THE DEVELOPMENT OF ALGINATE-BASED MATRICES LOADED WITH RIFAMPICIN FOR WOUND HEALING

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THE DEVELOPMENT OF ALGINATE-BASED MATRICES LOADED WITH RIFAMPICIN FOR WOUND HEALING (Abstract): The proposed study aims to develop polymeric systems based on alginate and N-vinylcaprolactam (NVCL) coated with poly(D,L-lactide-co-glycolide) (PLGA) as vehicles for Rifampicin (RIF) as active substance. Rifampicin is an agent used for tuberculosis treatment, but the present study proposes to be used for wound healing. The proposed topic is a complex study comprising a lot of data; herein information on Rifampicin loaded matrix and its antimicrobial activity against the main strains of the following bacteria: S. aureus, MRSA, E. coli, P. aeruginosa. Materials and methods: Alginate and NVCL were used as main materials for polymer preparation then coated with PLGA; the polymeric matrix was loaded with Rifampicin as active substance. The preparation of polymer consisted of various steps such as polymerization technique, water in oil in water emulsification and then matrix lyophilization for characterization in solid state. Chromatography was used to indicate the presence of the drug (TLC) and then HPLC was used to quantify the amount of it. The antimicrobial activity was tested by culturing the tested substances against some bacteria e.g. S. aureus, MRSA, E. coli, P. aeruginosa. Results: The tested matrix showed positive results in terms of drug loading and antimicrobial activity and will be further investigated for IC50 and cytotoxicity. Conclusions: Based on the obtained results, the proposed rifampicin-loaded alginate-based matrix shows potential in wound treatment, including surgical wound care. Keywords: RIFAMPICIN, ALGINATE MATRICES LOADED, WOUND HEALING.

Advancements in medical technology have led to improvements in patient care, compliance, and outcomes. Significant strides have been made in the development of materials used during or after surgical procedures. These procedures typically
Involving incisions with specialized instruments in an operating theatre, often requiring anesthesia and/or respiratory support. Surgical materials, including adhesives, sealants, hemostatic agents, wound covers, absorptive sponges, and sutures, are now expected to meet specific criteria. They should be non-toxic, biocompatible, and supportive of cellular proliferation for tissue regeneration, while also possessing sufficient mechanical and physical properties for durability. Naturally sourced biopolymers, which are biological macromolecules metabolized within the body, have gained recognition for their compatibility with the biological environment. Unlike synthetic polymers, they typically do not provoke chronic inflammatory or immunological reactions or toxicity due to their similarity to the extracellular matrix. Furthermore, modifying the structure or processing conditions of biopolymers can enhance their functionality, physical-mechanical properties, regenerative capabilities, adsorptive properties, and more. Biopolymers, which are naturally occurring polymers, are typically produced by living organisms and can be sourced from microbial systems, extracted from plants, or synthesized chemically from basic biological systems. They offer several advantages over synthetic polymers, including a well-defined and more intricate structure, biocompatibility, functionality, degradability, and renewability. However, they also have drawbacks such as poor mechanical properties, lower producibility, and a dependence on various environmental factors. Nonetheless, with appropriate functionalization, their properties can be significantly enhanced. The most utilized biopolymers in surgical materials include chitosan, alginate, hyaluronic acid as polysaccharides, collagen, gelatine, fibroin as proteins, and biocompatible polymers like polylactic acid derivatives. In the subsequent section, we will outline the general properties of these key biopolymers and their roles in surgical applications. Alginate, a polysaccharide extracted from brown seaweeds, possesses distinct physical properties that make it valuable as a rheology modifier in various applications such as food products, paper consumables, printing inks, and biomaterials for medical and pharmaceutical preparations. Its biocompatibility and biodegradability have sparked significant research interest. Like chitosan, alginites are copolymers comprised of repeating units of guluronic acid (G) and β-D-mannuronic acid (M), linked via 1,4 glycosidic bonds. In molecular terms, they are C5 epimers of each other. The orientation of the carboxyl group (−COOH) on the C5 carbon of the six-membered saccharide ring is above the plane of the ring in the M unit and below the plane in the G unit. The polymer chain of alginate contains blocks of guluronic acid, mannuronic acid, and alternating sequences thereof. The viscosity and gel-forming capacities are key characteristics of alginate, heavily influenced by the block structure and chain length.

