Poly(o-Phenylenediamine)/ Montmorillonite Nanocomposites: A Novel Strategy to Arrest Bacterial Pathogens Infestation

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Research Article

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Abstract

Antibacterial potential of NC were investigated by the broth microdilution and agar well diffusion methods. Further, effective concentration (EC_{50}) and minimum inhibitory concentration (MIC) of the NC were determined. Intercalation and in-situ polymerization of POPD within MMT was successfully obtained using sonochemical technique. The NC was then checked for antimicrobial activity against four bacterial strains and they showed potent antibacterial activity in both broth and on agar plate. Quantitative test in liquid media clearly showed that NC had viable cells reduction ability for testing strains. Results obtained here concluded that the NC can prevent the infestation of various bacterial pathogens effectively.

1. Introduction

The excessive use of antibiotics over the years is now under scrutiny of a subsequent propagation of antimicrobial resistance. Antimicrobial substances are capable of growth inhibition or even elimination of certain groups of microorganisms. The exploration of novel products with potent antimicrobial activity has acquired a particular interest in numerous operations, viz. raw materials for pharmaceuticals and cosmetics, clinical and veterinary products, etc. Indigenous population globally has consumed clay for remedial purposes throughout the history. Bandages of French green clay was in use for healing the Buruli ulcer (Williams et al. 2004) which is a necrotizing fasciitis caused by *Mycobacterium ulcerans*. French clays were proved to be of antibacterial nature (Haydel et al. 2008) whereas other French green clays augmented the bacterial growth compared to controls (Williams et al. 2008). Continued examination of clays globally has disclosed only a few numbers of deposits that possess antibacterial property. All deposits are mineralogically diverse but they belong to hydrothermally altered volcanic atmosphere; either transformed pyroclastic matter or bentonite (volcanic ash). To point out the antibacterial properties of an inert substance like clay, it is important to design nanocomposites which have exhibited bacteriostatic activity (Corrales et al. 2012). Clay minerals have been employed to produce organic-inorganic hybrid nanocomposites because of their ultra-fine particle size, intercalation property and large surface area.

Specifically, montmorillonite (MMT) is broadly used in this domain (Pontes et al. 2013; Hosseini 2011; Wang et al. 2010; Anuar et al. 2004; Chiu et al. 2014). MMT is well-known mineral for its hydrophilic nature which is placed in the general family of 2:1 phyllosilicates that are made up of piled up layers of silicon tetrahedrons and aluminium octahedron. Through the past decade, appreciable attention has been given to the generation and implementation of functionalized polymer/clay composites with the recent development in the arena of nanoscience and nanotechnology (Letaief et al. 2008; Ruiz-Hitzky 1993; Aranda and Ruiz-Hitzky 1992; Aranda et al. 2003; Ruiz-Hitzky et al. 2011). MMT has been employed as a promising filler for the moderation of thermal and mechanical features of polymer matrices (Biswas and Ray 2001; Wu et al. 2000; Yeh et al. 2003). For a better understanding for the clay's role, conducting polymer as a filler and as well as a matrix, in this research paper, the nanocomposites of poly(o-phenylenediamine) (POPD) and MMT were prepared by means of sonochemical intercalation with the

help of two methods, first by taking POPD to act in one case as a filler and matrix in another one. The investigation of spectral, fluorescence and spectral features of the synthesized nanocomposites led to explore the clay's optimum loading and conducting polymer for designing nanocomposites possessing controlled self-assembled morphology which can be utilized in optoelectronic devices (Riaz et al. 2016).

