Materials Science

Review

Protein based devices for oral tissue repair and regeneration

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Abstract: In the last decades, a goal of tissue engineering has been devoted to the design of devices with multiple micro- or nano-structures and loaded with bioactive molecules, to mimic the extracellular matrix (ECM) so generating a conducive microenvironment for new tissue replacement/regeneration. The ECM, naturally, is composed of fibrous proteins which provide structural support for tissues, mainly regulating cells behavior in terms of proliferation, growth, survival, shape, migration and differentiation by cell-matrix interactions. Several studies have been just investigated the fabrication of different platforms for the regeneration of teeth, oral mucosa, salivary glands, bone, and periodontium. In this context, many proteins—from a natural or biological source—have been used as instructive substances to in vitro guide tissue organization and functions. In particular, new advances in the definition of protein-based formulations currently represent a great challenge to promote a more effective regeneration of dental tissues to be transplanted into patients to replace damaged, diseased or missing tissues. Hence, the purpose of this review is to discuss the use of protein-based systems for the regeneration of oral tissues.

Abbreviations: ECM: Extracellular Matrix; EGF: Epidermal Growth Factor; FAK: Focal Adhesion Kinase; FN: Fibronectin; GBR: Guided Bone Regeneration; GTR: Guided Tissue Regeneration; HA: Hydroxyapatite; hMSC: human Mesenchymal Stem Cells; IFPs: Intermediate Filaments Proteins; MAC: Membrane Attack Complex; MSC: Mesenchymal Stem Cells; PDGF: Platelet-Derived Growth Factor; PDL: Poly-D-Lysine; PDLSCs: Periodontal Ligaments Stem Cells; PL: Polylysine; PLL: Poly-L-Lysine; PTFE: Polytetrafluoroethylene; RGD: Tripeptide Arg-Gly-Asp; SHED: Stem

Cells from Human Exfoliated Deciduous Teeth; VEGF: Vascular Endothelial Growth Factor; VN: Vitronectin

1. Introduction

The employ of biomaterials plays a crucial role in biomedical applications, allowing the fabrication of 3D frameworks able to guide the basic mechanisms of tissue regeneration. In this context, synthetic polymers have been historically preferred mainly for their capability to be easily manipulated by more diffused processing techniques in the form of porous structures to support the invasion of ex novo tissues, i.e., cartilage [1], bone [2], ligaments [3], and various other soft ones.

Only recently, the growing attention towards the understanding of cell materials interaction is addressing the investigation of new materials to more accurately reproduce the local biological microenvironment, with the final aim to improve cellular response and more efficiently modelling biological context.

For this purpose, starting from the innate attitude of natural polymers to guide the cell behavior through biophysical and biochemical cues, many researchers are exploring the use of natural proteins to design temporary platforms able to mimic the native extracellular matrix (ECM). Besides, from a structural point of view, ECM is basically composed of protein fibres with diameters ranging from tens to hundreds of nanometres [4], concurring to support and preserve the mechanical integrity of tissues and organs. Fibrous proteins such as collagen, elastin, keratin, laminins, fibronectin and vitronectin with elongated three-dimensional structure (Table 1) are primary components that directly interact with cells similarly to the bioactive component of the native ECM.

Table 1. Summary of proteins for scaffold manufacturing in oral tisse engineering.

Protein	Attributes	Biological properties	References
Collagen and gelatin	Right-handed triple helix, composed of three α-chains (Gly-X-Y) RGD motifs	High biocompatibility Structural properties adhesion, proliferation and differentiation signal	[5–7]
Keratin	RGD and LDV motifs Cysteine content	Cell adhesion High mechanical strength, inertness, and rigidity	[8,9]
Silk	Disulfide bonds	High mechanical properties Biocompatibility	[10,11]
Zein	Alcohol-soluble Rich in glutamic acid, and non-polar amino acids (leucine, proline and alanine)	High chemical stability Low degradation rates	[12,13]

In the last years, several efforts have been spent to identify natural proteins, manipulating them by micro- and nano-technologies to improve their structural and functional stability in order to more efficiently mimic the native tissue microenvironments, as well as triggering new functionalities for cells for ex novo tissue replacement.

