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Research Article

Design of a New Coating Agent Based on Graphene Oxide and Antimicrobial/Spermicidal Peptide (Sarcotoxin Pd) for Condom Coating: New Strategy for Prevention of Unplanned Pregnancy and Sexually Transmitted Infections

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In this study, sarcotoxin Pd-functionalized graphene oxide (GO-Pd) was synthesized as a new condom-coating agent. Antimicrobial activity was evaluated by radial diffusion assay (RDA) and absorbance-based methods. Sperm motility and morphology were assessed in different concentrations of designed nanostructures. Peptide stability on the GO structures was assessed by the CD technique. GO-Pd showed the highest contact angle. The results approved that GO-Pd had broad-spectrum antimicrobial activities against examined pathogens, especially vaginal infections such as *Candida vulvovaginitis*. This antimicrobial activity was more than pristine peptides, vancomycin, and fluconazole. GO-Pd also had a higher inhibitory activity on the sperm motility and viability than pristine peptides. GO-Pd had high stability and activity in all examined conditions. But, naked peptides had low stability and activity after incubation in acidic pH and high temperatures (>38°C). In all tests, GO-Pd showed a significant difference compared to naked peptide. Based on the results, GO-Pd can be used as a condom coating to prevent unplanned pregnancy and sexually transmitted infections.

1. Introduction

Sarcotoxin Pd is an antimicrobial peptide purified and identified from *Paederus dermatitis* [1]. In our previous studies, these peptides have potent antimicrobial and spermicidal activity [1, 2]. Sarcotoxin Pd has been introduced as a powerful contraceptive agent in preventing unplanned pregnancy and sexually transmitted infections (STIs) [2]. The limitation of applying these peptides is low stability in various environments (with different pH and temperatures) [3–5]. Different strategies have been developed to increase the metabolic stability of peptides, such as cyclization, substituting amides with sulfonamides, etc. [6–8]. In between, nanotechnology can be used to design new biocompatible and biodegradable systems to enhance peptide stability, slow-release, targeted delivery, maintenance of peptide's

structure and function, and so on [9–12]. Among all nanostructures, carbon nanostructures have potent properties as drug delivery systems, especially graphene oxide (GO) [13–15]. GO, as a derivation of graphene, can enhance the beneficial properties of natural and synthetic materials due to its unique characteristics, e.g., tensile strength, elasticity, conductivity, and more [16]. Functionalizing GO with natural compounds can increase these agents' local concentration and efficacy [16, 17]. Covalent interaction (peptide bond) between GO and peptides may establish the peptide structure [18, 19]. Due to the antimicrobial activity of GO, functionalized GO with antimicrobial peptides can lead to enhancement of this activity. On the other hand, spermicide-coating condoms are used for effective pregnancy and STIs prevention [20]. Studies showed that spermicide condoms are ~99% effective in preventing pregnancy [21, 22]. Nonoxynol-9 (N-9) is known as the main spermicide. However, regular and spermicide condoms can reduce the risk of STIs, but there is no evidence to show that these condoms increase that protection [23–25]. Using a compound with dual function (a compound with both spermicide and antimicrobial effect) in the design of condoms can double the effectiveness of condoms in preventing pregnancy and transmission of infection [26]. Sarcotoxin Pd has this dual function [1, 2]. Therefore, if the stability of this peptide is somehow increased, its use in condom design can be more effective than the existing condoms. So, this study aims to increase peptide stability by using GO and designing a dual-function coating (spermicidal and antimicrobial) for condoms. The design of a new cover with a double function for condom coating is the innovation of this study.

2. Materials and Methods

Based on previous studies, GO was synthesized using a modified Hummers method [16, 17]. Briefly, graphite powder was mixed with $NaNO_3$ and H_2SO_4 at 0°C. Then $KMnO_4$ was slowly added. Mixture temperature was increased to 40°C in this stage. The acquired solution was diluted with deionized water and stirred for 10 min. The reaction was stopped by adding H_2O_2 (30 wt%). The final mixture was centrifuged, and precipitation was washed with HCl solution and deionized water several times. This solution was dried at room temperature and in vacuum condition. The microwave method was used for GO functionalization to form a covalent bond between GO and peptide. GO powder was dissolved in deionized water and sonicated by probe sonication. Synthesized sarcotoxin Pd was dissolved in phosphate buffered saline (PBS). These two solutions were mixed on a shaker. After 10 min, this solution was irritated in an industrial microwave (with an output power of 700 W) at 150°C for 20 min. Centrifugation was used to remove unbound peptides. Acquired precipitations in this stage were dispersed in deionized water on the shaker. Characterization was done by TEM and FTIR methods. Contact angle measurement was also done by a contact angle analyzer.

