SYNTHESIS AND CHARACTERIZATION OF HBPEI-Ag-CHITOSAN HYBRID MEMBRANES: THEIR POTENTIAL AS GENTAMICIN CARRIERS

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ABSTARCT

HBPEI-Ag-Chitosan hybrid systems with different weight ratios were produced. Firstly, a HBPEI-Ag complex in aqueous media was formed and then several amounts of chitosan, which was initially dissolved in 1% acetic acid, were added in order to reduce the toxicity of HBPEI. These nanoparticles are expected to have not only the combined properties of Chitosan (Chit) and Hyperbranched poly(ethylene imine) (HBPEI) but improved colloidal stability in neutral aqueous environment or physiological salinity as well. TEM, UV-Vis, XRD, and FTIR techniques were used for the characterization of the colloids. TEM and UV-Vis characterization revealed the formation of spherical silver nanoparticles with a mean diameter at around 10 nm. The ability of the optimum hybrid system to encapsulate and release antibiotic agents such as gentamicin was also studied in terms of UV-Vis. Additional tests were conducted, in order to estimate the toxicity of the specimens and assess a possible bactericidal capacity.

INTRODUCTION

Over the past few decades, polymers possessing cavities with groups capable of intermolecular interactions have emerged in the frontier of the advancement of drug delivery systems by providing controlled release of biomolecules in constant doses for a long time, and the ability to carry and release both hydrophilic and hydrophobic pharmaceutical compounds [1]. Dendritic polymers have many exceptional properties i.e. high degree of branching, manifoldness, high surface area and spherical architecture that make them promising materials for drug delivery [2-4]. Polymers encapsulating antimicrobial drugs have been applied as coatings in several areas such as biomedical devices, food industry, filters etc. The use of cationic polymers with excess of protonated amino groups can eliminate bacterial colonization of implanted devices, thus, this fact makes it antiseptic and therefore less capable of transmitting bacterial infections [5-6].

Polymers with antibacterial activity are divided in two classes, polymeric materials that have antibacterial efficacy by their own and polymeric materials modified with antibiotic drugs [7]. Hyperbranched poly (ethylene imine) (HBPEI) is a cationic polymer with many cavities and high dense of functional groups in its surface which are capable of physically or chemically binding with various pharmaceutical compounds. However, HBPEIs' application in biomedical fields is limited by its toxicity due to high local concentrations of amino groups at the periphery, especially at molecular weights 25.000 and beyond. A commonly used strategy to avoid this problem is to develop modified HBPEI derivatives [4,8-9]. Chitosan, a non-toxic natural polymer, has attracted the interest of researchers due to its biocompatibility, antimicrobial and mucoadhesive properties, low cost as well as its potential for ease chemical modification. This polymer has the capacity to bind to negatively charged bacterial cell walls, with subsequent modification of cell envelope structures and permeability and inhibition of DNA replication[10-12]. The combination of the above mentioned

polymers and the incorporation of silver nanoparticles which are well-known antibacterial agents[13-15] could result in novel systems with superior properties, which would be increased biocompatibility due to the presence of chitosan, buffering capacity, polydispersity, efficacy etc. Furthermore, the incorporation of an antibiotic agent, such as gentamicin, which was used as model antibiotic in the present work, could further increase the efficacy of the nanocomposite. Gentamicin (GM) is used for the treatment of a wide range of bacterial infections such as topical, orthopaedic and ocular. It belongs to the aminoglycosides and its antibacterial activity is attributed to its ability to irreversibly bind to ribosomes and hamper proteins synthesis [16].

MATERIALS & METHODS

Synthesis of HBPEI-Ag-Chitosan and HBPEI-Ag-GM-Chitosan membranes

HBPEI-Chitosan-Ag films have been developed through the solvent cast method. Firstly, the reduction of silver ions took place by using HBPEI as the reduction agent. Subsequently gentamicin (GM) was optionally added. Then, appropriate amounts of a chitosan-acetic acid solution were added to the above suspension in order to reduce the toxicity of HBPEI, which is attributed to the high positive charge of the amino groups, due to its high molecular weight. The as-prepared materials were coded as $HBPEI_{0.1}Ag_{0.25}Chit_{0.9}GM$, $HBPEI_{0.2}Ag_{0.25}Chit_{0.8}GM$ and $HBPEI_{0.3}Ag_{0.25}Chit_{0.7}GM$.

