β-N-acetylglucosaminidase grafted on mesoporous silica nanoparticles. A bionanoantibiotic system against *Staphylococcus aureus* bacteria

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ABSTRACT

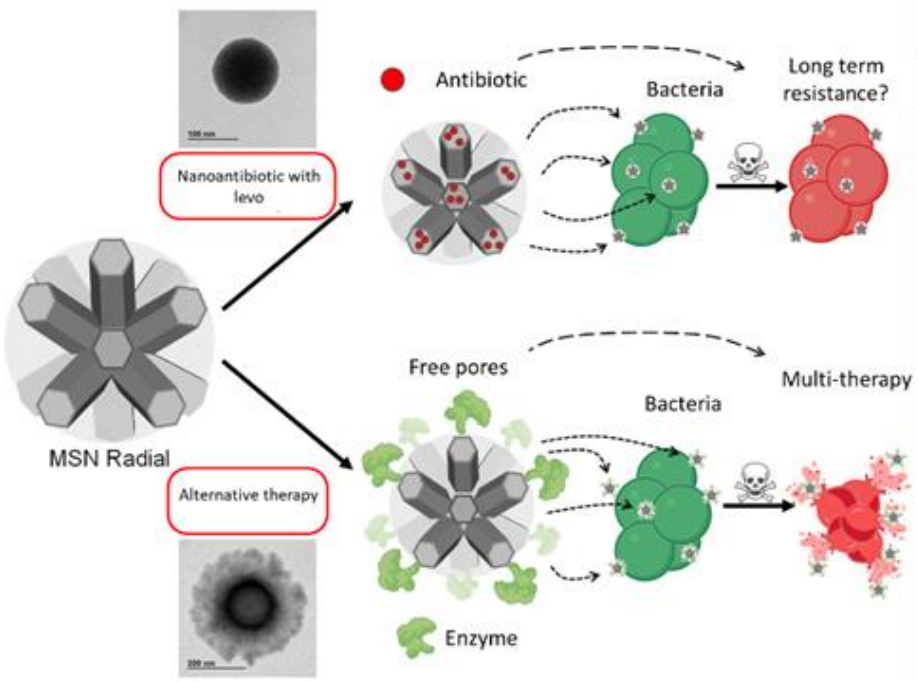
A bionanoantibiotic system based on β-N-acetylglucosaminidase (Ami) and Lysozyme (Lyz) enzymes grafted on the external surface of amino functionalized mesoporous silica nanoparticles, having a radial arrangement of pores (MSN_r-NH_2), was prepared and fully characterized. Before the enzyme grafting the nanoparticles were also loaded with the antibiotic drug levofloxacin (Levo) to explore the possible synergic effect with the enzymes. MSN_r-NH_2-Lyz-Levo and MSN_r-NH_2-Ami-Levo did not show any activity against *S. aureus*. On the contrary, in the absence of the antibiotic, both Lyz and Ami immobilized on MSN_r were able to destroy *S. aureus* cells, suggesting an inhibiting action of the antibiotic on the enzymes. Although the loading of immobilized Lyz was higher than that of Ami (76 *vs.* 20 mg/g, respectively), the highest antibacterial efficacy was found for MSN_r-NH_2-Ami nanoantibiotic. Moreover, MSN_r-NH_2-Ami was active against *S. aureus* even at very low concentration (12.5 μg/mL) with a bactericidal activity (79 %), higher than that determined for MSN_r-NH_2 loaded with levofloxacin (54%). These results suggest the possibility of using enzyme grafted MSN_r as a bionanoantibiotic drug with high efficiency even at low nanoparticles concentration.

KEYWORDS: Antibiotic resistance; bionanoantibiotic system; Mesoporous silica nanoparticles; β-N-acetylglucosaminidase; Lysozyme.