2.1. Antimicrobial Assay. The antimicrobial assay was done by two methods: radial diffusion assay (RDA) and absorbance-based methods. One Gram-positive bacterium (*Staphylococcus aureus*), one Gram-negative bacterium (*Escherichia coli*), and one fungal strain (*Candida vulvovaginitis*) were used for antimicrobial evaluation.

In the RDA method, bacteria were cultured for 18 hr at 37°C in 50 ml of tryptic soy agar (TSA) medium (w/v 3%). After culture medium solidification, some holes were created on a medium for sample loading (5 μg of the nanostructure is poured into the well). After 18 hr incubation at 37°C, the antimicrobial effects appear as bright halos around the wells (the diameter of the halo observed around the wells indicates the amount of antimicrobial activity of the samples). The diameter of the created growth inhibition halo was measured and reported as millimeters. A similar procedure was done for the fungal strain on potato dextrose agar (PDA) medium.

In the adsorption method, the bacterial and fungal suspensions were injected into the microplate wells. The first well is considered blank. Each well will be equivalent to 200 μl of solution. In the second well, regarded as a control, 180 μl of culture medium containing bacteria and 20 μl of PBS buffer was injected. In subsequent wells, 180 μl of culture medium was mixed with 20 μl of prepared nanostructure solution. The microplate was incubated for 18 hr at 37°C. After this time, the absorbance of the samples was read at 630 nm by an ELISA reader. The minimum concentration of nanostructures that have stopped bacterial growth is considered minimal inhibitory concentration (MIC). Vancomycin and fluconazole were used as positive control.

2.2. Spermicidal Activity. To evaluate spermicidal activity, acquired washed sperms from 10 healthy volunteers (people with normal sperm count and parameters) were treated with different concentrations of designed nanostructures (1,000, 800, 500, 250, 100, 50, and 25 μg/ml) and sperm motility and morphology were assessed at 0, 0.3, 5, 10, and 15 min. B2 medium and nonoxynol-9 (N-9) were used as a negative and positive control, respectively. The lowest concentration exhibited 100% sperm immobilization during 0.3 min was considered a maximal effective concentration (EC100).

2.3. Peptide Stability Evaluation. The stability of the peptide on the GO structures was evaluated by CD technique after treatment in different environments with different physical conditions. This determination was done by measurement of the CD signal at 220 nm.

3. Results

We prepared flexible nanocoating agents for condom design with antimicrobial and spermicidal activities by functionalized GO with sarcotoxin Pd.

3.1. Characteristics of Design Nanostructure. The results of FTIR are shown in Figure 1. Based on these data, the presence of –COOH groups was confirmed by absorption peak at 1,077 cm^{-1}. The absorption peaks at 3,331, 2,153, 1,958, 1,644, 1,566, 1,073, and 679 cm^{-1} indicated the peptide bond formation between the amine group of sarcotoxin Pd and the –COOH group of GO.

GO and GO-Pd were characterized by TEM, FTIR, and contact angle analyzer. In the TEM image (Figure 2), GO has a multilayered structure with a highly wrinkled shape. These