Among the polymers exhibiting a Lower Critical Solution Temperature (LCST) close to physiological conditions (32-34°C) are poly(N-vinylcaprolactam) and poly(N-isopropylacrylamide), rendering them suitable for biomedical applications. Poly(N-vinylcaprolactam) (PVCL) is a water-soluble, non-toxic, thermosensitive, and biocompatible polymer categorized under N-vinylamide polymers. In comparison, although poly(N-isopropylacrylamide) (PNIPAAm) is extensively studied as a thermosensitive polymer, PVCLC exhibits even higher biocompatibility due to the
The development of alginate-based matrices loaded with Rifampicin for wound healing

direct linkage of the amide group to the hydrophobic backbone chain (C–C). Consequently, the hydrolysis of the amide group in PNVCL does not yield small amide compounds, which are undesirable for biomedical applications and limit the biocompatibility of PNIPAAm (1, 2, 3).

The combination with biocompatible monomers such as N-vinylcaprolactam with alginate can lead to functional biocompatible materials which impart both the characteristics of alginate and NVCL. The present study aimed to prepare and characterize the nano/microparticles of alginate grafted from NVCL and covered with a shell of PLGA for applications like wound healing, particularly, healing after a surgical operation. Within the present study, there will be reported the loading of the selected model drug i.e. Rifampicin and the antimicrobial activity against 4 strains of bacteria such as S. aureus, MRSA, E. coli, P. aeruginosa.

MATERIALS AND METHODS

Chemicals

Alginic acid (AgA, Sigma Aldrich product) was utilized, along with N-vinylcaprolactam (NVCL), as monomer, achieved from Sigma Aldrich. The initiator system comprised ammonium persulphate (APS, Sigma Aldrich) and 50% hydrogen peroxide solution. The reaction was carried out in ultra-pure distilled water as the solvent. To create the nanoparticle suspension, a 0.5 wt.% acetic acid glacial solution was employed. Purification of the copolymer was achieved using a dialysis bag with a cut-off of 3.5 kDa. Rifampicin (RIF), 99% purity, with a molecular weight (Mw) of 822.95 g/mol sourced from S.C. Antibiotice S.A. Iasi, Romania. Poly(D,L-lactide-co-glycolide) 50:50 (PLGA), Sigma Aldrich product was used to form the Nano/microparticles. All other chemicals and reagents used in this study met analytical grade standards or exceeded them and were procured commercially.

Preparation of grafted biopolymer

The grafted biopolymer i.e. alginate grafted N-vinylcaprolactam (NVCL) was synthesized by using the free radical polymerization method in water by using as initiating systems of azobisisobutyronitrile (AIBN) under nitrogen flow within a ratio of 0.1% against the monomer (NVCL). The proposed mechanism was represented within the Fig. 1. The structure identification was assessed by FTIR spectroscopy.

![Fig. 1. The proposed mechanism for the synthesis of the polymeric matrix](image-url)
Preparation of PLGA coated alginate grafted from NVCL

The prepared biopolymeric matrix was used further to synthesize nano/micro-particles without and containing Rifampicin, the selected drug model for the postsurgery wound healing.

An amount of prepared biopolymer was dissolved in water within a concentration of 0.1 wt.% and then Rifampicin was added to the polymeric solution to ensure a good blending and formation of electrostatic bonds with the hydrophilic groups of the biopolymer. The theoretical amount used for the Rifampicin loading within the matrix was 0.5 wt.% against the polymer amount. The mixture was stirred for couple of hours then lyophilized.