Antibiotic resistance will be one of the major health challenges for the human kind in the next years [1]. Bacterial infections are one of the main issues correlated with bone implants. Indeed, the surface of the implant is an optimal environment for bacterial colonization by opportunistic pathogens of *Staphylococcus* genus such as *S. aureus* or *S. epidermidis* [2]. The antibiotic therapy has various adverse side effects for the patient, in addition to the inherent risk of development of new antibiotic resistances. Several attempts have been made to find substitutes to conventional treatments, such as the use of antibacterial surfaces or coatings on scaffolds for bone tissue regeneration and, more recently, the use of nano carriers [3,4]. Among them [5,6], mesoporous silica nanoparticles (MSNs) have peculiar characteristics such as superior surface area and easy surface functionalization [7–9]. Several antibiotics, such as gentamicin, ampicillin, or levofloxacin, have been successfully loaded on MSNs to obtain antimicrobial nanosystems [10,11]. However, to fight against antibiotic resistance, new therapeutic strategies must be explored. Cell walls are essential for bacterial survival and growth. Consequently, destruction of the cell wall prevents bacterial cell to carry out vital functions. Some hydrolases break covalent bonds in bacterial cell walls. In particular, among the five classes of hydrolases, glucosaminidases, muramidases (lysozymes) and transglycosylases, have shown a potential antimicrobial activity [12]. Hence, antimicrobial hydrolases could in principle be used instead of, or in synergy with, antibiotic drugs to kill bacteria. Due to its hydrolytic activity toward polysaccharides of bacterial cell wall, lysozyme (Lyz, E.C: 3.2.1.17) has been used as an antimicrobial agent [13]. Enzymes benefit of immobilization on solid supports as widely demonstrated in biocatalysis applications [14]. Lorente et al. [15] described the main advantages of using immobilized enzymes on MSN as responsive drug delivery systems. Li et al. [16] used lysozyme grafted on MSN-COOH as antibacterial agent against *E. Coli* both *in*

vitro and *in vivo*. Very recently, Devlin et al. [17] immobilized various enzymes such as "lysostaphin, serrapeptase and DNase I" on MSN for the eradication of *S. aureus*.

β-N-acetylglucosaminidase (Ami), also known as dispersin B (E.C. 3.5.1.4), catalyzes the hydrolysis of the glycan backbone of peptidoglycan [18]. Similarly to Lyz [19], the action of Ami is based on the damage of the dense peptidoglycan wall present in Gram positive bacteria [20]. Ami was studied to disperse bacterial biofilms [21]. While the effect of immobilized enzymes such as α-amylase, bromelain, lysostaphin, and papain on MSN has been evaluated for biofilm eradication and bacteria killing, the bactericidal activity of Ami immobilized on MSNs against bacteria has not been explored yet. Moreover, the possible synergic antibacterial effect of Ami immobilized on the external surface of MSNs, and an antibiotic, loaded within their pores deserves to be investigated.



Scheme 1. Schematic of radial MSN_r with antibiotic and MSN MSN_r-NH_2-Lyz/Ami as a possible bionanoantibiotic system.

Here, β-N-acetylglucosaminidase (Ami) was grafted on the external surface of radial MSN_r-NH_2 for the first time. Lysozyme (Lyz) was immobilized following the same protocol for comparison purposes. Additional samples were loaded with the antibiotic levofloxacin with and without successive enzyme grafting. All these samples were tested in planktonic as a possible innovative bionanoantibiotic drug to fight against Gram-positive *S. aureus* bacteria (Scheme 1).

Figure 1 A-B displays TEM figures of the synthesized mesoporous silica nanoparticles (MSN_r). MSN_r showed a regular spherical shape and a regular particle size distribution in the range of 100-125 nm. These silica nanoparticles show a radial distribution of pores. In this type of nanoparticles, the pore size increases slightly going from the center to the outer surface [22]. Unlike the most common hexagonal MSN_r, radially aligned pore channels nanoparticles have an improved mechanical strength and colloidal stability [23].