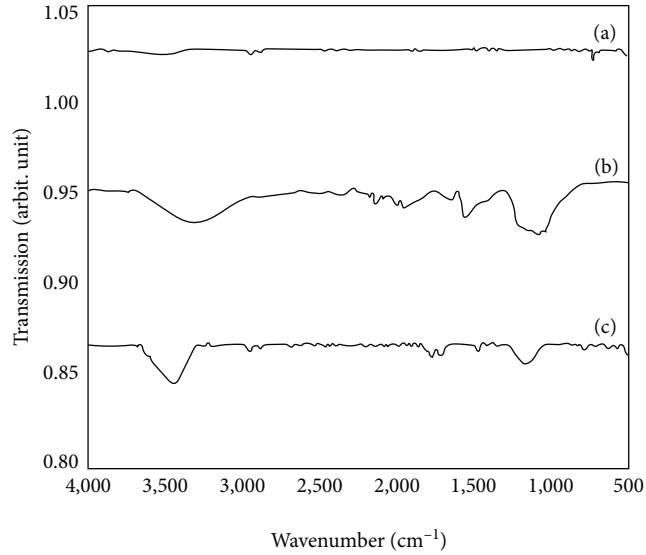
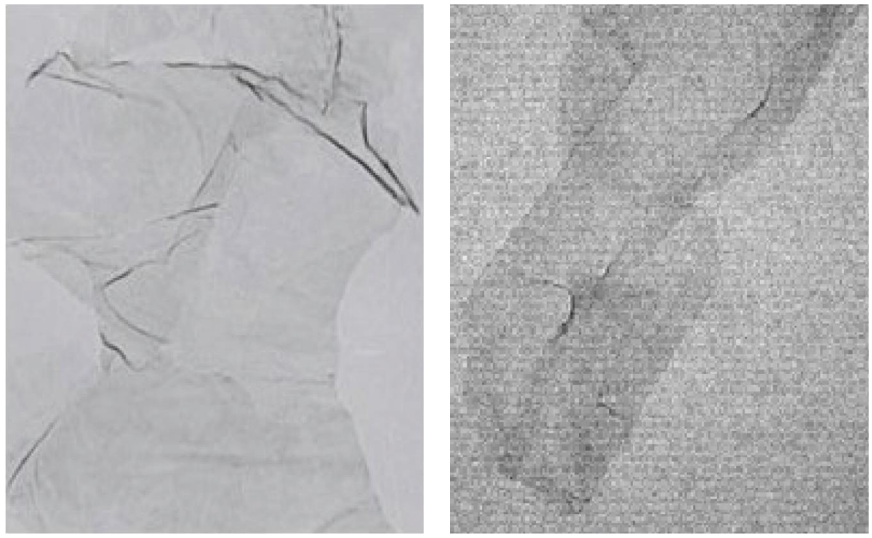


Figure 1: FTIR spectra for synthesized nanostructures, graphene (a); sarcotoxin Pd-functionalized GO (GO-Pd) (b); graphene oxide (GO) (c).



(a) (b)

Figure 2: TEM image of sarcotoxin Pd-functionalized GO (GO-Pd) (a) and graphene oxide (GO) (b).

pd properties were also observed in GO-Pd. In the structure of GO-Pd, the amount of shrinkage is more than GO, so graphene is almost crumpled.

The lowest (20°) and highest angles (150°) were observed for GO-Pd and graphite, respectively (Figure 3).

3.2. Peptide Stability. Peptide stability was evaluated by structure analysis with CD spectra (Figure 4). The change in peptide structure was slightly observed after functionalization on GO. It is indicated that covalent interaction between sarcotoxin Pd and GO leads to partially unfolding induction and change of α-helix structure. However, this helix reduction was insignificant compared to the naked peptide.

