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REVIEW PAPER

Recent advances in nanocarriers containing Bromelain: *In vitro* and *in vivo* studies

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ABSTRACT

Medicinal products of plant origin have long been considered the most affordable and accessible sources to treat different health problems. Bromelain (Br) is a mixture of enzymes derived from pineapple (*Ananas comosus* L.) with a wide field of applications including medicine, health, food, and cosmetics. Br has various therapeutic effects, such as antimicrobial, antioxidant, anticancer, wound healing, burn treatment, pain relief, anti-inflammatory, inhibition of platelet aggregation, and fibrinolytic activity. On the other hand, most proteins are susceptible to denaturation and structural changes that may reduce their activities. Encapsulation of drug molecules into nanoparticles (NPs) could increase their stability, bioavailability, and overcome other challenges in drug delivery and therapy. This review aimed to highlight various Br nano-formulations approaches, toward the improvement of Br therapeutic efficiency.

Keywords: *Bromelain, Nanoparticles, Pineapple, Stability, Therapeutic application*

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INTRODUCTION

Medicinal products of plant origin have long been considered the most affordable and accessible sources to treat different health problems. Bromelain (Br) is a mixture of enzymes such as proteases, phosphatases, and glucosidases, and compounds like glycoproteins and carbohydrates extracted from the stem and fruit of pineapples (*Ananas comosus* L.) [1]. Br has various therapeutic effects like antimicrobial, antioxidant, anticancer, wound healing, burn treatment, pain relief, anti-inflammatory, inhibition of platelet aggregation, and fibrinolytic activity. It could reduce the risk of cancer, diabetes, and various cardiovascular diseases in the long term. It can also increase bioavailability and reduces the associated side effects of antibiotics. Br also supports the immune system and elicits anti-inflammatory response results while used as an anti-metastatic and anti-edema medicine [2].

The storage and stability of Br, rich in papain and cysteine protease, is an important issue to enhance its bioavailability and efficiency [3]. Several methods have been reported for enzyme stabilization, including enzyme chemical modifications, protein engineering techniques, use of compatible osmolytes, and nanocarriers [4, 5]. Encapsulation of drug molecules into nanoparticles (NPs)

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could increase their stability against enzymatic denaturation and prevent their rapid clearance resulting in bioavailability enhancement. In addition, nanocarriers have the ability to overcome other challenges in drug delivery and therapy, including poor solubility, poor permeability, and high toxicity. NPs can also deliver drugs to target tissues in a controlled-release manner. Their small size and high surface-to-volume ratio allow them to easily enter cells and interact with biomolecules [6]. This review aimed to highlight various Br nano-formulation approaches toward improvement of Br therapeutic efficiency.

Research methods

The literature search was performed with PubMed and Google Scholar using the keywords nanoparticles, bromelain, and therapeutic applications. In this search, only articles related to nanobromelain and its medicinal and therapeutic effects were discussed and other articles were excluded. This search was carried out between 1990 and 2022 and 54 articles met the inclusion criteria.

Importance of bromelain in nanostructures

Enhancing the storage and operational stability of an enzyme is an important consideration for its therapeutic applications, as most proteins and/or enzymes are fragile, and even small structural changes may decrease their activity. Nanoparticles (NPs) as drug delivery systems could stabilize active ingredients against denaturation and increase their biopharmaceutical applications [7]. Br was applied to synthesize NPs, overcome the mucus barrier against NPs, and enhance their stability, particle uptake, and activities [8-13]. In Table 1, the importance of Br in some nanostructures has been summarized.

Silica nanoparticles

Mesoporous silica nanoparticles (MSNs) are biocompatible materials, with large surface area and pore volume, providing great potential for drug adsorption and loading in their pore channels. In a study by Parodi *et al*., the surface of MSN was functionalized by Br and its diffusion efficiency in the tumor extracellular matrix was evaluated. In *in vitro* studies, Br-MSN increased particle uptake of NPs in endothelial, macrophage, and cancer cell lines with minimal effect on cellular viability. In addition, these modified NPs showed higher diffusion throughout the tumor mass in breast cancer-bearing BALB/c mice compared with unmodified MSN [8].

Gold nanoparticles

Gold NPs with the possibility of surface functionalization have presented various biomedical applications. Despite their advantages, the common production methods might have an important effect on their toxicity and instability properties. Therefore, the search for the synthesis of new gold NPs molecules has recently been taken into consideration. It was shown that Br-capped Gold NPs containing levofloxacin and covered by Br could enhance the levofloxacin antibacterial effects compared with drug molecule alone against *Staphylococcus aureus* and *Escherichia coli* [9]. Khan *et al*. proposed a novel method in which Br was used in the synthesis of Gold NPs and effectively controlled the size of those NPs. Also, the gold NPs synthesized by this method were stable for several months at room temperature (10).