In recent years, clay mineral-based antibacterial complexes have been prepared by a series of processes between the clay minerals and antibacterial substances. Clay minerals are being scrutinized because of the nontoxic and eco-friendly properties and an effortless production through intercalation with the use of antimicrobial organic modifiers (Bertagnolli et al. 2011). Recent investigations have revealed that the reason behind the antimicrobial property is because of the contact between organoclay and the cells, moreover the activity is reinforced by intercalation of polymers with positive charges present on the clay as they nullify the relocation of biocidal cationic surfactant. In the recent time, scarce numbers of reports are available in the open literature regarding biocompatibility and application of antimicrobial nanocomposites as implantable biomaterials (Kaya and Oren 2005; Özdemir et al. 2010; Rivera-Garza 2000). Recently, Rivera-Garza et al. reported the elimination of the infectious microorganisms, *Escherichia coli* and *Enterococcus faecalis* from water by silver-loaded clinoptilolite/heulandite mineral. Their study revealed that antibacterial activity was a role exchange level in the exchanged samples against *Pseudomonas aeruginosa* and *E. coli* (Rivera-Garza 2000). In another work, a vermiculite–copper hybrid material shows strong antibacterial action against *S. aureus* (Drelich 2011). This work aimed to develop novel approaches in clay medication for the improvement of interactions between clays and polymer and additionally to formulate nanocomposites comprising antimicrobial property sufficient to develop new antibiotics. The investigation examined POPD/MMT and MMT/POPD nanocomposites for the antimicrobial activity against various bacterial strains. The minimum inhibitory concentration was also determined to prevent the visible bacterial growth.

2. Materials And Methods

K-10 montmorillonite (MMT) (Sigma Aldrich, St. Louis, Missouri, United States), o-phenylenediamine, Luria Bertani (LB) broth, LB Agar and Ampicillin (Himedia, India), was used without any further purification. All other reagents and chemicals used were of analytical grade and procured from Merck, Thermo Fisher Scientific etc. The tested bacterial strains were identified and obtained from NCCS, Pune, India. These included *Staphylococcus aureus* (MTCC 902), *Escherichia coli* (MTCC 443), *Bacillus subtilis* (MTCC 736) and *Pseudomonas aeruginosa* (MTCC 2453). The stock of bacteria was kept on nutrient agar slants at 4°C.

2.1 Synthesis and characterization of NC by sonochemical dispersion method

The NC were prepared as per our previously published protocol (Riaz et al. 2016). POPD/MMT NC was synthesized by dissolving MMT in distilled water followed by the addition of POPD. Whereas, MMT/POPD based NC was synthesized by dissolving MMT in distilled water followed by the addition of

o-phenylenediamine and ferric chloride (oxidant). The NC were then characterized for their size and morphology using TEM and further evaluated for their antimicrobial potential. The powdered NC were soluble in double distilled water, dimethyl sulfoxide (DMSO), N-Methyl-2-pyrrolidone (NMP) upto 5 mg/ml.

3. Characterization

3.1 Assessment of antibacterial activity

Antibacterial potential of NC were calculated as per the method of Shamsi et al. with minor modification (Shamsi et al. 2016). Antibacterial activity was tested against various bacterial strains such as *E. coli* (ATCC 25922), *S. aureus* (MTCC 902), *P. aeruginosa* (MTCC 2453), and *B. subtilis* (MTCC 736). The preparation of overnight cultures was done in Luria Broth (LB) media via inoculation of a single colony collected from agar plates and kept for incubation for 12 hours and at physiological temperature. Overnight culture was diluted with fresh LB media to approximately 10^4 colony-forming units (CFU) and incubated at 37°C for 12–14 hrs in the presence of NC. The growth of bacterial culture obtained (test) was compared to the growth obtained in the presence of ampicillin (negative control). The experiment was performed in triplicates. For each replicates, the average values were recorded. The growth of positive control culture was also determined where only media and primary culture was taken. Bacterial growth was determined spectrophotometrically by taking absorbance at 600nm. The % mean growth inhibition (%MGI) was calculated by using the formula:

$MGI (\%) = (A_c - A_s / A_c) \times 100$, where A_c and A_s represent absorbance of control and treated sample strains respectively.

The EC_{50} value (concentration causing 50 % reduction in the bacterial growth) was calculated using Microsoft office Excel 2007, based on the readings.

3.2 Determination of Minimum Inhibitory Concentration (MIC)

The determination of MIC was carried out by technique of Broth dilution as explained in method M07-A9 from the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2012). Briefly, 300µl of media was placed in each well of 96 wells plate. Further, 150 µl of the NC were added to the first well and then sample was serially diluted. The extra sample from last well was discarded to make final volume as 300µl in all well). ~10µl of bacterial cell culture (normally to an OD₆₀₀ 0.1) was added to each well and OD₆₀₀ was measured after 12 hours of incubation at 37°C at incubator. The MIC₈₀ was defined as the concentration at which ~ 80% of the bacterial growth was inhibited.