3.3. Antimicrobial Activity. RDA test showed that naked peptide, GO, and GO-Pd had antibacterial and antifungal activities. Among these three compounds, GO-Pd had higher microbicidal activity. GO-Pd showed higher inhibitory activity on Gram-positive bacteria, Gram-negative bacteria, and fungus. The sequence for antimicrobial activity was GO-Pd > sarcotoxin Pd > GO for all examined strains. The results for MIC determination by absorbance method conformed to the RDA data. MIC values for GO-Pd were lower than naked peptides and GO. In between microbial strains, the lowest MIC of GO-Pd was observed for *Escherichia coli*, *Staphylococcus aureus*, and *Candida vulvovaginitis*. These data were similar to RDA results (Figure 5).

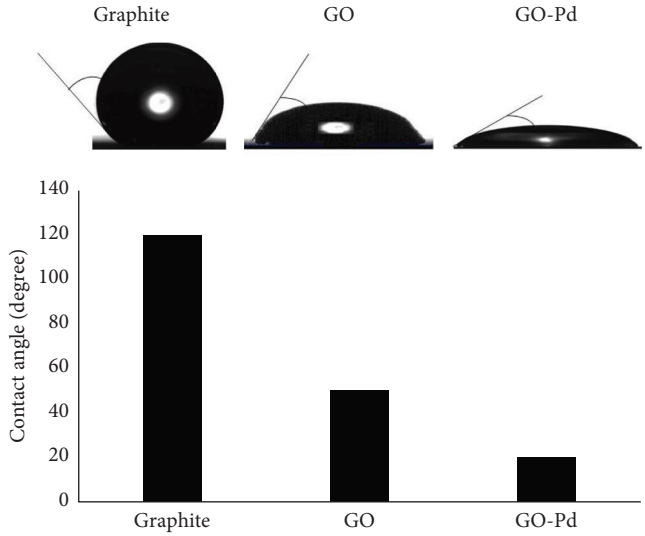


Figure 3: Water contact angle (WCA) between water droplets with graphite, graphene oxide (GO), and sarcotoxin Pd-functionalized GO (GO-Pd).