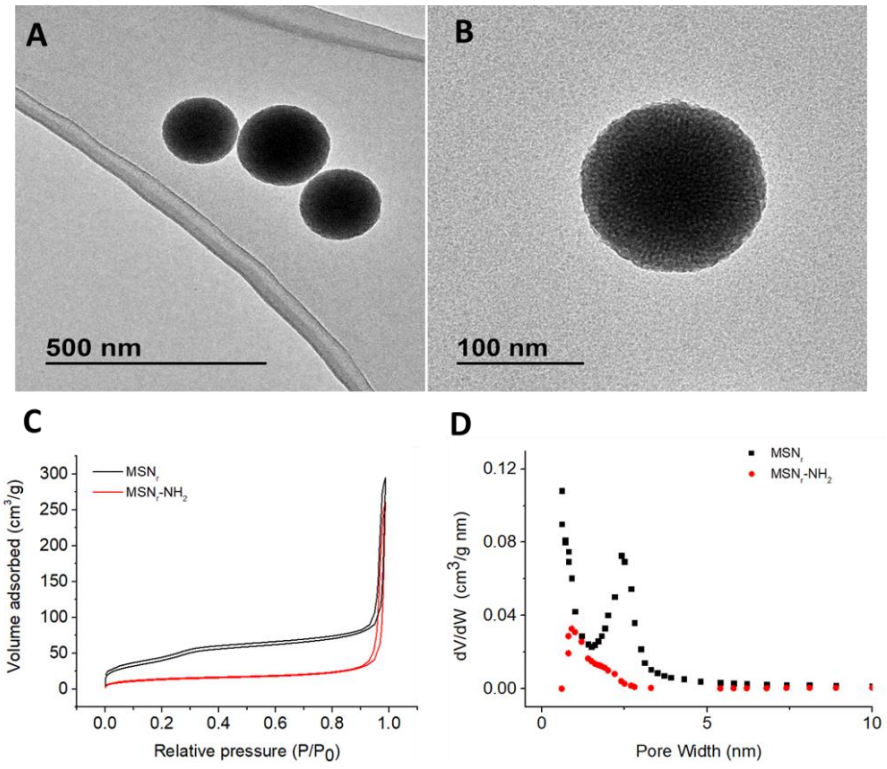


Figure 1. A-B) TEM images of MSN_r with radial pore orientation at different magnifications, C-D) N_2 adsorption desorption isotherm and pore distribution of MSN_r and MSN_r-NH_2

N_2-adsorption/desorption isotherms and BJH analysis, shown in Figure 1C-D, allowed to obtain the surface area (S_{BET}), volume (V_P) and diameter (D_P) of MSN_r pores and functionalized MSN_r-NH_2 samples. All these parameters changed due to aminopropyl functionalization. Specifically, S_{BET} decreased from 375.3 m^2/g to 47.2 m^2/g, V_p from 0.20 cm^3/g to 0.03 cm^3/g, and Dp from 2.6 nm to 2.3 nm upon MSN_r functionalization to obtain MSN_r-NH_2 (Table 1). Lysozyme (Lyz) and amidase (Ami) enzymes were both grafted on MSN_r-NH_2 by mean of the bifunctional reagent glutaraldehyde to obtain MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami, respectively. Lyz was also immobilized on MSN_r-NH_2 to compare its antimicrobial activity with that of Ami. The size distribution of bare and functionalized MSN_r was determined by DLS (Figure S1, Supporting Information file). Bare MSN_r showed a hydrodynamic size of 122.4 nm that increased, due to enzyme grafting, to 220.2 nm for both MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami samples. The occurrence of the enzymes on the MSN_r surface was investigated by zeta potential (ζ) measurements (Table 1). Indeed, the values of ζ were +29 mV for MSN_r-NH_2-Lyz (pI Lyz ~11) and -8 mV for MSN_r-NH_2-Ami (pI Ami ~ 5). The successful enzyme grafting was investigated also by FTIR. The incorporation of Lyz on MSN_r was evidenced by the appearing of the bands of amide I and amide II at 1650 and 1526 cm^{-1}, respectively. The incorporation of Ami resulted in the appearance of the same peaks with a much lower intensity likely due to a lower enzymatic loading on MSN_r respect to Lyz.