Characterization techniques

HRTEM (Jeol 2100HR), UV-Vis (Carry 100), XRD (Bruker D8 Focus) and FTIR (Nicolet Magna-Ir550) techniques were used for the characterization of the colloids. The ability of the optimum hybrid system to encapsulate antibiotic agents such as gentamicin was also studied in terms of UV-Vis. Additional tests were conducted, in order to estimate antibacterial and antibiofilm properties of the as-prepared membranes.

RESULTS & DISCUSSION

Figure 1a shows the UV absorption spectra of three HBPEI-Ag-Chit colloidal solutions containing 0.25 g of silver precursor that differ in the mass ratios of HBPEI:Chitosan. For comparison reasons the spectrum of $HBPEI_{0.2}Chit_{0.8}$ solution, that does not contain any silver nanoparticles, is shown as well. The single absorption band peak presented at 420 nm indicates the formation of spherical silver nanoparticles[17-19]. Furthermore, it can be observed that by increasing the HBPEI amount, the intensity of the absorption peak increases due to the higher amount of amino groups which are available for the coordination and reduction of silver ions. In addition, it must be pointed out that the silver colloid systems were very stable as even after several days they remained unchanged. From the X-ray analysis (Fig. 1b), silver and silver acetate phases were detected for all HBPEI-Ag-Chit samples. Several researches [20-21] have shown that except for silver nanoparticles, silver ions have also antibacterial properties.

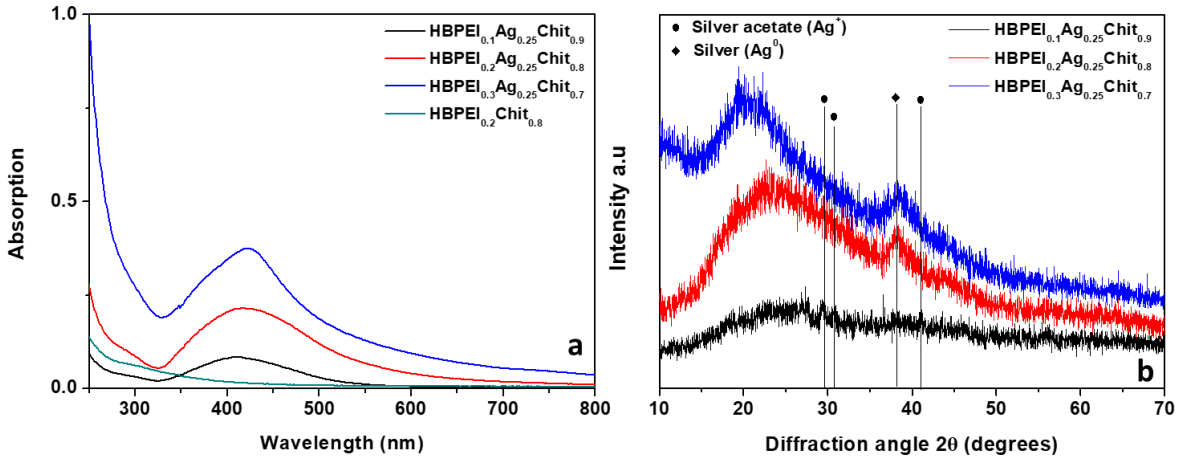


Figure 1: *UV-vis spectra of HBPEI$_{0.2}$-Chit$_{0.8}$ and HBPEI-Ag-Chit (a) and XRD patterns of HBPEI-Ag-Chit films with 0.25 gr silver precursor (b).*

Several tests concerning the films' resistance in aqueous environment have been conducted. Specifically, the as prepared films were immersed in water for 24 hours. The results showed that the HBPEI:Chit mass ratio has a critical point where the final films do not have resistance in water as only the HBPEI$_{0.2}$Ag$_{0.25}$Chit$_{0.8}$ membrane maintained, at a very satisfactory level, its morphology after its immersion in water. Hence, HBPEI$_{0.2}$Ag$_{0.25}$Chit$_{0.8}$ was selected for further characterization and incorporation of the biomolecules.

The morphology and particle-size distribution of the HBPEI$_{0.2}$-Ag$_{0.25}$-Chit$_{0.8}$ suspension are shown in Figure 2a. It can be seen that the silver nanoparticles' size is at around 10 nm with a spherical shape. In Figure 2b, the as-received HBPEI$_{0.2}$Ag$_{0.25}$Chit$_{0.8}$ film is shown, while in Figure 2c the EDS spectrum is given, where silver and carbon, due to the presence of polymers, are detected.