The biopolymeric matrix without Rifampicin was dissolved in twice distilled water within a concentration of 0.1 wt.%. The oil phase solution was prepared by dissolving 0.1 g PLGA (50/50) in dichloromethane (DCM) and Span 80 was added to ensure a good stability of the emulsion. The oil phase was probe sonicated for 10 minutes then the water phase was added dropwise under strong stirring under vortex to prepare water-in-oil emulsion. The first emulsion was again probe sonicated then added dropwise in the second water phase consisting of 5 wt.% polyvinyl alcohol low molecular weight, Tween 80 and salt to give the final water-in-oil-in water (W/O/W) dispersion. The solvent was evaporated at room temperature, and the system was kept under stirring overnight. The final dispersion was then centrifugated at 15000 rpm under a temperature of 5°C to collect the nano/micro-particles of N-vinylcaprolactam grafted alginate (AgA-g-NVCL) coated with PLGA. The schematic representation of the procedure was illustrated within second figure.

The formation of particles containing Rifampicin was performed similarly except the fact that in the water was dissolved the matrix loaded with Rifampicin. The drug content was determined by means of high-performance liquid chromatography (HPLC).

Fourier transform infrared spectroscopy (FTIR) analysis

The structure was confirmed by FT-IR spectra of the copolymer films which were recorded with a Perkin-Elmer Spectrum-100 ATR-FTIR instrument by scanning in
The development of alginate-based matrices loaded with Rifampicin for wound healing

the range of 600-4000 cm⁻¹.

**Thin layer chromatographic chemical screening (TLC)**

Thin Layer Chromatography (TLC) was used to separate components in the analyzed compounds. After separation, each compound shows horizontal bands at different points; each band has a specific retention factor (Rf) which be determined by using equation (1):

\[
R_f = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent}} \quad (1)
\]

The biopolymer loaded Rifampicin was dissolved in ethanol with a concentration of 1 mg/mL. Chloroform: methanol (9:1) solvent mixture used as mobile phase to screen and determine the separation. Approximately 10 mL of mobile phase was placed into a rectangular glass tank and covered with a glass lid; the solvent-based mixtures were allowed to saturate for 10 min before use. 2 μL of each sample were placed with a capillary tube on the DC Kieselgel 60 F₂₅₄ silica gel (0.2 mm thickness was purchased from Merck), total 5 drops. As a reference sample was used the pure drug marked as standard and placed onto silica gel as well. After separation, spots were detected at 254 nm.

**HPLC method**

The amount of Rifampicin was analyzed by using an Agilent 1260 high-pressure liquid chromatograph (HPLC) system. The system was equipped with UV detector and Chrompack C18 (250×4.6 mm) column, Santa Clara, CA, USA. UV detection was performed at 238 nm for Rifampicin and the HPLC conditions were applied according to the HPLC method developed by Chellini et al. (4). Gradient elution was carried out with a mobile phase A of 20 mM monobasic sodium phosphate buffer with 0.2% triethylamine (pH 7.0) and acetonitrile and mobile phase B was acetonitrile, at a flow rate of 1.5 mL/min. The total run time was 12 min, and the re-equilibration time was 5 min. Gradient elution was carried out as follows: 100% mobile phase A was first held for 5 min, then mobile phase B was raised up to 52% in 7 min, mobile phase B was held at this level until 12 min, and at 12.01 min, mobile phase A was switched back to 100% until 17 min (re-equilibration). The flow rate was 1.5 mL/min, and the injection volume was 25 μL. Limit of detection (LOD) and limit of quantification (LOQ) were evaluated. For the determination of Rifampicin amount, a calibration curve was done by preparing serial standard solutions within a concentration range of 0-1000 µg/mL and recorded the absorbance obtained at 238 nm, specific for RIF. For this purpose, approximately 20 mg polymeric matrix loaded with Rifampicin was weighted, then sonicated and dissolved in 250 μL CH₃OH; the solution was filtrated and 100 μL were transferred to a new vial and injected 25 μL into the chromatographic column.

