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Natural Rubber Latex Biomaterials in Bone Regenerative Medicine

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Abstract

Natural rubber latex (NRL) is a white and milky solution that exudes from *Hevea brasiliensis* bark when perforated, and it has been appointed as a new promising biomaterial. NRL has been proven to be a very biocompatible material, and several new biomedical applications have been proposed. NRL has been proven to stimulate angiogenesis, cellular adhesion and formation of extracellular matrix, besides promoting replacement and regeneration of tissue. NRL also can be used as an occlusive membrane for guided bone regeneration (GBR) with promising results. Therefore, the aim of this chapter is to review NRL studies and to present NRL membrane as a promising biomembrane for use in bone trauma and injury.

Keywords: natural rubber latex, biomembranes, biomaterial, bone regeneration

1. Introduction

Hevea brasiliensis, popularly known as rubber tree, is a plant species that belongs to the Euphorbiaceae family. This plant synthesizes latex by a system of laticiferous rings, organized as paracirculatory vessel systems, in the inner bark of the plant. Latex is the cytoplasm of the laticiferous cells, and its composition resembles the composition of common cells, except for having 30–45% of natural rubber [1]. The latex of *H. brasiliensis* exudes from the bark when it is perforated (**Figure 1**).

Most of the harvested latex is coagulated for the manufacture of “dry rubber” products, including automotive tires. The latex of *H. brasiliensis* can be stabilized in an uncoagulated form with the use of ammonia, which allows the latex to be used for the manufacture of other products, such as surgical gloves (**Figure 2**) [2].



Figure 1. Latex of *Hevea brasiliensis* on exuding of bark after drilling.



Figure 2. Products made by the latex of *Hevea brasiliensis*.

Natural rubber latex (NRL) from *H. brasiliensis* is a colloidal anionic system formed by rubber particles (1,4-*cis*-polyisoprene) stabilized by phospholipids and proteins molecules (**Figure 3**) [4, 5]. One-third of the weight of *H. brasiliensis* latex is made of natural rubber, but 1–2% of its weight consists of hundreds of proteins [5]. Other constituents such as lipids, Quebrachitol, ribonucleic acids and organic salts are also present [6].

In the last years, researchers have been publishing about NRL membranes (**Figure 4**) that has been proven to be an important inductor of wound healing, inductor of esophagic wall regeneration and tympanic membrane regeneration, by mechanisms involved with angiogenesis [7–9]. NRL has also been studied for reconstructing temporal muscle fascia [9] and as arterial prosthesis in animal models, healing of ocular conjunctiva and neoangiogenesis in rabbits [10].

Besides forming biomembranes, which represent a complex colloid made of rubber particles and lutoids bodies suspended in a protein-rich media, NRL can be centrifuged in high speed and separated in fractions that mainly consist of a superior phase of rubber particles, an aqueous phase called centrifuged serum (C-serum) and an inferior phase called bottom serum (B-serum) (**Figure 5**) [12–14].

The rubber particles represent 25–45% of the fresh NRL and they have a medium diameter of 50 Å to 3 μm [15, 16]. The main proteins found in the rubber particles surface are Hev b 1 and Hev b 3 [17]. Centrifuged serum is the solution composed by carbohydrates, electrolytes, proteins and amino acids. This solution is composed by the cytoplasm of the laticiferous cells and contains a great amount of proteins related to the cell metabolism [18]. This phase is implicated with most of the biological properties of NRL [19]. Bottom serum is mainly constituted of lutoids, spherical

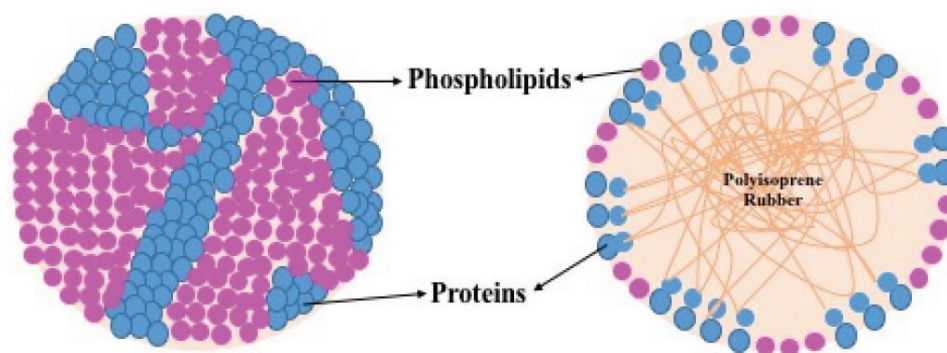


Figure 3. Rubber particles surrounded by a layer of protein-phospholipid (adapted from Ref. [3]).