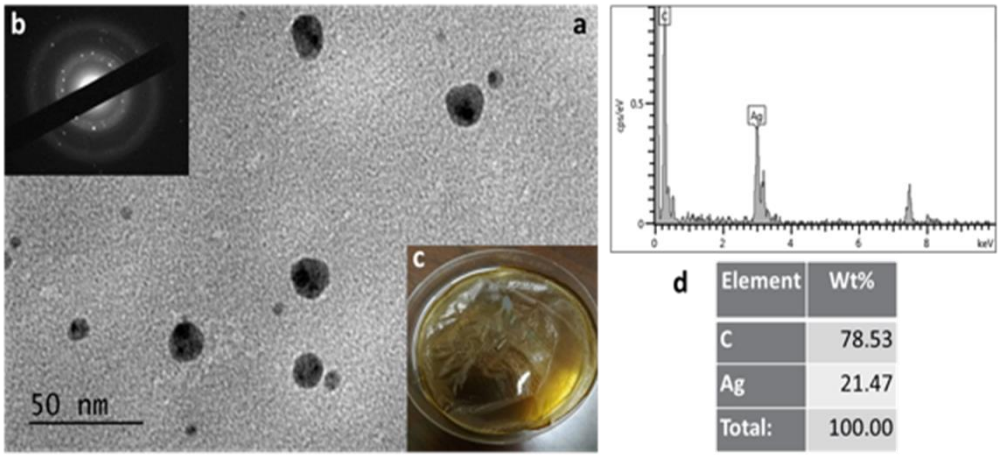


Figure 2: *TEM image (a), SAED pattern (b) as-received film (c) and EDS analysis (d) of HBPEI$_{0.2}$Ag$_{0.25}$Chit$_{0.8}$ film.*

FTIR measurements were conducted for the synthesized materials with and without the biomolecules. Figure 3a depicts the FTIR spectra of HBPEI$_{0.2}$Chit$_{0.8}$ and HBPEI$_{0.2}$Ag$_{0.25}$Chit$_{0.8}$. In the spectrum of HBPEI$_{0.2}$Ag$_{0.25}$Chit$_{0.8}$, the peaks are similar with those of HBPEI$_{0.2}$Chit$_{0.8}$. However, two peaks in the spectrum of HBPEI$_{0.2}$Ag$_{0.25}$Chit$_{0.8}$ assigned to NHx^{+} 1633.6 cm^{-1} and C-N stretching

vibrations of NH_2 at 1400.7 cm^{-1} are blue shifted to 1627 cm^{-1} and 1320.5 cm^{-1}, respectively. This can be attributed to the interaction of nitrogen atoms of amine groups with silver nanoparticles, since they reduce deformation vibration intensity of the N-H. [12] Figure 3b presents the FTIR spectra and the vibrational assignments of HBPEI-Ag-Chitosan and HBPEI-Ag-Chitosan-GM membranes. The FTIR spectrum of HBPEI-Ag-Chitosan-GM contains a shifted peak of gentamicin from 2886 to 2872 cm^{-1} which is attributed to the C-H stretching vibrations of gentamicin methyl group implying that it has been incorporated successfully into HBPEI-Ag-Chitosan films [22].

