) as radical initiators. The polymer was purified