**Antimicrobial activity**

There were used 4 bacterial strains (S. aureus, MRSA, E. coli, P. aeruginosa) isolated from patients with common infections. The pure and fresh culture (18 hours) of each of the 4 bacterial strains were tested against Rifampicin-loaded nanoparticles (RNPs), Rifampicin pure substance (RIF) and unloaded nanoparticles (NPs), following the Kirby-Bauer disk diffusion protocol (10⁸ CFU/mL bacterial suspension, homogeneous inoculation of Mueller Hinton blood
agar (5). The tested antimicrobial substances were prepared to respect the classical antibiotic discs: 6 mm in diameter. The diameter of inhibition area was measured in mm, in transmitted light, after 18-24 hours incubation at 35°C.

RESULTS AND DISCUSSION

Matrix characterization

The structure of the synthesized biopolymer was analysed by means of FTIR spectroscopy and FTIR spectrum of the prepared polymer was represented within fig. 3.

![Fig. 3. FTIR spectrum of AgA-g-NVCL](image)

During the synthesis procedure, NVCL as monomer, containing vinyl groups was polymerized and connected with alginate via ester bonds. Exploration of the FTIR spectra of the final grafted copolymer is in fact verifying whether the monomer has transformed into polymer and ensuring that the characteristic peak of vinyl groups at 1626 cm\(^{-1}\) attributed to the \(-\text{C}=\text{C}-\) stretching vibration has disappeared, showed that polymerization occurs by opening the carbon–carbon double bond without any change in the caprolactam ring. The peaks at 2926 cm\(^{-1}\) and 2856 cm\(^{-1}\) correspond to the aliphatic C–H stretching. The peak of C–N stretching vibration was observed at 1421 cm\(^{-1}\) and the peak at 3282 cm\(^{-1}\) can be assigned to N–H stretching vibrations. Similar results were found in the case of a NVCL homopolymerization, the spectrum showing similar trendline (6).

Thin layer chromatography

chemical screening (TLC)

The qualitative assessment of the drug loading was performed by TLC chemical screening. The loaded sample was compared with the pure drug as standard. The Fig. 4 described the UV images recorded at 254 nm of the sample with Rifampicin and pure Rifampicin as standard. Using a silica gel plate and ethyl acetate as the development solvent, the pure drug as standards and by comparison, the loaded sample with Rifampicin showed spots having Rf's of 0.55 and 0.58, and 0.52 the latter one. Comparing these Rf polymeric matrix loaded with Rifampicin with the Rf's of standard, a similarity between the elution spots could be observed; the difference being just in the intensity due to the concentration differences.
The development of alginate-based matrices loaded with Rifampicin for wound healing

Considering the hydrophobic character of Rifampicin, the identification of the spot belonging to the drug was facile, the results confirming the presence of Rifampicin within the polymeric matrix. Similar results were found by Abdelwahab et al. (7), where values of 0.44 for Rf were determined but similar trendline of elution column.

**Fig. 4.** The chromatographic separation of the standard substance (Rifampicin) and the loaded matrix with Rf (left). UV images at 254 nm

**HPLC method**

The quantitative assessment of the drug loading was performed by HPLC analysis by using the method described with the section 2. The retention time of Rifampicin using the described method was found to be around 12.6 minutes (fig. 5).

**Fig. 5.** Representative chromatogram of Rifampicin with its characteristic peak and the calibration curve based on HPLC analysis
The determined limit of the detection LOD and limit of quantification LOQ were found to be 0.7132 and 2.1623 μg/mL respectively. The amount of Rifampicin found in 20 mg was determined by using the calibration curve of being 160 μg/mL, a 26.66% loading as compared with the amount loaded initially. This result was considered for further studies in vitro (e.g. cytotoxicity, IC50 determination, etc.).