3.4. Spermicidal Activity. Acquired data indicated that all concentrations of naked peptide and GO-Pd have inhibitory effects on sperm motility. After 0.3 min, the 100% inhibitory concentration (EC100) was 100 and 250 μg/mL for sarcotoxin Pd and GO-Pd, respectively. GO showed no significant effects on sperm motility (Figure 5). Inhibitory effects of sarcotoxin Pd and GO-Pd on sperm motility were dose-dependent. The highest concentration of naked peptides and GO-Pd had the best inhibition. At EC100 of sarcotoxin Pd (100 μg/mL) and GO-Pd (250 μg/mL), peptide immobilization occurred after 30 s (Figure 6).

4. Discussion

Condoms are the most important contraceptive vehicles [27]. The development of new condoms with more effective preventive effects can be beneficial for reproductive health. Using spermicidal compounds in the design and manufacture of condoms can double its effectiveness in preventing unwanted pregnancy [28, 29]. In addition to preventing pregnancy, preventing the transmission of infection between couples during sex is also of double importance [30]. Compounds with dual spermicidal and antimicrobial activity can effectively achieve the two mentioned goals. This study used an antimicrobial peptide, sarcotoxin Pd, for this goal. Based on previous studies, this peptide has antimicrobial effects on various microorganisms, mainly STIs. This peptide also has spermicidal activity in low concentrations [1, 2]. We loaded this peptide on the GO surface. This interaction led to the enhancement of peptide stability in various temperatures and acidic conditions. With this strategy, the limitation of low peptide stability was overcome [3, 4]. N-9 has spermicidal and antimicrobial activity. However, N-9 harms the vaginal epithelium with low effective antimicrobial activity [31, 32]. The physiology of sperm function has not been considered in the design of spermicidal compounds such as N-9 [33]. Paying attention to this issue in the design of contraceptive compounds is vital. In this context, the mode of spermicidal and antimicrobial activity of sarcotoxin Pd has been determined. In addition, our study also considered another positive approach. We used GO for peptide loading and coating fabrication. Graphene condoms are thinner than regular condoms. Due to the high strength of the graphene layer, it is possible to design a thinner condom [34, 35]. This point causes the heat to be better directed, and couples experience more sexual pleasure in addition to ensuring pregnancy prevention. The high flexibility of the graphene layer also makes it easier to use in condoms [36]. Designed GO-Pd includes the mentioned positive points: the presence of a strong, flexible, and thin graphene layer that is functionalized with a spermicidal and antimicrobial compound. Based on reported studies, graphene in rubber latex enhances tensile strength and thermal conductivity. These two changes are useful for the materials for skin-contacting applications, such as male and female condoms [37, 38]. Maintaining the secondary structure of peptides and proteins after loading on nanostructures and other substrates is one of the most important challenges. It is reported that all nanostructures had no similar effects or patterns on structural change of peptides and proteins [39, 40]. For carbon nanostructures, interaction sites, peptide/protein stability, and the shape of nanostructures are primary factors for structural changes after functionalization [41]. Similar articles showed that the interaction of SWCNTs, MWCNTs, graphene, and their derivatives with various peptides/proteins leads to unfolded structures by destroying secondary structures [42–44]. Based on a previous study, structure prediction showed that the secondary structure of sarcotoxin Pd is α-helix with the amphipathic and amphiphilic amino acid arrangement. CD spectra in the current study proved this structure. Our result also showed that GO leads to partially unfolded induction and changes of α-helix structure. However, this helix reduction was not significant compared to the naked peptide. The results of this section are similar to related studies [45–48]. The less spermicidal activity of GO-Pd than sarcotoxin Pd may also be due to this slight change in the peptide's secondary structure after loading GO. The sequence for spermicidal activity was sarcotoxin Pd > GO-Pd > GO. This result can be justified according to the CD results. Sarcotoxin Pd with an α-helical structure has an amphipathic structure with two hydrophilic and hydrophobic faces and a net charge +6. These properties have a crucial role in peptide interaction with sperm membranes and the destruction of sperm cells [1, 2]. Preservation of these two facial α-helical structures after binding to GO led to the spermicidal effect of functionalized GO. A comparison of antimicrobial activity showed a different order than spermicide activity. The sequence for spermicidal activity was GO-Pd > sarcotoxin Pd > GO. According to these data, the higher antimicrobial effect of GO-Pd than sarcotoxin Pd may be due to the synergistic effect of these compounds. The antimicrobial effect of GO and sarcotoxin Pd has been demonstrated in previous studies [1, 2, 49, 50]. The synergistic effect led to a multiplication of antimicrobial effects. Although partial degradation of the peptide structure can reduce its antimicrobial activity, the presence of the antimicrobial effect