3.3 Agar well-diffusion method

Agar well-diffusion method was used to determine the antibacterial activity by the method as described in method M07-A9 from the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2012). Ampicillin

(stock, 2 mg ml^{-1}) was used as standard antibacterial drug. The log phase bacterial cultures (secondary culture) were spread on LB agar medium plates using a sterile spreader in order to get a uniform bacterial growth on test plates. Wells were punched over the agar plates using sterile cork borer. Stock solution of each nanocomposite was prepared at a concentration of 2 mg ml^{-1} in distilled water. Approximately 40–50 μl NC ($\sim 80\text{ }\mu\text{g}$) and Ampicillin solution was added with the aid of a sterile syringe into the wells and diffusion was allowed to carry out at room temperature for 2 hours. Control experiments comprising LB media was also set up. The plates were incubated at 37°C for 18–24 h. The diameter of the inhibition zone (mm) was measured. Experiment was done in triplicates. The results (zone of inhibition) were compared with the activity of the standards, ampicillin (2 mg ml^{-1}).

3.4 Statistical Analysis

The experiments were performed in triplicates ($n = 3$). Graph Pad Prism Software was used to prepare Graphs and to calculate the statistical parameters such as mean, standard error of mean (SEM) etc. The data obtained were expressed as 'mean $\pm$ SEM'.

4. Results

In this study, the nanocomposites of POPD and MMT were successfully synthesized via intercalation and in-situ polymerization of o-phenylenediamine within MMT using sonochemical technique by taking the former to be as filler in one case and as matrix in the other. The results confirmed the formation of NC which is published in our previously paper (Riaz et al., 2016).

4.1 Characterization of NC

4.2 TEM analysis

TEM micrograph of the different ratios of POPD:MMT and MMT:POPD (i.e. 1:0.25, 1:0.50, 1:1) exhibited particle size in the range of 20–90 nm and showed the spherical chain like structure (Fig. 1). POPD has shown the formation of core and MMT was found to encapsulate the dense POPD particles like a shell. The POPD:MMT-1:1 and MMT:POPD-1:1 NC showed clustering and aggregation of the spherical nanoparticles of 50–90 nm range (Fig. 1d and 1g). The results obtained were in compliance with as described in our previously published paper (Riaz et al., 2016).

4.3 Assessment of antibacterial activity

To check the efficacy of NC against bacterial strains, antibacterial tests were performed by two independent methods viz. broth micro-dilution and well diffusion assay.

4.4 Broth microdilution Assay

The effects of different ratios of MMT/POPD and POPD/MMT and standard antibiotic drug (ampicillin) on bacterial strains were determined by measuring postincubation absorbance readings at 600 nm and

%MGI was calculated. The results were compared with standard ampicillin as shown in Fig. 2. NC had exhibited various degree of growth inhibition against tested bacterial strains such as *E. coli*, *S. aureus*, *B. subtilis*, *P. aeruginosa* etc. Among the all bacterial strains tested, *B. subtilis* was the most sensitive and *S. aureus* was the least sensitive strains towards the NC. When MMT: POPD – 1:0.25, MMT: POPD – 1:0.5 and MMT: POPD – 1:1 were tested, they showed the highest resistance against *B. subtilis* i.e. %MGI were 31.609 ± 0.349 , 86.565 ± 0.440 and 77.733 ± 0.749 respectively whereas they showed least % inhibition against *S. aureus* which were 18.235 ± 1.014 , 47.819 ± 0.793 and 38.403 ± 0.943 respectively (Fig. 2).

When bacterial cells were incubated with POPD: MMT – 1:0.25, POPD: MMT – 1:0.5, and POPD: MMT – 1:1, they showed the highest susceptibility towards *B. subtilis* and hence high mean growth inhibition was observed i.e. %MGI were 38.866 ± 0.974 , 79.591 ± 0.429 and 77.971 ± 1.026 respectively whereas *S. aureus* showed least sensitivity towards these POPD/MMT NC. The % mean growth inhibition against *S. aureus* was observed approximately 15.524 ± 0.427 , 61.878 ± 0.347 and 37.616 ± 0.896 respectively. The results were quite comparable with standard ampicillin which showed highest % inhibition against *B. subtilis* (%MGI- 78.798 ± 0.366) and least against *S. aureus* (%MGI 56.236 ± 0.364) (Fig. 3).