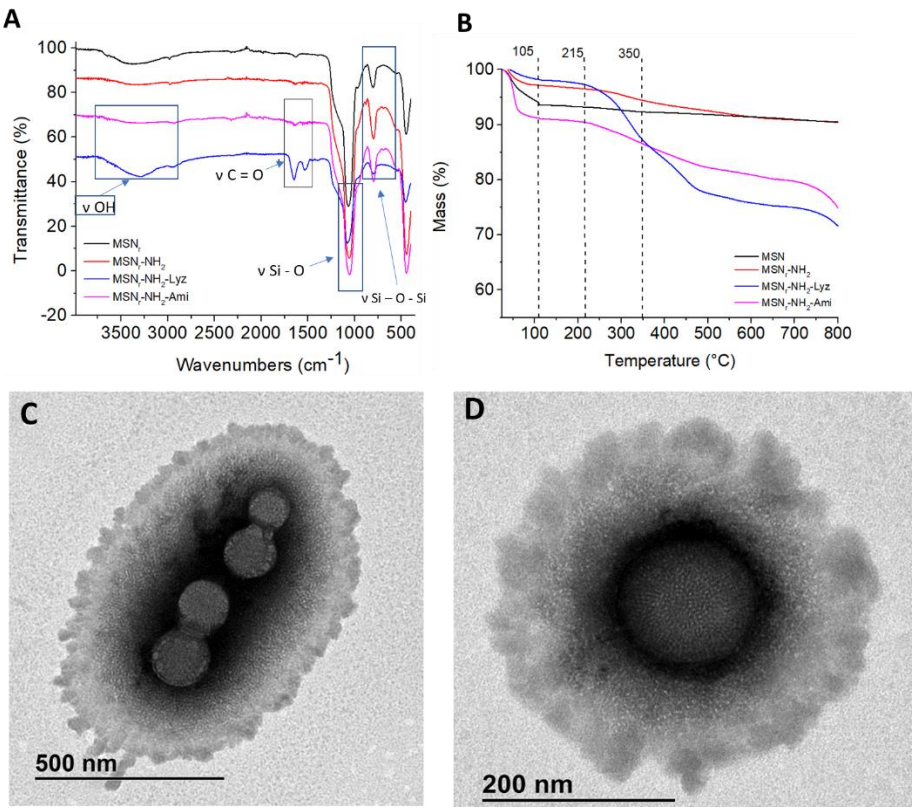


Figure 2. FTIR (A) and TGA analysis (B) of MSN_r, MSN_r-NH_2, MSN_r-NH_2-Lyz, and MSN_r-NH_2-Ami. C-D) TEM images of MSN_r-NH_2-Ami dyed with PTA at different magnifications.

TGA curves (Figure 2B) show a mass loss of 2.4 % in the range 215-350 °C due to the presence of the aminopropyl functionalization for MSN_r-NH_2. In the same temperature range a mass loss of 10% and 4% was obtained for MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami, respectively. This allowed us to quantify the amount of grafted enzyme (Table 1 and Table S2) which was 76 and 20 mg/g for MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami, respectively. Figure 2C and 2D show a layer of organic matter surrounding the nanoparticles due to PTA staining. These results together with CHN analysis (Table S1, Supporting Information file) confirmed the successful enzyme grafting on MSN_r external surface.

Table 1. Structural characterization of bare and functionalized MSN_r. Surface area (S_{BET}), pore volume (V_p) and pore size (D_p) obtained from N_2 adsorption/desorption measurements. Hydrodynamic size (d_H) and zeta potential (ζ) and determined by DLS and ELS measurements in water. Amount of grafted functional groups and enzyme loading (mg/g) calculated by TGA.

Sample	S_{BET} (m^2/g)	V_p (cm^3/g)	D_p (nm)	d_H (nm)	ζ (mV)	Loading (mg g^{-1})
MSN_r	375.3	0.20	2.6	122.4	-10 ± 1	-
MSN_r-NH_2	47.2	0.03	2.3	164.2	+27 ± 2	-
MSN_r-NH_2-Lyz	-	-	-	220.2	+29 ± 3	76
MSN_r-NH_2-Ami	-	-	-	220.2	-8 ± 1	20

Levofloxacin antibiotic loading was performed by an impregnation method on MSN_r-NH_2 right before the enzyme grafting. Levofloxacin (Levo) is a broad-spectrum fluoroquinolone drug used against a wide range of bacteria especially in respiratory diseases [24]. The enzyme functionalized MSN_r with and without impregnated levofloxacin were then tested in planktonic against the Gram-positive *S. aureus* bacteria cultures. The bacteria cells were counted before and after 24 h of incubation with the appropriate bionanoantibiotic system. In the absence of both the antibiotic and the enzyme, MSN_r and MSN_r-NH_2 samples, used as controls, did not affect the bacteria survival at all the assayed concentrations (Figure 3A, Figure S3). The antibacterial properties of Lyz are mainly attributed to the destruction of residues in peptidoglycan [25]. MSN_r-NH_2-Lyz was effective already at a nanoparticle concentration of 12.5 μg/mL reaching a bacterial cell death of 45 %. The percentage of bacterial death increased to 80% at 25 μg/mL (Figure 3B). In a previous work [26], Lyz immobilized on chitosan nanoparticles was used against *S. aureus* resulting in a very good MIC (minimum inhibitory concentration) of 0.15 mg/mL but at a very high loading of 600 mg/g. Very recently, Arpanei et al. [27] showed that the antibacterial activity of immobilized Lyz depended more on the immobilization method rather than the amount of enzyme attached on carboxyl-functionalized silica nanoparticles. Results in Figure 3 A-B showed that MSN_r-NH_2-Ami had a better performance than MSN_r-NH_2-Lyz. Although Ami had a lower loading than Lyz on MSN_r (20 *vs* 76 mg/g, respectively) it was more efficient against the bacteria even at the lowest