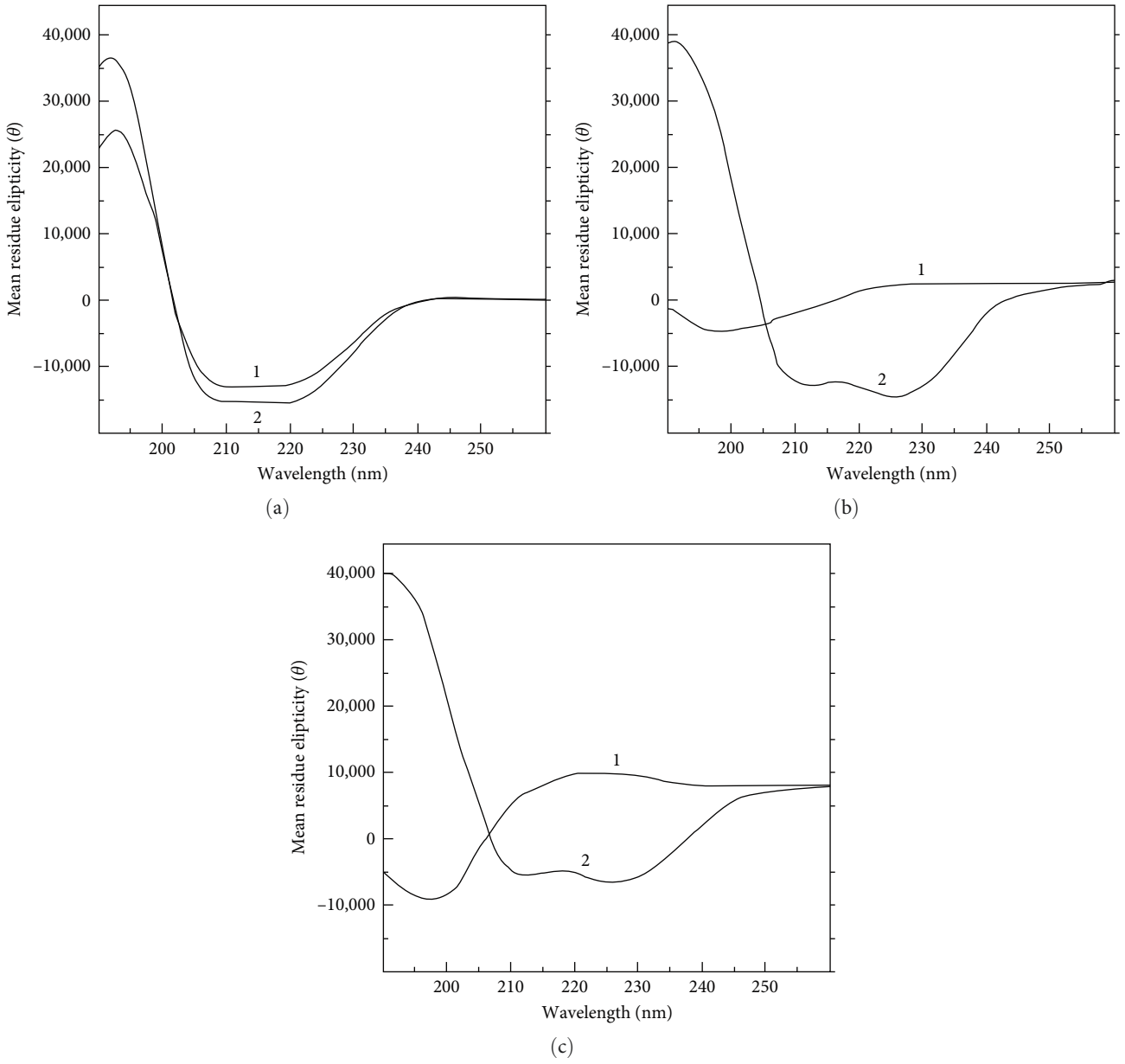


Figure 4: The CD spectra for peptide stability in physiologic conditions (a); high temperature (40°C) (b); and acidic pH (4.6) (c). 1: naked peptide, 2: sarcotoxin Pd-functionalized GO (GO-Pd).

of GO compensates for this reduction. Two facial α-helical structures sarcotoxin Pd also justifies its antimicrobial effect. Like other antimicrobial peptides, sarcotoxin Pd inhibits microbial growth by barrel-stave mechanism [51, 52]. This peptide interacts with negative phospholipids of the membrane by a cationic face and disrupts the membrane with the help of a hydrophobic face. By covalent interaction between sarcotoxin Pd and GO, two faces of the peptide are free for disruption of microbial membrane. So, a similar antimicrobial mechanism is used in naked peptides and sarcotoxin Pd-functionalized GO. However, for sarcotoxin Pd-functionalized GO, the antimicrobial effect of GO is also added and doubles its effect. Graphene and its derivation (i.e., graphene oxide (GO)) have attracted significant research interest in biomedicine due to their excellent physical and chemical properties. There are two critical strategies for a load of peptides on GO: covalent and noncovalent interaction [53]. In our study, covalent interaction was used. Sukumar et al. [34] showed that the functionalization of graphene with the right ingredients (e.g., surfactant/compatibilizer) could increase its dispersion and aid in good interfacial interaction to achieve the desired properties. This point is not excluded from using graphene in the design of condoms. A decrease in contact angle indicates an increase in the moisture content of the compound [54]. In our study, reduction of WCA was proven for GO and functionalized GO. The lowest and the highest angles were observed for graphite and GO-Pd, respectively. Functionalization of GO with sarcotoxin Pd led to a significant increase in wettability. So, the hydrophilicity of GO and GO-Pd is higher than graphite. This hydrophilicity