Figure 4. NRL membrane.

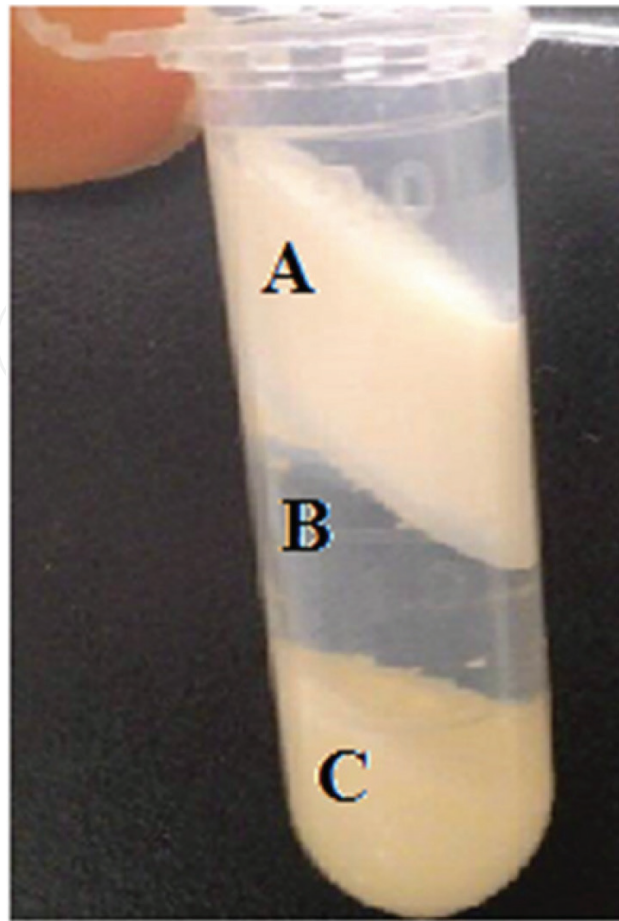


Figure 5. NRL fractions after high speed centrifugation: (A) rubber particles, (B) centrifuged serum and (C) bottom serum.

vacuoles that are osmotically sensitive and induce the latex flow to stop [20, 21]. Hevein is the main protein found in bottom serum, and it is implicated with allergic reactions [18].

Since many biological and biomedical properties can be implicated with NRL from *H. brasiliensis*, the aim of this chapter is to review and explore the studies showing the applications of this biomaterial in bone regenerative medicine.

2. Bone remodeling

Bone is an organ capable of replacing old and disrupted tissue through a remodeling process. Mechanical changes required by skeletal functions make the remodeling process indispensable for the bones. The cells responsible for this process are osteoblasts and osteoclasts, and the first promote bone formation and the latter bone resorption. Osteoblasts are derived of the osteogenic differentiation of mesenchymal stem cells. Other important skeletal cells that have their origin in mature osteoblasts are the osteocytes, and these cells in particular are surrounded by extracellular matrix and have the ability to regulate osteoblast and osteoclast activities to maintain bone homeostasis [11].

Bones protect vital organs and provide storage for calcium and phosphate. Bone compartments designated for mechanical functions are called cortical bone, and the bone compartments designed for metabolic functions are called trabecular bone. Bones can also be distinguished by their formation. When the formation occurs in a direct way, it is called intramembranous ossification and it is characterized by the condensation of mesenchymal stem cells that become osteoblasts. This type of process occurs in the flat bones of neuro- and viscerocranium and in part of the clavicle [22]. When bone formation occurs in an indirect way, it is called endochondral ossification, and it is characterized by the differentiation of mesenchymal stem cells into cartilage first and this cartilage is later replaced by bone [23]. Endochondral ossification occurs in long bones, in vertebrae and in the skull base and the posterior part of the skull [22].