nanoparticle concentration of 12.5 μg/mL, obtaining 45% and 79% of bacterial death for MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami, respectively (Figure 3B). Dispersin B glycosylhydrolase from *A. actinomycetemcomitans* was able to disperse 85% of a *S. Epidermis* biofilm when used in combination with Ag [28]. Compared with the free enzyme, immobilized Ami on carboxymethyl chitosan nanoparticles [29] was able to promote the inhibition and a significant detachment of several biofilms including *S. aureus*. Ami immobilized on magnetic nanoparticles was able to remove 50% of bacterial biofilm at a loading of 12.5 mg/g [30]. The antibiotic performance of the Ami-grafted MSN_r increased with the concentration of nanoparticles reaching 100% of bacterial removal at MSN_r-NH_2-Ami concentration of 200 μg/mL.

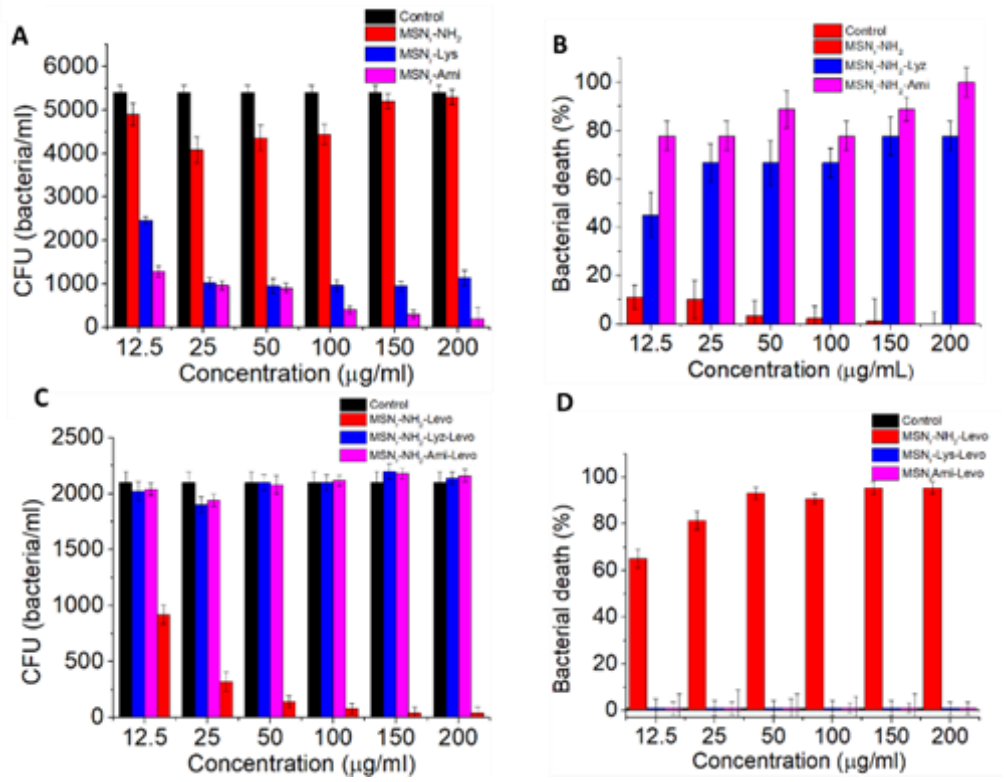


Figure 3. In vitro survival bacterial assay in CFU (colony forming unit) of colonies (control), MSN_r-NH_2, MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami against *S. aureus* in the absence A) and in presence C) of the antibiotic levofloxacin. B) Bacterial death percentage of *S. aureus* colonies (control), MSN_r-NH_2, MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami *S. aureus* in the absence B) and in presence D) of the antibiotic levofloxacin.

The in vitro antimicrobial activity of free Ami, was studied revealing a weak efficacy when used in combination with DNase and a wide spectrum antibiotic, tobramycin [31]. In the presence of levofloxacin MSN_r-NH_2 allows the death of bacteria starting from a concentration of nanoparticles of 12.5 μg/mL with a bacterial death of 65% (Figure 3C). Increasing the MSN_r-NH_2 concentration the bacterial death reached the 84% for nanoparticles concentration of 50, 150 and 200 μg/mL. On the other hand, the antibiotic seems to have no effect for the systems grafted with the two enzymes (Figure 3C). Surprisingly, the combination between the loaded antibiotic and the grafted enzyme on the nanoparticles did not show an improvement in antibiotic activity, but in fact a decrease of it, reaching almost no bacterial death (Figure 3D). The endurance of the bacteria in the presence of the enzyme and levofloxacin suggests a negative interaction between the drug molecule and the enzyme. Indeed, fluoroquinolone drugs have showed inhibition effects on enzymes, such as DNA gyrase [32]. Waryah et al.[31] studied the activity of Ami and DNase in the presence of the antibiotic tobramycin. Similarly, to our results, the combination between the drug and the enzymes did not result in a synergic antimicrobial effect.