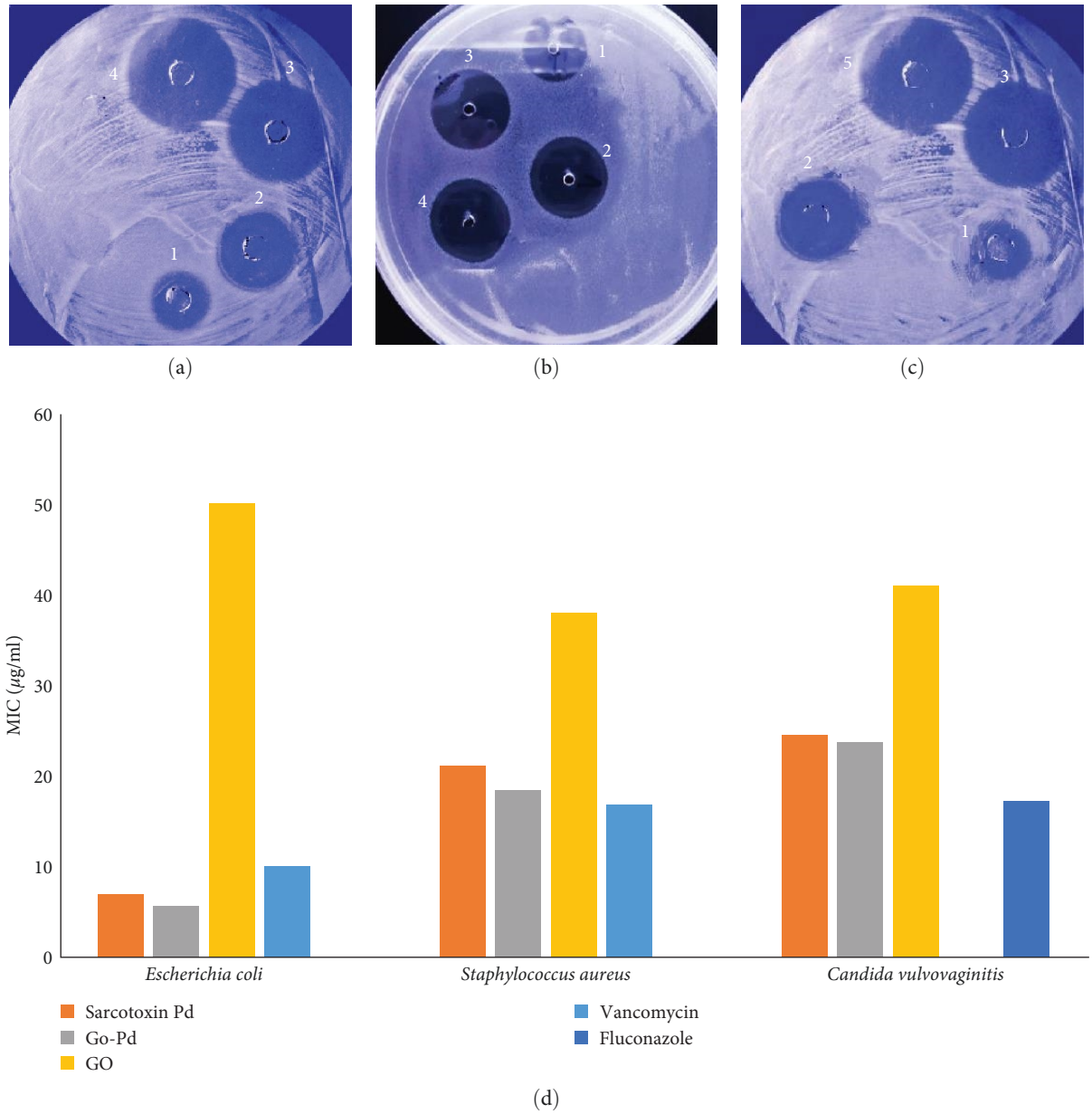


Figure 5: Antimicrobial activity of the naked peptide, graphene oxide (GO), and sarcotoxin Pd-functionalized GO (GO-Pd) on examined pathogens by RDA (a–c) and MIC test (d). 1: GO, 2: sarcotoxin Pd, 3: GO-Pd, 4: vancomycin, and 5: fluconazole. (a) *Escherichia coli*, (b) *Staphylococcus aureus*, and (c) *Candida vulvovaginitis*.

improves biological and biomedical applications [3, 14, 55]. Structural stability and changes are the most critical challenge in the functionalization of nanostructures with peptides and proteins [52, 56]. This high wettability of the designed GO-Pd also increases the better lubricating properties of the condom.

5. Conclusion

The main aim of the study was to enhance the stability of the antimicrobial peptide, sarcotoxin Pd. This aim was achieved. GO-Pd has higher stability than a naked peptide. Other related purposes were also completed. Our results showed that GO-Pd has strong antimicrobial and spermicidal activities. These designed nanostructures also have suitable flexibility and malleability. So, our study showed that GO-Pd could be used as a condom coating for creating a new spermicidal and antimicrobial condom. This new condom can effectively prevent unplanned pregnancies and STIs. Although GO has better solubility in a polar solvent such as water than graphene, the low solubility was one of the main challenges in using GO. Functionalization with peptide led to the higher solubility of GO. The high price of peptide purification is also one of the significant problems. To solve this challenge, in this study, we used synthesized peptides. The use of polar functional groups can lead to an increase in GO solubility. Therefore, it is suggested to use polar groups-functionalized GO to load peptides and investigate their antimicrobial and spermicidal effects in future