S. aureus was the only strain which was less susceptible to the antibacterial effect of the all NC and ampicillin as compared to the other strains. NC exhibited different degree of growth percentage inhibition among tested bacterial strains which were presented in the form of EC_{50} values .

Both the assays confirmed that NC is capable to inhibit the growth of bacterial strains in a range of $15.625\text{--}2000 \mu\text{g ml}^{-1}$. The results showed that *B. subtilis* is the highest sensitive strains among all NC and exhibited the highest % inhibition i.e. sensitivity towards all the ratios of NC that's why MIC was performed for this strain only in presence of all NC. However, the results also proved that NC was bacteriostatic to *B. subtilis* at its MIC_{80} concentration where its 80% growth was inhibited and the clear solution was obtained. Inhibitory concentration of *B. subtilis* was evaluated by determining MIC_{80} as depicted in Figs. 4 and 5.

The antibacterial activity of NC showed that, *B. subtilis* displayed susceptibility to all NC ranging from $15\text{--}2000 \mu\text{g ml}^{-1}$. This method is based on a broth micro dilution method in 96-well microtitre plates. The MIC (MIC_{80}) of MMT/POPD and POPD/MMT against the *B. subtilis* was shown below. No matter whichever the NC were tested, all of them exhibited good antibacterial activity and a broad spectrum of activity.

Determination of zone of inhibition by agar well-diffusion method

NC which was synthesized from MMT and POPD has shown good zone of inhibitions which were provided in and Fig. 6, 7, 8. The antibacterial activity was conducted against the pathogenic bacterial strains such as *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis*. The well-diffusion method also showed the highest antimicrobial activity against *B. subtilis* followed by *P. aeruginosa*, *E. coli*, and *S. aureus*. The

stock concentration used for different ratios of MMT:POPD and POPD:MMT were 2 mg ml^{-1} and approximately 40 μl of the NC were diffused into each well.

NC had showed various degree of zone of inhibition against tested bacterial strains and among them, *B. subtilis* showed the highest inhibition zone whereas *S. aureus* possess the minimum zone towards the NC. When MMT: POPD – 1:0.25, MMT: POPD – 1:0.5 and MMT: POPD – 1:1 were diffused into the wells, they showed the maximum zone of inhibition in *B. subtilis* i.e. 30, 35 and 32 mm respectively whereas they showed minimum inhibition zone in *S. aureus* which were 21, 24 and 23 mm respectively (Fig. 6).

When ~ 40 μl of POPD: MMT – 1:0.25, POPD: MMT – 1:0.5, and POPD: MMT – 1:1 was diffused into each well of agar plate of separate bacterial strains, they showed the highest susceptibility towards *B. subtilis* and hence maximum zone of inhibition was observed i.e. 20, 25 and 24 mm respectively whereas *S. aureus* showed minimum zone of inhibition on agar plate towards these POPD/MMT NC. The observed inhibition zone in *S. aureus* was approximately 16, 20 and 17 mm respectively. The results were quite comparable with standard ampicillin which showed maximum zone of inhibition in *B. subtilis* (34 mm) and minimum zone of inhibition in *S. aureus* (30 mm) (Figs. 7 and 8). The positive control where the LB broth was used, there were no zone of inhibition produced suggesting that the NC are antibacterial agent as they have cleared the bacterial growth zone on agar plate.

The synthesized NC was energetically involved in the antibacterial activity against *B. subtilis*, *P. aeruginosa*, *E. coli* and *S. aureus*. *B. subtilis* had the maximum zone of inhibition among all NC tested and *S. aureus* had the minimum zone of inhibition because of the maximum resistant capacity of the bacterial isolates and having lowest growth inhibition with all NC .