**Antimicrobial activity**

*S. aureus, MRSA, E. coli, P. aeruginosa* are bacterial strains with a high potential to produce infections difficult to be treated, if only *mecA* gene of *S. aureus* and extended-spectrum β-lactamase (ESBL)-producing *E. coli* is good to be mentioned. Other reports are mentioning the high prevalence of infected wounds with similar strains as we tested (8, 9, 10), which underline the importance of developing new therapeutical strategies. Our results are promising, but to be implemented in clinical practice we should validate our developed therapeutical drugs using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline (11).

The antimicrobial activity was evaluated and RNPs was found to have a good antimicrobial action against all 4 microbial strains. Fig. 6 described the images of cultured blood agar plates inoculated with *S. aureus, MRSA, E. coli, P. aeruginosa*, tested at RNPs, RIF and NPs.

The diameters of inhibition areas are summarized in first table.

As observed both from table I and figure 6, the greatest area of inhibition is against MRSA and comparable to those against *S. aureus* and *P. aeruginosa*. NPs have a good antimicrobial action against MRSA and *E. coli*, which recommends this type of matrix for its use in therapy after loading with antimicrobial substances, to which it will give added efficacy. It should be noted that on *P. aeruginosa*, neither Rifampicin has antimicrobial action and even less so NPs, which was to be expected, while RNPs have good antimicrobial action, and so they are particularly valuable for use in healing superinfected wounds or for preventing superinfection in immunocompromised patients.

![Fig. 6. Images of blood agar plates inoculated with S. aureus, MRSA, E. coli, P. aeruginosa, tested at RNPs, RIF and NPs](image-url)
The development of alginate-based matrices loaded with Rifampicin for wound healing

**TABLE I.**
The diameters of inhibition areas for the Rifampicin-loaded nanoparticles (RNPs) comparative with the Rifampicin pure substance (RIF) and the unloaded nanoparticles (NPs)

<table>
<thead>
<tr>
<th></th>
<th>RNP</th>
<th>RIF</th>
<th>NPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>S. aureus</td>
<td>35</td>
<td>40</td>
<td>complete inhibition</td>
</tr>
<tr>
<td>MRSA</td>
<td>41</td>
<td>45</td>
<td>1/2 inhibition</td>
</tr>
<tr>
<td>E. coli</td>
<td>24</td>
<td>26</td>
<td>30</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>32</td>
<td>36</td>
<td>-</td>
</tr>
</tbody>
</table>

RIF was expected to have very good efficacy, as proven on *S. aureus* (complete inhibition) and *MRSA* (1/2 inhibition), and against *E. coli*, but the side effects of high concentrations of Rifampicin are not to be neglected and therefore RNPs are a viable and highly effective alternative in the care of postoperative wounds/sores/burns in general.

In all cases the zone of inhibition increased slightly after 48 hours, which indicates maintenance of the antimicrobial activity for a long time, from which it follows that the application to the wound of the pharmaceutical form with RNPs (as ointment, dressing, patch, etc.) can be done at a long interval and will thus improve patient compliance to proper wound care.

**CONCLUSIONS**
The study had the purpose to prepare materials suitable for the treatment of post-surgical wounds by using Rifampicin as the active substance. The first step within this complex study was to select a proper matrix, i.e. polymeric matrix which has the role not only to protect the active substance but also to release it at the target site without creating a burst delivery. Moreover, the matrix should be biocompatible and non-toxic for the human body. Considering the data from literature a copolymer of alginic acid and N-vinylcaprolactam seemed a good choice and it was tested from the perspective of loading and matrix compatibility with Rifampicin and its antimicrobial activity against very powerful bacteria commonly found in post-surgical infections (e.g. *S. aureus*, *MRSA*, *E. coli*, *P. aeruginosa*). Rifampicin loading in the PLGA-coated alginate and PNVCL copolymer matrix was demonstrated qualitatively by TLC and quantitatively the drug loading was found to be 26.66% using the HPLC method. As regarding its antimicrobial activity, it was found out that the inhibition against the bacterial agents increased slightly even after 48 hours, indicating the persistence of the antimicrobial activity for a long time, making the systems suitable for wound healing, including post-surgical wound treatments, when this time of 48 hours is extremely important.

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