Bone modeling occurs during the growth process, and bone remodeling occurs during lifetime. These two processes take place under the control of various substances such as parathyroid hormone (PTH), calcitonin, vitamin D, growth hormone (GH), steroids, soluble cytokines and growth factors (i.e., macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear κ B ligand (RANKL), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) family) [11].

Microfractures or factors related to bone microenvironment generate different stimuli that induce osteoblast to produce RANKL, which interacts with its receptor expressed by osteoclasts. This interaction activates the polarization of the osteoclasts that secretes enzymes required for bone resorption. Osteoblasts synthesize type I collagen (that represents 90% of the proteins in bone matrix) and procollagen I N-terminal peptide (PINP) that is considered a marker of bone formation [24].

Type I collagen together with other fibrillar collagens, bone proteins (osteopontin, bone sialoprotein and osteocalcin), proteoglycans, fibronectin and glycosaminoglycans compose unmineralized osteoid. The development of the osteoblast along with the stimulation of the osteogenic genes and mineral deposition are dependent on the pigment epithelium-derived factor (PEDF) [25]. Osteoblast phosphatases are responsible for the mineralization process, since they release phosphates that along with calcium form hydroxyapatite crystals $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ [26]. The osteoblasts that remain trapped inside the mineralized matrix acquire a stellar shape after morphologic change and form a network that produces signaling through bone tissue. These cells are called osteocytes.

2.1. Bone formation and bone remodeling molecular pathways

During osteogenesis, bone morphogenetics proteins (BMPs) and WNT signaling pathways are very important. The BMP pathways activate intracellular proteins called SMAD that control the expression of the gene RUNX2 (runt-related transcriptional factor 2), and this gene codifies a transcriptional factor that stimulates the mesenchymal stem cells to differentiate into osteogenic lineage [27]. WNT pathway is formed by proteins that are involved in many other biological processes, and it also regulates the expression of RUNX2 gene [28].

Depending on the level of differentiation of progenitor cells, WNT classic pathway induces or inhibits osteoblast formation. It also controls bone resorption when increasing the ratio of osteoprotegerin (OPG)/RANKL proteins [28]. These proteins are specifically produced by osteoblast

to either inhibit (OPG) or enhance (RANKL) osteoclasts activity [29]. **Figure 6** schematically represents the complex network of molecular signaling pathways during osteogenesis.

Besides the pathways described above, systemic hormones also regulate osteogenic commitment or differentiation of mesenchymal cells. Examples of these hormones are PTH, glucocorticoids and estrogens. Local growth factor signaling, such as bone transforming growth factor- β (TGF- β 1/2), insulin-like growth factor (IGF), fibroblast growth factor 2 (FGF-2), VEGF, cytokine modulators (prostaglandins) and mitogen-activated protein kinases (MAPK), also regulates the differentiation of mesenchymal stem cells [30]. Other enzyme that plays an important role in bone regulation is Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (PIN1), which interacts with RUNX2, SMAD1/5 and β -catenin proteins [31].

Osteogenesis is also regulated by epigenetic factors such as DNA methylation, microRNAs (miRNAs), histone acetylation and deacetylation, and chromatin structure modification [32, 33]. Especially short noncoding miRNAs have been proven to affect both osteoblast (bone formation) and osteoclast (bone resorption) lineage. Some miRNAs regulate osteoblastogenesis by posttranscription regulation of RUNX2 (e.g., miR-34c, miR-133a, miR135a, miR-137, miR-205, miR-217, etc.), Osterix (OSX) (e.g., miR-31, miR-93, miR-143, miR-145, etc.) and type I collagen (e.g., miR-29, miR-Let7) proteins [34].