The organic-inorganic hybrid nanocomposites generation using clay minerals was aided by their properties like large surface area, ultra-fine particle size, and intercalation property. Although, in construction of these nanocomposites comprising controlled properties the interaction between matrix and filler serves as a substantial feature. Hence, for a better understanding of the role played by polymer as well as conducting polymer as a matrix and filler, in this research paper nanocomposites of poly(o-phenylenediamine) (POPD) and MMT have been synthesized using sonochemical intercalation methods via two methods – by keeping the former as a filler in one case and as matrix in other. To navigate the optimum loading of clay and conducting polymer for the construction of nanocomposites with property of self-assembled morphology so that they could be employed in optoelectronic devices, the spectral, fluorescence, and morphological properties were investigated (Pontes et al. 2013; Hosseini 2011; Wang et al. 2010; Anuar et al. 2004; Chiu et al. 2014; Riaz et al. 2016). The antimicrobial property of such nanocomposites makes it a versatile material in various bacterial mediated disorders. In this regard, Rhim et al. (Rhim et al. 2006) reported that the nanocomposite films containing certain organically modified nanoclay offers strong antimicrobial function against both Gram-positive and Gram negative bacteria. They postulated that the quaternary ammonium groups present in the organically modified clays are responsible for the antimicrobial function of nanocomposite films (Rhim et al. 2009).

Some of the chemical antimicrobial agents are irritant and toxic, while plants are easily available, safe, and nontoxic in most cases, but do not have antimicrobial potential as effective as other chemical agents. Therefore, there is vital need and much interest in finding ways to formulate new types of safe and cost-effective biocidal materials. This study helped us to use these NC as safe and strong antimicrobial material. It may be due to the strong adsorption and immobilization capacity of modified layered silicates (Guo et al. 2005; Hu et al. 2005). It is a well-established fact that at pH 7.4, a net negative charge is exhibited by the parent layered silicate (Nzengung et al. 1996). Bacteria possessing negative charge will not be adsorbed notably onto the clay under these conditions. Nevertheless, after modification of POPD, nanocomposites were synthesized with several degrees of hydrophobicity. The diameters exhibited by inhibition zone fell between 10 and 14 mm. Furthermore, Gram-positive bacteria displayed a greater effect than the Gram-negative bacteria. The basic difference is the presence of an outer membrane in Gram-negative whereas absent in Gram-positive bacteria. Dissimilar barricades serves as the explanation of variation in their sensitivity as their capability to avoid the entrance of microbicides varies. Antimicrobial agents kill bacteria through various means depending on the type of bacteria. Most antiseptics and disinfectants kill bacteria immediately on contact by causing the bacterial cell to burst, or by depleting the nutrients preventing bacterial reproduction such as bacterial conjugation. Antimicrobial polymers might kill bacteria by adsorbing onto the bacterial cell wall. Most bacterial surfaces are negatively charged; therefore, the adsorption of polymeric cations has proved to be more effective than adsorption of polymeric anions. The antimicrobial agent must then diffuse through the bacterial cell wall and adsorb onto the cytoplasmic membrane. This might lead to the disruption of the cytoplasmic membrane and subsequent leakage of cytoplasmic constituents leads to the death of the cell (Nonaka et al. 2003; Uemura et al. 1999).

Conclusion

From the executed study, it can be inferred that NC showed potent antimicrobial activity and hence it can be concluded that the NC may be used as novel antimicrobial agents against wide range of microbes over conventional antibiotics that combined the benefit of organic materials with the excellence of inorganic materials prepared via simple solution intercalation technique as they have shown good antimicrobial efficacy. MMT/POPD and POPD/MMT NC have shown the %MGI in the order of *B. subtilis* > *P. aeruginosa* > *E. coli* > *S. aureus*. In the future, nontoxic and biocompatible NC may replace some antibiotics or medicines to destroy pathogenic bacterial strains in the gastrointestinal tract of animals. Results concluded that with the development of multidrug-resistant strains of bacteria, NC could be good alternatives in the prevention of MDR strains infections because of their dual role i.e. the NC are good nanocarriers and the carrier itself will act as strong antibacterial agent. Hence, this study open the new gateways for future research such as mechanism of action of these NC, drug carrying capability and inhibition of MDR strains.