The antibiotic release from radial nanoparticles was studied from bare, functionalized, and Lyz grafted MSN_r in physiological conditions that is PBS 10%, 37 °C and pH 7.4 [33]. Levofloxacin was released in the first 5 h with a higher released amount from MSN_r-NH_2 than MSN_r-NH_2-Lyz (Figure S2). Interestingly, the levofloxacin action released from MSNr-NH_2 achieved only 60 % of bacterial death at the lowest MSN_r concentration (12.5 μg/mL) whereas 79% was reached with MSN_r-NH_2-Ami. The good antibiotic performance of MSN_r-NH_2-Levo can be explained by release trends shown in Figure S1. The highest levofloxacin release shown with MSN_r-NH_2 could be due to the easy release from the MSN_r pores. Ami molecules have a size of 4.5×3.4×5.7 nm [34] and likely lock the entrance of the pores which have a smaller size (2.3 nm). Although Lyz is

a smaller enzyme (1.9×2.5×4.5 nm) [35], it is big enough to hinder the antibiotic release thus showing a worse performance as bactericidal. MSN have been studied in vivo to assess their biosafety. MSN are known to be toxic in the bloodstream for concentrations above 25 μg/mL [36]. This concentration limit increases with higher concentrations tolerated due to the presence of grafted biomaterials (such as proteins) on the surface [37,38]. Here, MSN_r-NH_2-Ami showed a better antibacterial activity (79 vs 60%) respect to MSN_r-NH_2-Levo at the lowest MSN_r concentration (12.5 μg/mL) suggesting its actual use as an efficient bionanoantibiotic system against *S. aureus* bacteria. A previous study has evaluated the cytotoxicity of Ami and it does not appear to be toxic to human cells [39]. However, this aspect should be further evaluated. Seeing the antimicrobial potential of both enzymes, these nanosystems could be employed as a substitute to common antibiotic therapy in bone or implant-associated infections. Indeed, Lyz has already been evaluated on MSNs as a potential antibacterial with encouraging results for food storage purposes [40].

In summary, Lyz and Ami antimicrobial enzymes were immobilized on radial amino functionalized MSN_r to obtain MSN_r-NH_2-Lyz and MSN_r-NH_2-Ami, respectively. To explore the possible synergic effect between enzyme and antibiotic drugs, the nanoparticles were loaded with the antibiotic levofloxacin before enzyme grafting. Unlike the antibiotic free samples, MSN_r-NH_2-Lyz-Levo and MSN_r-NH_2-Ami-Levo did not show any activity against *S. aureus*. This result suggests an inhibiting action of the antibiotic on the enzymes. However, in the absence of the antibiotic, the enzymes immobilized on MSN_r were able to destroy *S. aureus* cells. Despite the highest enzyme loading obtained with MSN_r-NH_2-Lyz (76 mg/g *vs* 20 mg/g) MSN_r-NH_2-Ami showed a 79% antibacterial activity even with a very low concentration of 12.5 μg/mL and reached 100% of bacterial death at 200 μg/mL. Differently by the common use of MSNs, that is to load

antibiotic drugs, a new antimicrobial nanodevice could be based on the use of enzyme grafted MSNs loaded with osteoregeneration compounds. Future work will explore this possible strategy for the treatment of bone infections.

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Author contributions

C.C.: Conceptualization, Formal analysis; Investigation; Methodology; Visualization; Writing - original draft; Writing - review & editing. J.L.P.; Formal analysis; Investigation; Methodology; Validation; Data curation; Writing - original draft. A.R.A.: Formal analysis; Methodology; B.G.: Investigation; Methodology; Data curation; Resources, Supervision. I.I.B.: Investigation; Methodology; Resources, Supervision. M.C.: Investigation; Methodology; Resources, Supervision. A.S.: Investigation; Formal analysis; Validation; Methodology; Resources; Supervision; Writing - original draft, Writing - review & editing. M.M.: Validation; Funding acquisition; Writing - review & editing, Supervision. M.V.R: Validation; Funding acquisition; Supervision.

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