Herein, we propose an overview of recent studies involving structural and functional proteins used for the regeneration of hard and soft tissues in the oral cavity (Figure 1). After a punctual excursus of natural proteins with different functionalities (i.e., structural, bioactive), and their possible interaction mechanisms with cells, it will be provided a concise classification of studies to explore their ability to trigger the regeneration of native tissues in different compartment of the oral cavity (i.e., teeth, periodontal ligament, pulp, gum).
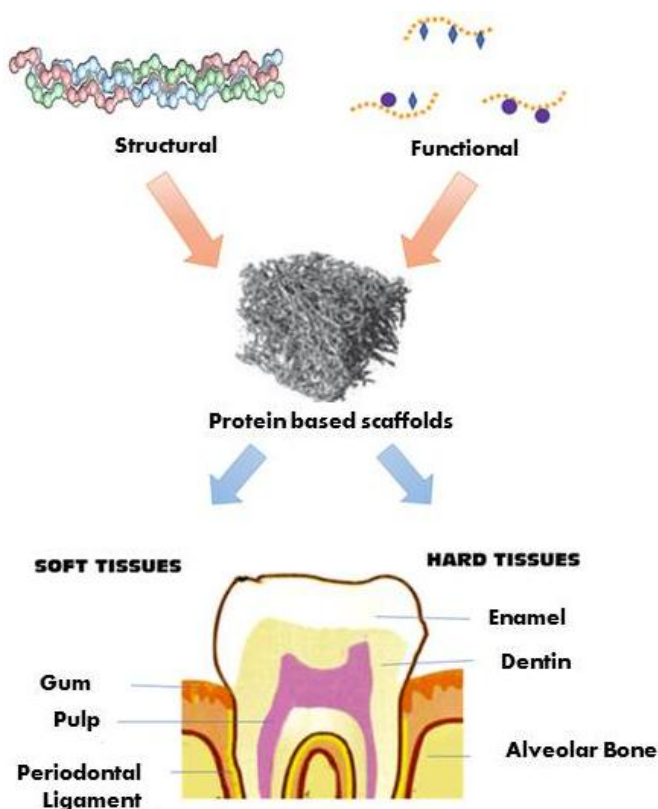
Figure 1. Scheme of oral tissue engineering by using structural and functional proteins.

2. Structural proteins

2.1. Collagen and gelatin

Collagen is the most abundant protein in the human body and the main component of extracellular matrix (ECM) in soft and hard tissues. More than twenty types of collagen have been identified, and the most common is type I. In the field of tissue engineering, collagen and its denaturized form, i.e., gelatin, have been widely studied for the fabrication of scaffolds. The principal functions of collagen are as a scaffold for cell attachment and influence cell behavior, as well the maintenance of the structure and integrity of tissues by providing specific mechanical properties [5]. Collagen primary structure consists of triplets of Gly-X-Y, where X and Y are frequently proline and 4-hydroxyproline respectively [6]. Collagen is degraded enzymatically by collagenases such as metalloproteinases. Their degradability can be regulated by diverse methods as physical crosslinking techniques (i.e., ultraviolet (UV) or dehydrothermal (DHT) crosslinking) or

chemical modifications (i.e., glutaraldehyde; isocyanates) [14,15]. Gelatin, a fibrous protein extracted from denatured native collagen shares a similar structure, composition and biological properties of native collagen. According to denaturation hydrolysis process, there are two types of gelatin: type A, it means acid process; type B, which is the alkali breaking. Alone or in combination with natural or synthetic polymers, gelatin has been widely used for the fabrication of *in vitro* stable bioactive scaffolds for tissue engineering [7,16,17].

2.2. Keratin

Keratin is a fibrous protein, constituent of hair, wool, feathers, nails, and horns of mammals, reptiles and birds. Keratin proteins are classified as α-keratins, known as intermediate filaments proteins (IFPs) and β-keratins, both embedded in an amorphous keratin matrix. The α-keratins are in the fiber cortex; are low in sulfur content, with 40–60 kDa of average molecular mass [18]. The matrix proteins are globular, have low weight, and high content of cysteine, glycine and tyrosine residues; surrounding the IFPs and interact with them through intermolecular disulfide bonds [8]. The high mechanical strength, inertness and rigidity of α-keratins depend on crosslink between IF-matrix composite. The β-keratins and their function are to protect keratin fibers from physical and chemical damage [19]. Keratin has been explored for biomaterials applications because its mechanical properties, that are related with the high content of cysteine residues to form disulfide bonds; and biological properties, such as providing support the cytoskeleton of cells and tissues, adhesion, as well cell transport, regulation of protein synthesis and cell differentiation [8,9].