After a bone fracture, an inflammatory response is activated and it is crucial for the process of bone regeneration, bone remodeling and healing. This inflammatory response involves the secretion of tumor necrosis factor- α (TNF- α) and interleukins (IL) IL-1, IL-6, IL-11 and IL-18

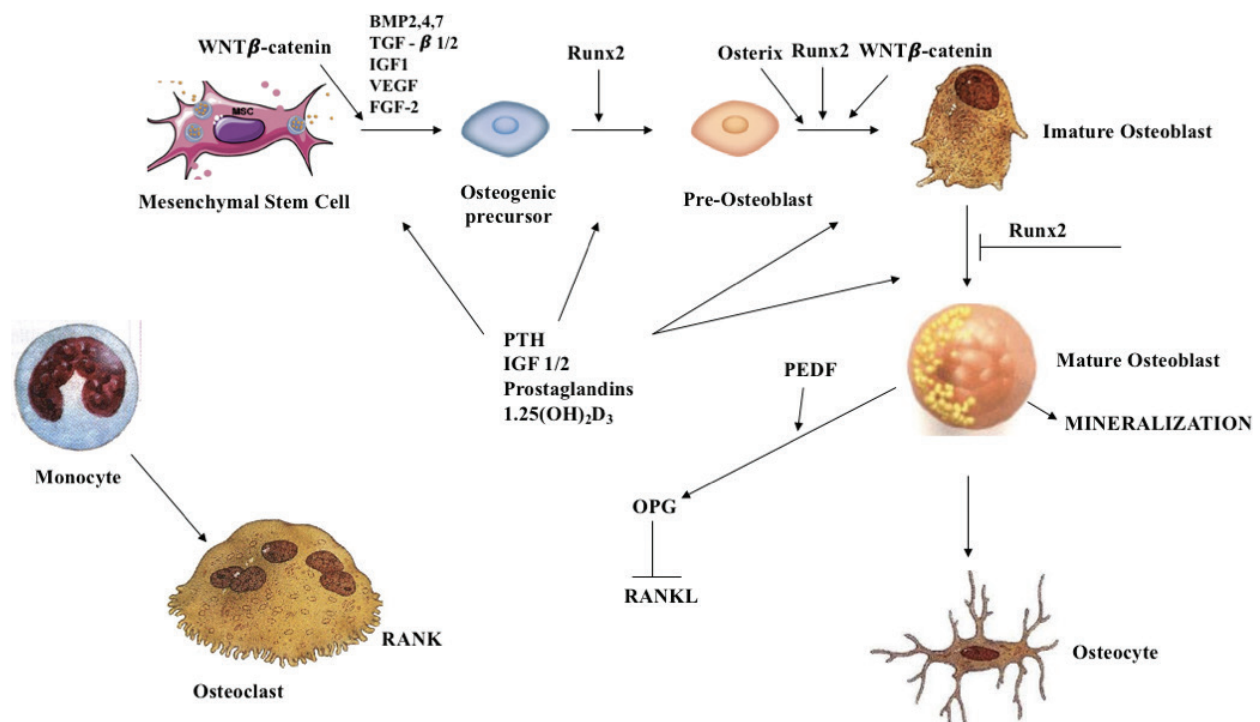


Figure 6. Schematic representation of cells and regulatory molecules involved in osteoblastogenesis and bone formation (adapted from [11]).

by macrophages, inflammatory cells and mesenchymal cells [35]. TNF- α stimulates osteoclast cells and promotes mesenchymal stem cells to start endochondral bone formation. TNF- α also stimulates apoptosis in hypertrophic chondrocytes, and a delay in the reabsorption of mineralized cartilage stops bone formation. When TNF- α is overproduced, as in diabetics, the cartilage is removed in a premature way, delaying bone formation and healing [36]. Right after the injury, during osteoclastogenesis, RANKL and OPG expressions are elevated. In bone remodeling, the expression of these proteins is diminished [37].

During bone remodeling, IL-1 and IL-6 are the most important IL. IL-1 is produced by macrophages and promotes the formation of the primary cartilaginous callus and also promotes angiogenesis in the site [38]. IL-1 also stimulates the production of IL-6 by the osteoblasts, and IL-6 also stimulates angiogenesis by the production of VEGF. It induces the differentiation of osteoblasts and osteoclasts [39].

In bone remodeling, mesenchymal, osteoblasts and chondrocytes cells produce BMPs that can work independently or in collaboration with each other and with other members of the TGF- β family to start osteoclastogenesis [40]. BMPs are structurally and functionally related, but they exhibit different patterns of expression during bone remodeling. Murine studies have shown that BMP-2 expression is more elevated in the first 24 h after the fracture, suggesting its primary function in the beginning of the bone repair. This BMP is also related to the maintenance of the normal bone mass [41, 42].