PAA nanoparticles

Poly (acrylic acid) (PAA) is a biocompatible hydrophilic polymer that can be chemically modified to create particulate systems [14]. In a study by de Sousa *et al*., Br and papain as mucolytic enzymes were covalently conjugated to PAA through carbodiimide coupling reaction, and NP formulation was synthesized using

Table 1. Bromelain in nanostructures

Nanostructures	Role of bromelain	Ref.
Mesoporous silica NPs	Increased diffusion of NPs in the tumor extracellular matrix	[8]
Gold NPs	Enhanced antibacterial effect of levofloxacin	[9]
	Control the size and stabilize Gold NPs	[10]
PAA NPs	Enhanced mucus permeating properties of NPs	[11]
PLGA NPs	Enhanced permeation of NPs to intestinal mucus	[12]
Chitosan NPs	Decreased cohesiveness and instability index of the NPs	[13]

NPs: Nanoparticles; PAA: Poly acrylic acid: PLGA: Poly (lactide-co-glycolide) acid

the ionic gelation method. The maintained enzymatic activity was 43% and 76% for PAP and Br, respectively. In addition, Br-loaded NPs showed higher potential than PAP NPs as mucus permeating drug delivery systems [11].

PLGA nanoparticles

Poly (lactide-co-glycolide) acid (PLGA), a copolymer of lactic and glycolic acid, is one of the most widely used synthetic polymers for biomolecule encapsulation. Suitable biocompatibility, biodegradability, safety and controlled release kinetics properties of PLGA led to its FDA approval. The most common method used to prepare PLGA NPs is the double emulsion solvent evaporation method using dichloromethane or ethyl acetate as the polymer solvent [14, 15]. PLGA NPs were functionalized with proteolytic enzymes including trypsin, papain, and Br through the two-step carbodiimide coupling reaction. The results showed that the enzymatic activities of conjugated enzymes were preserved. The synthesized NPs effectively disrupted the gel structure of mucin, confirming the permeability of enzyme-functionalized NPs. NPs with papain and Br showed higher permeation to porcine intestinal mucus (three-fold) in comparison whit NPs with trypsin (two-fold increase) [12].

Chitosan nanoparticles

Chitosan is a mucopolysaccharide obtained by deacetylation of chitin, the main component of the exoskeleton in crustaceans [16]. Different methods can be used to produce chitosan NPs like emulsion, ionic gel, reverse micellar, and self-assembly. These methods can directly affect particle size, particle formation, and aggregation [17]. Ataide and Gerios in 2019 synthesized Br-chitosan NPs and evaluated the effect of polysaccharide sources of chitosan on its physiochemical properties. They showed that more than 79% of Br enzymatic activity remained in Br-chitosan NPs. The highest encapsulation efficiency was seen with the low molecular weight of chitosan. Moreover, adding Br to chitosan, resulted in decreased cohesiveness of the NPs' suspension as well as demonstrating a low instability index (<0.3) [13].

Therapeutic applications of nanoparticles containing bromelain

Encapsulation of drug molecules into NPs could increase their solubility, stability, and permeability as well as prevent their rapid clearance resulting in enhanced bioavailability. NPs can also deliver drugs to target tissues in a controlled release manner [6].

Mala and Anal employed hydrogel beads based on starch and pectin to provide Br gastrointestinal delivery. They used the extrusion gelation method for thermal processing protection. Using this complex resulted in the controlled and extended release of Br [18].

In a study by Britto and colleagues [19], Br that was immobilized on gold NPs promoted its partial digestion of type I collagen membrane, but in a differentiated way concerning the free enzyme. In addition, Br- gold NPs showed a significant increase in sensitivity to inhibition by protease inhibitor E-64. Therefore, immobilization of Br in gold NPs resulted in higher stability and specific activity.

In the following, it will highlight various Br nano-formulations approaches, toward the improvement of Br therapeutic efficiency.