2.3. Silk

Silks fibers are natural proteins produced by species of arthropods, as spiders, scorpions, silkworms, mites, and bees. Natural silk is composed of two self-assembled proteins: a filament core of silk fibroin, surrounded by sericin. Among the different types of silks, those extracted from silkworm *Bombyx mori* (*B. mori)* are the best characterized. The amino acid composition of silk fibroin consists primarily of glycine (43%), alanine (30%), serine (12%) and tyrosine (5%) [10,20]. Silk fiber filament is composed of two chains: heavy chain (H-chain), and a light chain (L-chain) linked by a single disulfide bond. Silk is commonly used in textile industry, recent years have been studied as biomaterial for tissue engineering due to its superior mechanical properties, cell biocompatibility, and degradability [11,21]. Recent reports show that silk protein promotes osteoblast differentiation of multipotent cells including primary bone marrow cells [22].

2.4. Zein

Zein is a vegetable protein, the main component of the endosperm in corn to provide resistance to microbial attack, approved as a generally safe food-grade ingredient by the U.S. Food and Drug Administration [12]. Zein is an alcohol-soluble protein, which amino acid composition is particularly rich in glutamic acid, and non-polar amino acids, as leucine, proline and alanine, but deficient in basic and acidic amino acids [23]. Zein consists of highly homologous repeat units and has a high α-helix content. There are several studies about the molecular structure of the zein, cylindrical model, ribbon-like model, hairpin model, superhelical structure model [13]. It has been studied for its

potential as implant material because its compatibility in vivo. Zein materials have been shown to have a proper porous structure and mechanical properties for cell adhesion, migration, proliferation and tissue ingrowth.

3. Bioactive proteins

There are a few types of biologically active proteins, with physiological properties that are useful for the regeneration of tissues, described in the paragraphs below.

3.1. Poly-L-Lysine

Polylysines (PL) is a homo-polypeptide belonging to the group of a family of polycationic dendritic macromolecules that could come from nature or artificial synthesis. This dendritic molecule is known for the highly branched macromolecular architecture with several reactive ending groups. The dendrimer chirality results in Poly-L-Lysine (PLL) and Poly-D-Lysine (PDL). Both are water-soluble, stable and are promising candidates in tissue engineering due to their biodegradable properties [24,25]. PLL has an enormous and different application in cell biology, and biomaterials for tissue engineering because enhance cell adhesion based on the interaction between the positively charged of the dendrimer and the negatively charged of the cell membrane, provoke the cell spreading, proliferation and differentiation of several kind of cells [26,27]. Now in tissue engineering is gained interest because is cheaper and could modify the material interaction with all environmental of cells, especially when is carried as drug and gene delivery [28,29].

3.2. Fibronectin

Fibronectin (FN) is an adhesive protein, which is free in plasma and is one of the main components of ECM. The FN is a glycoprotein formed by an asymmetric molecule consisting of two similar subunits of 220 kDa, linked by disulfide bridges near their carboxy-terminal region. It is in soluble in the dimer form while it is insoluble in the case of high molecular weight multimers generally packed into fibrillar components as in the native ECM [30,31]. Several biochemical studies have allowed to identify and purify their functional domains and therefore determine the structural characteristics of the molecule [32,33]. These studies have shown that FN can interact with a wide variety of macromolecules, including: gelatin, collagen, fibrin, factor XIIIa, heparin and proteoglycans [34]. Moreover, in biomedical and tissue engineering field have attracted the attention because FN promotes the cell adhesion, spreading pattern on materials functionalized with it, regulate migration, proliferation and differentiation [35–37].

3.3. Vitronectin

Vitronectin (VN) is a plasma glycoprotein and extracellular matrix protein, encoded by the VTN gene and consisting of somatomedin B, hemopexin, heparin-binding domains and an RGD motif [38,39]. VN is found circulating in plasma at a concentration of 0.2 µg/ml, in two molecular forms, a single chain of 75 kDa and another formed by two subunits of 65 kDa and 10 kDa, involved in diverse biological processes including regulation of coagulation pathways, on the fibrinolytic

system, formation of the membrane attack complex (MAC), antimicrobial properties and, also play an important role on cell attachment, proliferation, migration, wound healing and tissue remodeling when several surface biomaterials are coated and could be used to maintain the pluripotency of human mesenchymal stem cells (hMSC) and induce the adhesion of several cell lines [40–43].