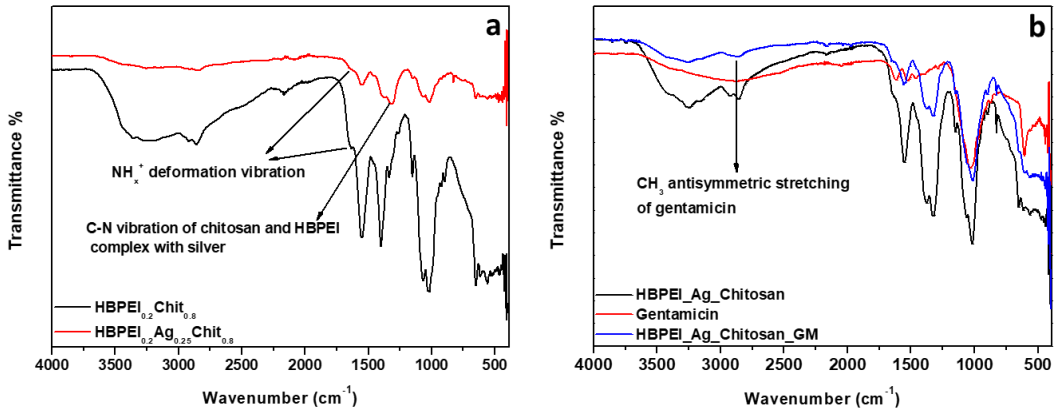
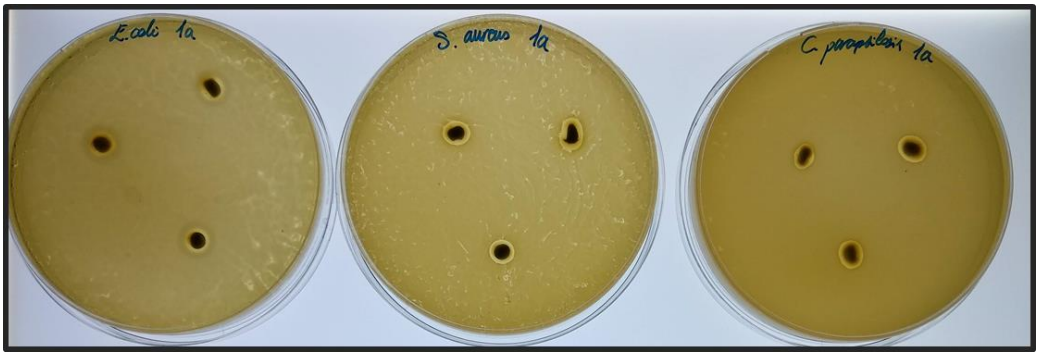


Figure 3: *FTIR spectra of $HBPEI_{0.2}Chit_{0.8}$ and $HBPEI_{0.2}Ag_{0.25}Chit_{0.8}$ films (a), and FTIR spectra of $HBPEI_{0.2}Ag_{0.25}Chit_{0.8}$, Gentamicin and $HBPEI_{0.2}Ag_{0.25}Chit_{0.8}$Gentamicin (b).*

The agar diffusion method has been used in order to investigate the membranes' antibacterial activity. The membranes were cut out in 6 mm disks, using a sterilized paper hole punch. TSA plates were inoculated with the three different species (*E. coli, S. aureus and C. parapsilosis*) by glass beads at concentrations of 108 UFC/mL for *E. coli* and *S. aureus* and 106 for *C. parapsilosis*. These plates have been analyzed by direct observation and measuring the diameters of the inhibition zones caused by the membrane disks. A control plate bearing only glass beads was added.



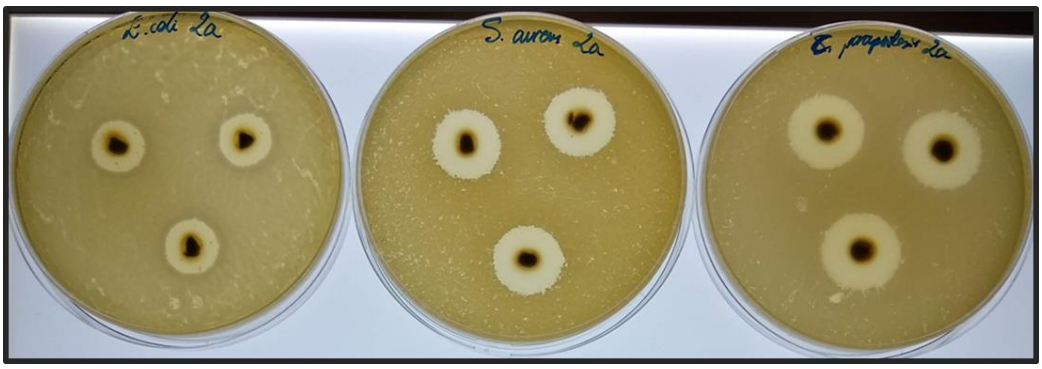


Figure. 4: *Images of agar plates showing the antibacterial activity of $HBPEI_{0.2}Ag_{0.25}Chit_{0.8}$ film (up) and $HBPEI_{0.2}Ag_{0.25}Chit_{0.8}$Gentamicin (down) against E. Coli, S. aureus and C. Parapsilosis.*

As it is evident from Fig.4, the free-drug membrane $HBPEI_{0.2}Ag_{0.25}Chit_{0.8}$ showed an inhibition zone between 0,6 <x≤ 1 for all the bacteria, whereas in the presence of gentamicin, the inhibition zone increases becoming >1.5 in the case of *S. aureus* and *C. Parapsilosis* and between 1 < x ≤ 1,5 in the case of *E. Coli.*

The membranes were also examined for their antibiofilm activity against S. aureus and P. aeruginosa. Small pieces of membranes were cut. 50 μl of bacterial suspended (106 UFC/ml) in adequate culture medium were inoculated. The plates were incubated overnight at 37°C. Membranes were washed and the bacteria adhered to the membrane were counted in LB agar plates. Both membranes have antibiofilm activity as no colony former units (CFU) per cm^2 were observed.