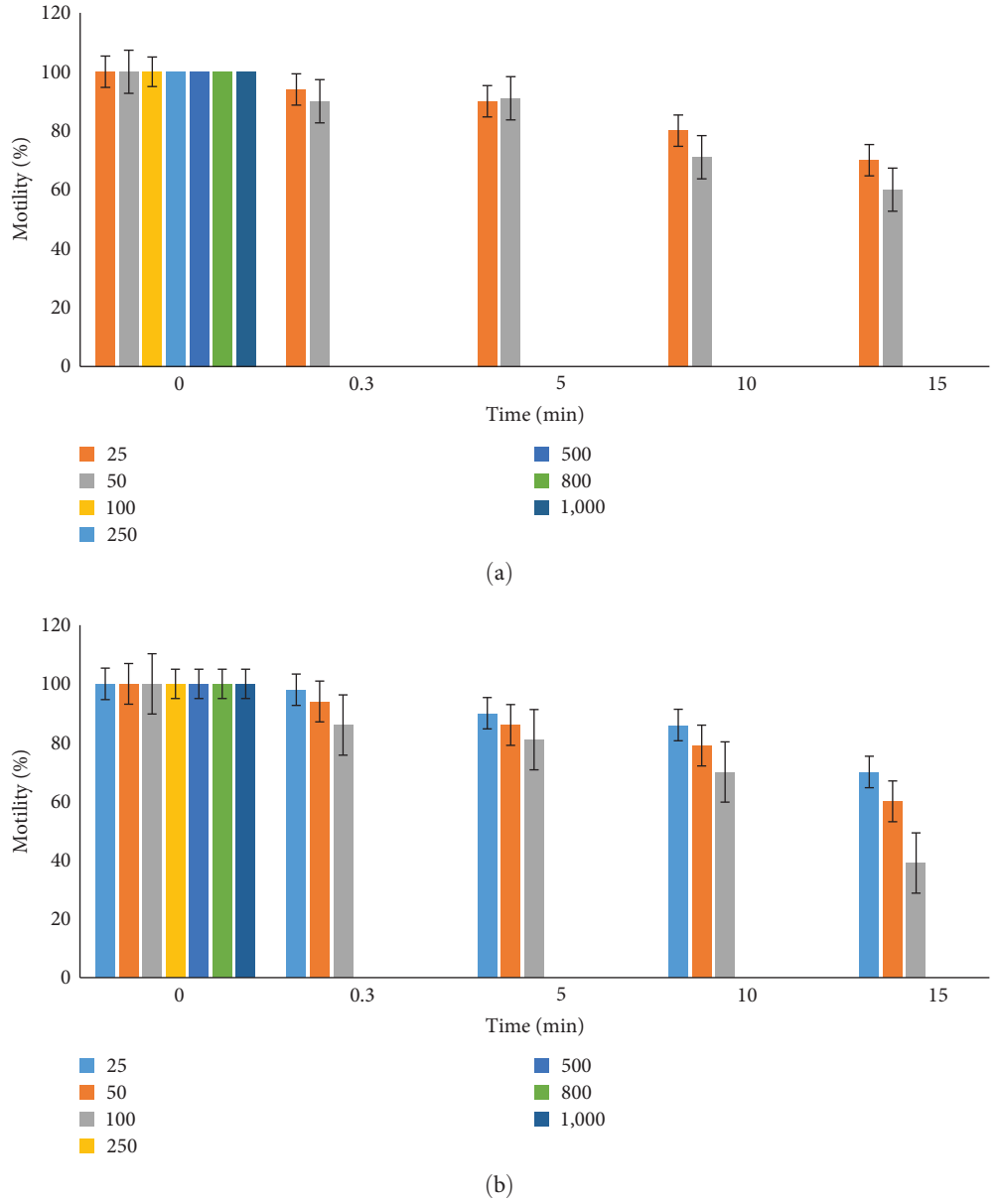


Figure 6: Sperm motility after treatment of sarcotoxin Pd (a) and sarcotoxin Pd-functionalized GO (GO-Pd) (b). At EC100 of sarcotoxin Pd (100 μg/mL) and GO-Pd (250 μg/mL), sperm immobilization occurred after 30 s.

research. It is also recommended to examine the impact of changing the amino acid sequence of the peptide on its antimicrobial and spermicidal activities and its stability through bioinformatics and computer modeling and use the designed peptide to make the condom coating.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

The study was approved by the Ethics Committee of Abadan University of Medical Sciences, Abadan, Iran (AIR.ABADA-NUMS.REC.1399.215).

Disclosure

The abstract of this study has been presented as conference in "Proceedings of the Fourth National Conference on Protein and Peptide Sciences" according to the following link: https://conf.ui.ac.ir/fa/article.php?lrId=40&cnfId=12.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

In this study, all authors contributed to the manuscript's design, writing, and review. HZZ contributed to experimental sections, including material synthesis and animal

treatment. AA led the execution of laboratory and fieldwork for the entire project and managed and supervised the experiments and results. MZ contributed to the biological assessment of nanostructures and article editing. HS did the synthesis of nanomaterial and biological tests. HG contributed to material synthesis. MF did the statistical analysis of the data. SAH contributed to biological tests as well as article editing. ND contributed to data collection and analysis.

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