Wound healing and burn treatment

TWound healing consists of several stages, starting with inflammatory responses, followed by proliferative and maturation stages. The enzymatic debridement effect of Br has been proven [20-22]. Br could degrade collagen, elastin, laminin, and fibronectin, as well as damaged extracellular matrix components using Escharase which has no hydrolytic enzyme activity against normal proteins. The protective effect of Br on the skin with burn wounds and the effective elimination of eschar is probably due to its escharase activity [23]. In addition, there are other mechanisms involved in removing necrotic cells using Br, including the modulatory effect on the expression of tumor necrosis factor-alpha (TNF-α) and transforming growth factor beta (TGF-β) [24]. Ghensi and Cucchi found out that Br could activate mesenchymal stem cells and increase the activity of IL-10 hence promoting anti-inflammatory and wound-healing effects [25, 26]. The fibrin clot on the wound and the damaged components of the extracellular matrix such as collagen, elastin, and laminin are hydrolyzed by Br proteases and lead to release of growth factors and angiogenesis in the matrix and activation of chemokines and bioactive cytokines [27, 28].

To date, numerous studies have been conducted to increase the stability of Br and

improve its burn treatment activity. Miranda *et al*. synthesized membranes based on carboxymethyl cellulose/acetylated arrowroot starch containing free Br, Br-loaded EUDRAGIT® S 100 NPs, and Br-loaded liposomes. Their results showed that the combinations of Br with carboxymethyl cellulose maintained its proteolytic activity. On the other hand, Br loaded in NPs and liposomes kept the moisture of the wound, reduced evaporation, and also had the best tensile strength during rupture. In an animal study, Br-NPs showed only 13.28% wound contraction higher than that for membranes containing free Br. In treated groups with Br-loaded liposomes, more accelerated healing was observed compared with other treated groups. Br loading in liposomes prevented initial inactivation, degradation, and dilution of Br in blood circulation [29].

In another study, chitosan nanofibers encapsulated with Br showed a significant accelerating effect on wound healing compared with the chitosan nanofibers alone assessed by wound area reduction and histological findings (28). Bayat *et al*. evaluated the burn treatment effects of Br loaded in sodium-alginate NPs incorporated into chitosan hydrogels in an animal model. The formulation containing 10% Br showed the highest debridement effect in comparison with other treated groups [30]. In a similar study, Rachmawati *et al*. evaluated the debridement effect of Br in a nanoemulsion delivery system with various types of oil phases to increase its stability and efficiency. Using New Zealand rabbits as animal models, it was found that Br nanoemulsion containing Vit E acetate contributed to better stability and healing activity compared with other oils and the control group [31]. Hasannasab and colleagues applied Br in a complex with zinc oxide (ZnO) on silk fibroin nanofibers for the treatment of second-degree burns via the anti-inflammatory effect of Br and the antibacterial effects of ZnO. The nanofiber complex showed no fibroblast toxicity and induced epithelization superior to nanofiber alone, and hair follicles, sebaceous glands, and well-organized collagens had been seen in an *in vivo* model [32].

Cancer therapy

Br properties such as its proteolytic and immunomodulatory effects are likely contributing to inhibition of tumor cell proliferation and metastasis. Previous studies suggested that Br affects the expression of many genes involved in apoptosis, proliferation, or metastasis of cancer cells and can be employed as a possible future anticancer agent. The mechanism of action of bromelain against tumors is likely at 3 levels of cellular metabolism; 1) by expression modulation of crucial genes for cell differentiation and proliferation (like; MAPK signaling pathway, Akt, Cox-2, NF-κB), 2) inducing cell death by apoptosis/autophagy, and 3) blocking the cell cycle by inhibition of necessary cyclins [25].

In a study by Ataide *et al*., chitosan-Br NPs were produced by the ionic cross-linking method and lyophilized using glycine and maltose as lyoprotectors. Their results showed that chitosan-Br NPs had higher enzymatic activity with a corresponding amount of free Br activity. Freeze-drying effectively improved the stability of Br and maintained its enzymatic activity for 90 days. Moreover, the encapsulation rate was increased and resuspension time was shortened in freeze-dried chitosan-Br NPs [33]. Similar research by this group was conducted in 2021. They studied the anti-oxidant, anti-proliferative, and anti-migration activities of previously synthesized chitosan-Br NPs. The antiproliferative effect of free Br was seen in glioma, breast, ovarian, prostate, colon adenocarcinoma, chronic myeloid leukemia tumor cell lines and not for keratinocyte cells, whereas inhibition with chitosan-Br NPs was only observed in chronic myeloid leukemia at higher concentration. They proposed that decrease in the anti-oxidant and anti-proliferative activity of Br in chitosan-Br NPs was possibly due to the slow protein release from NP. On the other hand, using keratinocyte cell scratch assay, more than 90% wound retraction was seen at 24 hr compared with free form [33]. Chitosan NPs modified with lactobionic acid (LA) and Br were used as dual-functional carriers for the delivery of Doxorubicin (Dox). Based on *in vivo* results, the penetration and diffusion of synthesized NPs into the tumor were enhanced due to Br hydrolytic property on the extracellular matrix (ECM). Moreover, the enzymatic activity of Br remained relatively appropriate and was about 28.3% [34].