BMP-3, BMP-4, BMP-7 and BMP-8 are expressed during the healing time (14–21 days after the injury) when there is reabsorption of the calcified cartilage and osteogenesis [43]. It has been proposed that BMP-7 is the most potent inductor of differentiation in mesenchymal stem cells to osteoblasts [44].

2.2. Vasculature involved in bone formation and bone remodeling

Bone is a connective tissue that possesses high vascularization. During bone endochondral and intramembranous ossification, regeneration and remodeling, the vasculature plays an important role [45]. Ten to fifteen percent of total cardiac output goes to skeletal system [46]. The role of the blood vessels in the bone is not only to supply the bones with oxygen and nutrients but also to provide them with growth factor, hormones and neurotransmitters (e.g., brain-derived serotonin), maintaining the bone activity and cell survivor [47, 48].

Osteogenesis can occur through endochondral or intramembranous ossification; in both ways, angiogenesis is a critical stage and it is associated with the production of VEGF by hypertrophic chondrocytes or mesenchymal stem cells, respectively [49]. VEGF attracts endothelial cells acting as a chemotactic molecule that controls differentiation and function of osteoblasts and osteoclasts, participating in bone modeling and bone remodeling. A loss of the VEGF leads to incomplete bone vascularization and automatic disturbed endochondral ossification [50]. On the other hand, overexpression of VEGF can lead to osteosclerosis, highly increased bone formation that is a result of intense osteoblast differentiation, ending in altered bone morphology [51].

In endochondral ossification, cartilage vascularization begins with the formation of a primary vessel that is projected from the perichondrial vascular network to the adjacent cartilage. Then, a capillary glomerulus is formed at the leading edge and the entire vascular unit grows. Following elongation, a backward expansion of the capillary network occurs and it tightly surrounds a pair of main vessels composed by an arteriole and a venule [52].

In intramembranous ossification, capillaries of a small diameter move into thin avascular layer of mesenchyme that surrounds the mesenchymal condensation center. In this center, mesenchymal cells secrete VEGF that attracts endothelial cells. At the start of mineralization of the bone, the first blood vessels associate with an extensive external network of blood vessels [53].

During bone remodeling and bone regeneration, the blood vessels play an important role. Osteoclasts form a cutting cone that moves forward resorbing dead bone or damaged/old bone matrix. A blood vessel follows this cutting cone, delivering nutrients, hormones and growth factors to osteoblast that will produce new bone [48]. OSX-expressing osteoblast precursors are involved in the process of remodeling, accompanying the blood vessels, positioning themselves in a perivascular localization, showing the tight relationship between osteogenesis and angiogenesis [54].

3. Bone remodeling and latex

To clinically manage situations such as bone loss, injury or disease, researchers have been engaged for a long time to find a biocompatible material that is innocuous, promotes osseointegration, is manageable and has low cost for the people who need it. Natural rubber latex (NRL) extracted from *Hevea brasiliensis* has been used in the industrial manufacturing of several products, such as gloves, condoms, balloons and parts of medical and dental equipment [55].

Based on a new manufacturing process, several new biomedical applications have been proposed since NRL has been shown to be very biocompatible, stimulating cellular adhesion, the formation of extracellular matrix, and promoting the replacement and regeneration of tissue [8]. This new manufacturing process is based on the production of a biomembrane of NRL that has been used to replace vessels, esophagus, pericardium and abdominal wall [7]. In all of these experiments, the biomembrane promoted rapid tissue repair and elicited an inflammatory response that resembled the inflammatory response of normal healing process and not one of rejection process [8].

The membrane of NRL was also tested for the repair of bone defects in dental alveoli of rats [56], and the histological examination of the extraction sockets revealed a pattern of normal repair with characteristics similar to those reported for other materials [57]. Histometric evaluation of the areas close to the implant during the initial 7 days demonstrated progressive and accelerated osteogenesis by a decrease in the thickness of the fibrous capsule. At long-term, NRL implants did not induce the formation of foreign-body reaction nor persistent

inflammatory reaction, and after 42 days, the NRL implants were making close contact to the bone at many sites.