Declarations

Authors' Contribution

R.P, U.R. and S.F. developed the original idea and the protocol. T.N.S. and A.V. contributed to the experimental work. R.P. and T.N.S. have written, reviewed and edited the manuscript. S.F. supervised the experimental work.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Figures

Figure 1

TEM micrographs of (a) pure POPD (b) POPD:MMT-1:0.25, (c) POPD:MMT-1:0.5, (d) POPD:MMT-1:1, (e) MMT:POPD-1:0.25, (f) MMT:POPD-1:0.5, (g) MMT:POPD-1:1

Figure 2

%Mean Growth Inhibition of MMT:POPD. Bar diagrammatic representations of in vitro antibacterial activity of different NC where MMT was used as a filler (MMT: POPD -1:0.25, MMT: POPD -1:0.5 and MMT: POPD -1:1). The bars represent the percentage mean growth inhibition obtained in the presence of NC when tested against 4 bacterial strains. Bacterial strains were grown in LB medium and then incubated with NC. The incubation period was 12 h at 37 °C. The absorbance of test sample and control was recorded at 600 nm and the MGI% was calculated. Positive control contained only inoculum and media. Ampicillin was used as a reference drug.

Figure 3

% Mean Growth Inhibition of POPD:MMT. Bar diagrammatic representations of in vitro antibacterial activity of different NC where POPD was used as a filler (POPD: MMT – 1:0.25, POPD: MMT – 1:0.5, and POPD: MMT – 1:1). The bars represent the percentage mean growth inhibition obtained in the presence of NC when tested against 4 bacterial strains. Bacterial strains were grown in LB medium and then incubated with NC. The incubation period was 12 h at 37 °C. The absorbance of test sample and control was recorded at 600 nm and the MGI% was calculated. Positive control contained only inoculum and media. Ampicillin was used as a reference drug.

Figure 4

MICs of MMT:POPD. MIC of different NCs where MMT was used as a filler (MMT: POPD -1:0.25, MMT: POPD -1:0.5 and MMT: POPD -1:1) against *B. subtilis* (MTCC 736) was determined by the broth microdilution method. Turbidity was measured by recording absorbance at 600 nm. Graph was plotted between OD₆₀₀ vs different concentration of NC (µg ml⁻¹).

Figure 5

MICs of POPD:MMT. MIC of different NC where POPD was used as a filler (POPD: MMT – 1:0.25, POPD: MMT – 1:0.5, and POPD: MMT – 1:1) against *B. subtilis* (MTCC 736) was determined by the broth microdilution method. Turbidity was measured by recording absorbance at 600 nm. Graph was plotted between OD₆₀₀ vs different concentration of NC (µg ml⁻¹).

Zone of inhibition produced by ampicillin in agar plates of *E. coli* and *S. aureus*

Zone of inhibition produced by ampicillin in agar plates of *P. aeruginosa* and *B. subtilis*

Figure 6

Agar well diffusion assay showing zone of inhibition in separate agar plate for all four strains where positive control (LB broth) and negative control (ampicillin) were diffused into well.

Zone of inhibition produced by MMT: POPD -1:0.25, MMT: POPD -1:0.5 and MMT: POPD -1:1 in agar plates of *E. coli* and *S. aureus*

Zone of inhibition produced by MMT: POPD -1:0.25, MMT: POPD -1:0.5 and MMT: POPD -1:1 in agar plates of *P. aeruginosa* and *B. subtilis*

Figure 7

Antibacterial activity of NCs (MMT: POPD -1:0.25, MMT: POPD -1:0.5 and MMT: POPD -1:1) against pathogens by agar well diffusion method. Well diffusion assay showing zone of inhibition for each strain.

Zone of inhibition produced by POPD: MMT – 1:0.25, POPD: MMT – 1:0.5, and POPD: MMT – 1:1 in agar plates of *E. coli* and *S. aureus*

Zone of inhibition produced by POPD: MMT – 1:0.25, POPD: MMT – 1:0.5, and POPD: MMT – 1:1 in agar plates of *P. aeruginosa* and *B. subtilis*

Figure 8

Antibacterial activity of NC (POPD: MMT – 1:0.25, POPD: MMT – 1:0.5, and POPD: MMT – 1:1) against pathogens by agar well diffusion method. Effect of each NC showed zone of inhibition on each strain.