Nasiri *et al*. in 2017 engineered superparamagnetic NPs and conjugated them with Br and folic acid (FA) as targeting moiety. In *in vitro* and *in vivo* evaluations, NPs containing Br had superior anti-tumor properties in comparison with neat Br, and NPs with FA, however, the anti-

tumor activity was increased when FA was added to the complex [35]. Bhatnagar *et al*. synthesized Eudragit-coated Br-PLGA NPs for oral delivery of Br. Their results showed appropriate Br encapsulation efficiency and Br release profile, in addition to enhancement in antitumor effect on different cancer cell lines (HEK293, MCF-7, HeLa, and A549). Using western blot analysis, Eu-Br-PLGA-NPs were able to change the protein marker expression for apoptosis and cell death significantly higher than free Br. This formulation released Br in a sustained manner. Moreover, increased lifespan and reduced tumor size of Ehrlich ascites carcinoma (EAC) were seen in Swiss albino mice compared with the free Br control group [36]. It was shown that Br formulated with PLGA NPs protected skin cells against carcinogenicity caused by 7,12-dimethylbenz and 5Q1 anthracene in 2-stage skin tumorigenesis mice model. Induction of apoptosis inducer genes (P53 and Bax) and reduction of cell survival-inducing genes (Akt and Erk) were attributed [37]. Karimian Rad and Ramezani studied the physiochemical properties of Br in Magnetic carbon NPs (MCNPs) along with its anti-tumor effect in breast cancer cell line (MCF-7). They realized that the highest adsorption on NPs was 44 mg/g at pH 5, 35 °C and these NPs can be used as a possible tool for Br removing. Moreover, MCNPs can effectively inhibit breast cancer cells *in vitro* [38]. In a similar study, Montazeri *et al*. encapsulated Br in magnetic carbon nanotubes (MCN) to evaluate its anti-tumor effect against tumor cells (HT-29 colorectal cancer cells). The effective IC 50 concentration of Br was determined to be 100 µg/ml after 24, 48, and 72 hr against the HT-29 cell line using MTT assay. However, encapsulated Br showed the same decrease in cell viability only after 48 and 72 hr, which may be due to a slow release of the drug over time [39].

Anti-inflammation

Inflammation is a required biological response, but sometimes this process may be excessive [40]. As shown in Fig. 1, Br could act on acute inflammation with different mechanisms. Br decreases the expression of cyclooxygenase -2 (COX-2) and prostaglandin E2 (PGE-2) genes, involved in tumor angiogenesis and immune system suppression [41]. Br also causes activation of inflammatory mediators such as interleukins (IL-1β and IL-6), Interferon-gamma (INF-γ), and tumor necrosis factor-alpha (TNF-α) in mouse macrophages and human peripheral blood mononuclear cells (PBMC) [42]. Br controls cell stress by reducing advanced glycation end products as key mediators in inflammation and oxidative stress. The cell surface marker CD44 (cluster of differentiation), which is expressed by cancer and immune cells, is directly involved in cancer growth and metastasis. Furthermore, CD44 regulates lymphocyte requirement at the site of inflammation [43, 44]. Br could decrease the expression of CD44 levels in mouse and human tumor cells and modulate the expression of TGF-β as one of the main regulators of inflammation in patients with osteomyelofibrosis and rheumatoid arthritis [45]. Moreover, Br activates natural killer cells and increases the production of granulocyte-macrophage colony-stimulating factors, IL-2 and IL-6, and reduces the activation of T-helper cells [46].