The induction of angiogenesis is crucial for bone regeneration and remodeling, and a study using chick embryo chorioallantoic membrane (CAM) showed that NRL membranes possess angiogenic properties. The experiment also showed that the angiogenic capacity of the NRL membranes remains active and increases in temperatures ranging from 65 to 85°C. These results showed that the heating used to prepare NRL membrane does not affect its biological properties. The same study used centrifuged latex to demonstrate that the poly-isoprene is not the part of latex responsible for the biological properties [4].

Large fractures can mean significant reconstructive problems and sometimes require special procedures for regeneration. And this regeneration is dependent on blood clot stability, local vascularization, defect size and protection against invasion of competitive nonosteogenic tissues [58, 59]. Guided bone regeneration (GBR) was a technique developed to enhance bone repair, and in this procedure, an occlusive membrane is used to provide to the osteogenic cells better conditions to perform bone remodeling [60].

Different types of membranes have been tested for use in GBR, but resorbable membranes represent the most interesting alternative since they avoid removal surgery [61]. NRL membranes were tested as an occlusive barrier in GBR of large defects in rabbit calvaria. NRL membranes successfully enhanced bone regeneration process in the group of the treated animals, and it was shown by a statistically higher volume of mature bone in all periods of study that was up to 120 days. The NRL membranes worked as a passive barrier membrane that prevented epithelial and connective tissue migration, thus facilitating the proliferation and migration of regenerative bone cells into the wound. The NRL membranes used for GBR, as well as the ones used to treat dental alveoli defects, did not induce the formation of foreign body inflammatory reaction [62].

Another way to accelerate bone regeneration and remodeling would be incorporating BMPs to NRL membrane. As shown above, BMPs induce bone remodeling and many studies have tried to develop a BMP delivery system that could sustain gradual release of BMPs for dental and orthopedic use [63–66]. A study using bovine serum albumin (BSA) in the place of BMP (same molecular weight) and NRL membranes prepared at different polymerization temperatures showed that NRL membrane was able to release BSA for 18 days. This indicates a promising future of these membranes as active occlusive membrane in GBR and they could release BMPs for 18 days accelerating bone healing [55].

Based on previous studies, a protein was isolated from NRL (P-1) and its inductive bone repair properties were compared to recombinant human bone morphogenetic protein-2 (rhBMP-2), a commercial available human protein with good osteoinductive capabilities. To compare these two proteins, a carrier made of monoolein gel was used. P-1 is still being characterized to better understand its biological properties and its function in the laticifer cells of *Hevea brasiliensis*. However, associated with monoolein gel, P-1 was able to induce new bone formation on bone defect of rat calvaria [67].

This protein extracted from NRL (P-1) was used in combination with a fibrin sealant in the repair of rat tibial bone defects. This combination was successful, being immunoidentified by the presence of osteoblasts in the area, showing high osteogenic and osteoconductive capacity for bone healing [68].

Moura et al. [69] used different polymerized NRL membranes in rabbit calvaria with bone defect. These membranes were compared to polytetrafluoroethylene (PTFE) membranes, which are an extensively studied material considered gold standard for occlusive membranes [70]. In this study, NRL membranes performed their role as biological barriers and achieved a similar performance to the PTFE membrane. One of the polymerized NRL membranes was ammonia free, and these were the membranes that significantly improved the bone repair process producing higher bone formation, being more effective than PTFE membrane. These results were achieved since the method of preparation of these NRL ammonia-free membranes preserved the angiogenic stimulus of the membranes. These membranes also did not lead to bone tissue hypersensitization.

NRL was also coated with calcium phosphate (Ca/P) and tested for biomedical application. Biomaterials added with Ca/P present biological, chemical and mechanical properties very similar to the mineral phase of the bone besides the ability to bond to the host tissue. A hemolytic test was performed, and this material did not affect the blood cells, being so ready for animal tests [71].

These results showed above indicate NRL membranes as a promising future biomembrane that could be used to accelerate bone healing. More experiments are being done already in humans. Since NRL membranes present intense angiogenic activity and wound healing activity, NRL membranes are being commercialized in Brazil and other 60 countries around the world as a band-aid curative (BIOCURE®) for the treatment of ulcers in diabetic patients.

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