CONCLUSIONS

Polymer-silver membranes were developed in order to be used as coatings on medical devices. TEM characterization, in agreement with UV-Vis analysis, reveals the creation of monodispersed spherical silver nanoparticles with a mean diameter at around of 10 nm. An antibiotic agent called gentamicin was incorporated into the optimum membrane. The successful incorporation of it was confirmed in terms of FTIR analysis. Both the drug-free and the drug-loaded membranes show high antibacterial and antibiofilm properties against both gram-positive and gram-negative bacteria, with the drug-loaded membrane being much more effective. Thus, a very potential coating material for medical devices could be developed.

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REFERENCES

[1]. Liechty W. B., Kryscio D. R., Slaughter B. V., Peppas N. A., Annu. Rev. Chem. Biomol. Eng. 2010. 1:149–73.
[2]. Gillies E. R., Frıchet J. M.J., Drug Discovery Today, 10, 2005, 35-43.
[3]. Kannan R. M., Perumal O. P., Kannan S., 2007, Book chapter in Biomedical Applications of Nanotechnology, John Wiley & Sons, Inc.

[4]. Zhang L., Hu C.-H., Cheng S.-X., Zhuo R.-X., Colloids Surf., B, 76 (2010) 427–433.
[5]. Meng N., Zhou N.-L., Zhang S.-Q., Shen J., Appl. Clay Sci. 42 (2009) 667–670.
[6]. Larraza I., HBPEInado C., Abrusci C., Catalina F., Corrales T., J. Photochem. Photobiol., A, 224 (2011) 46–54.
[7]. Parisi O. I., Scrivano L., Sinicropi. S., Puoci F., Curr. Opin. Pharmacol., 36, 2017, 72–77.
[8]. Wong K., Sun G., Zhang X., Dai H., Liu Y., He C., Leong K. W., Bioconjugate Chem. 2006, 17, 152-158.
[9]. Kitsou I., Roussi E., Tsetsekou A., Ceram. Int., 43, (2017), 3861-3865.
[10]. Han R., Zeng J., Wang Y., Chang Q., Zhang X., Zhou J., Desalination and Water Treatment, 31-33, (2014), 5790-5795
[11]. Inphonlek S., Pimpha N., Sunintaboon P., Colloids Surf., B, 77 (2010) 219–226.
[12]. Biao L., Tan S., Wang Y., Guo X., Fua Y., Xu F., Zu Y., Liu Z., Mater. Sci. Eng. C, 76 (2017) 73–80.
[13]. Arkas M., Kithreoti G., Boukos N., Kitsou I., Petrakli F., Nano-Structures & Nano-Objects, (2018), 14, 138-148.
[14]. Saravanan M., Barik S. K., MubarakAlic D., Prakash P., PugazhendhiA., Microbial Pathogenesis 116 (2018) 221–226.
[15]. Shahverdi A., Fakhimi Ali, PharmD, Shahverdi H. R., Minaian S., Nanomedicine: Nanotechnology, Biology, and Medicine 3 (2007) 168– 171.
[16]. Perni S., Martini-Gilching K., Prokopovich P., Colloids and Surfaces A 541 (2018) 212–221.
[17]. Liu Z., Wang Y., Zu Y., Fu Y., Li N., Guo N., Liu R., Zhang Y., Mater. Sci. Eng. C, 42 (2014) 31–37.
[18]. Kumar-Krishnan S., Prokhorov E., Hernández-Iturriaga M., Mota-Morales J. D., Vázquez-Lepe M., Kovalenko Y., Sanchez I. C., Luna-Bárcenas G., Eur. Polym. J., 67 (2015) 242–251.
[19]. Regiel A., Irusta S., Kyzioł A., Arruebo M., Santamaria J., Nanotechnology 24 (2013) 015101 (13pp).
[20]. Sotiriou A. G., Pratsinis E. S., Environ. Sci. Technol. 2010, 44, 5649–5654.
[21]. Altinisik A., Yurdakoc K., J. Appl. Polym. Sci., 122, (2011), 1556–1563.
[22]. Liu Y., Ji P., Lv H., Qin Y., Deng L., Int. J. Biol. Macromol., 98, (2017), 550–556.