In research by Sherma *et al*., Br was used for oral delivery to treat rheumatoid arthritis

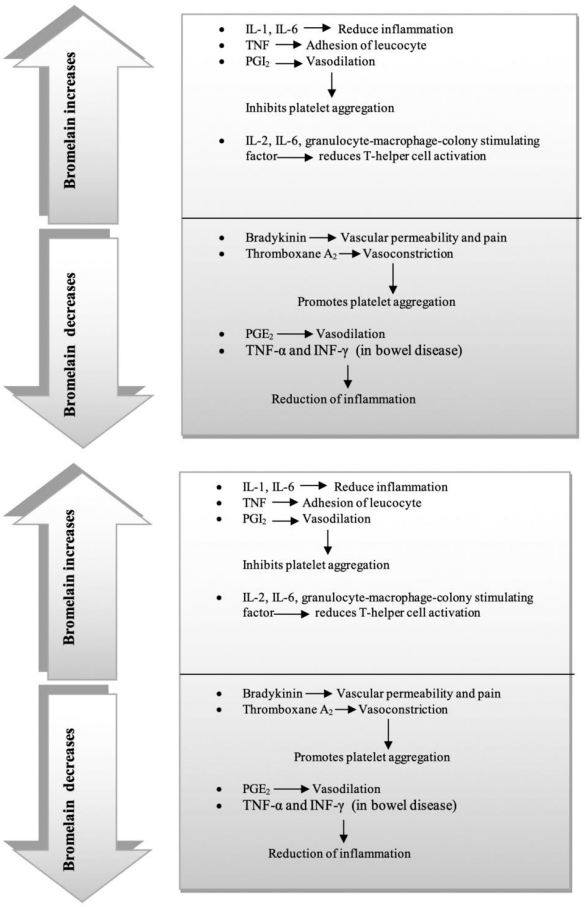

Figure 1. Effect of Bromelain on acute inflammation [47]

(RA). They used nanostructured lipid carriers to increase the stability of Br against gastric fluid and to achieve sustained release. The effectiveness of this treatment and delivery method was confirmed in Wistar rats using indicative parameters for RA-containing; paw edema, joint stiffness, and mechanical allodynia as well as oxidative stress markers. Moreover, radiological findings showed significant perseverance from joint deformity [48]. Bernela *et al*. (2016) synthesized Katira gum NPs containing Br using the ionotropic gelation method to inhibit induced inflammation of carrageenan using a rat paw model. The encapsulating Br in Katira gum NPs significantly increases its anti-inflammatory potential as a result of increased absorption and Br protection from proteases [49].

Treating of periodontal disease

Periodontal disease is an inflammatory disease with symptoms of periodontal tissue destruction. Shoba *et al*. investigated the 3D bilayer including Br-conjugated hydroxyapatite NPs containing magnesium (MG) for periodontal regeneration in mice. Their results showed that mechanical, physiochemical, and biological properties of the NPs were increased due to the imitation of the extracellular matrix (ECM) intricate. Br and MG were released in a sustained manner, which enhanced the wound healing process in the mouth through increased antibacterial effect, cell proliferation, migration, and angiogenesis using *in vitro* assay. Moreover, *in vivo* assay in Wistar rats, *in ovo* chicken chorioallantoic membrane assay, as well as *ex vivo* aortic ring assay, showed an increase in expression of wound healing marker that confirmed the efficacy of the formulation in terms of compatibility and inducing angiogenesis in the wounded area [50].

Blood coagulation and cardiovascular diseases

Br is an effective agent in the treatment of cardiovascular diseases, especially due to its antithrombotic and anticoagulant activities. In addition, Br is a proteolytic enzyme that can prevent the accumulation of blood platelets, ease glucose metabolism, and strengthen heart muscles. In a study, the efficiency of copper- Br NPs (Br-Cu NPs) was evaluated on diabetes mellitus and myocardial I/R injury in an animal model. Copper could facilitate glucose metabolism and strengthen cardiac muscle and antioxidant activity; Histological findings and cardiac biomarkers (CKMB) showed significant improvement against cardiac injury in rats with prophylactic treatment. Moreover, Br-Cu NPs reduced the release of inflammatory cytokines (IL-6 and TNF-α), and oxidative stress while improving nitric oxide bioavailability and lipid profile [51].

CONCLUSION

Bromelain (Br) is a mixture of extracted enzymes that is potentially used for the treatment of several diseases, including cardiovascular diseases, skin disorders, and inflammatory diseases. Based on its protein structure, it is susceptible to denaturation and structural changes that may reduce its stability and activity. Also, its delivery and application have been challenging. Nanotechnology overcomes some of these limitations of conventional delivery, such as poor water solubility and stability as well as limited bioavailability. It has been proved that encapsulation of Br is an efficient alternative to increase the enzymatic activity of Br and expand its medicinal and therapeutic applications. Br in all its applications combined with nanotechnology has significantly added properties and activities leading to better formulation results. However, further clinical trials are required in order to clarify their therapeutic efficacy and the possible toxic effects of these NPs containing Br.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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