4.   Proteins-cell interactions mechanisms

The functions of most part of cells are influenced in terms of adhesion as a function of the peculiar cytoskeleton arrangement. The surface receptors (i.e., integrins)—responsible for the cell response at the beginning step of cell adhesion—allow regulating all specific behavior. Integrins are cell surface receptors which sense and mediate the binding between ECM proteins cues to cells and regulated cell-cell, cell-material interactions, activating intracellular signaling pathways that regulate environment [44,45]. Most of the bioactive proteins in the ECM, show integrin-binding domains with specific sequences of few amino acids, e.g., RGD, that activate integrin receptors. Integrin receptors consist of two sub-units, a transmembrane glycoprotein α and another β that form a non-covalent complex. Each subunit contains a large extracellular domain and a small cytoplasmic domain. Both sub-units contain active centers contributing to the union. Currently 18 types of subunits α and 8 of type β are known, and their combination generates the 24 known integrin species and each specific heterodimer can recognize one or more ECM protein. Integrin have 2 major functional conformations that relate its affinity to ECM ligand: the inactive low affinity or bent conformation and the ligand bound high-affinity or extended conformation. Upon activation, integrin, provide to an intracellular scaffold for the assembly of cytosolic signaling complexes. These intracellular signaling events that follow extracellular stimulation are commonly described as "*outside-in*" signaling events, compared to intracellular events that lead to integrin activation, called "*inside-out*" signaling [46]. The combination of these adhesive interactions allows for distinct modes of integrin binding complexes frequently associated with the formation of so-called focal adhesions that may result in stable adherence, as at basement membrane sites, or may exhibit a transient character to facilitate cell locomotion and migration. The signaling pathways activated by integrin receptor engagement are extensive and show considerable similarities to initiated pathways through growth factors. The phosphorylation of distinct tyrosine residues has been shown to be an early and common event in integrin signaling. A signaling pathway through integrin is the activation of the non-receptor protein tyrosine kinase, FAK (focal adhesion kinase), localized in focal adhesions and quickly phosphorylated after the binding of integrin with extracellular components. The phosphorylation creates interaction points for other signaling molecules with SH2 domains, including members of the SRC family (which phosphorylate additional FAK points), PI3-kinase, phospholipase C-γ, and the Grb2-Sos complex. The recruitment of the latter mentioned triggers the activation of Ras, which binds the integrin to the activation of the ERK pathway. The activation of FAK and Src via integrin associates cell adhesion to the same pathways that regulate gene expression, cell proliferation and cell survival, activated by growth factors. In addition, integrins can interact and stimulate the activities of protein-tyrosine kinase receptors, such as EGF (epidermal growth factor), VEGF (vascular endothelial growth factor), and PDGF (platelet-derived growth factor) giving rise to the parallel activation of the pathways by factors and adhesion [47]. Thus, in tissue engineering the mechano-transduction of nanostructured mimicked ECM substrates have received attention for

enhance cell adhesion and tissue regeneration and as a strategy for new design of nanostructured system for fight against cancer [48–53].

5. Applications in oral tissue regeneration

Oral tissues repair is a complex process involving involves hybrid tissues—a combination of hard and soft matter. For example, to engineer the periodontium, it is required to stimulate, at the same time, the growth of alveolar bone, cementum and the periodontal ligament. The biological development of hybrid tissues and their interfaces currently represents a challenge over the simple engineering of single tissues. Different clinical approaches based on guided tissue regeneration (GTR) have been clinically investigated in the last years, basically including the use of barrier membranes with non-resorbable (i.e., polytetrafluoroethylene (PTFE)-based) or resorbable (collagen-based) properties [54]. Meanwhile, micro and nanostructured platforms have been developed through different processing techniques (i.e., electrospinning, self-assembly, and phase separation, among others) to re-create the natural micro-environment of cells in both, topographical cues and chemical composition, using natural proteins. Herein, we summarize all the main protein base methodologies used in oral surgery by distinguishing among the regeneration of hard or soft tissues.

5.1. Hard tissues

Guided bone regeneration (GBR) is a widely used treatment to heal and regenerate alveolar bone defects, based on the use of barrier membranes, to separate bone from epithelial and connective tissues. Collagen membranes are the most used due to their good biocompatibility, biodegradability, and osteoinductive properties, but have limited use by their limited mechanical properties. As a result, collagen-based scaffolds have been designed for GBR in periodontal research blended with other polymers and ceramics to enhance mechanical properties and cellular behavior [55,56]. Plastically compressed collagen gels are used as 3D scaffolds to seed mesenchymal stem cells (MSC), resulting a promising approach for bone healing process in craniofacial area [57]. Alternatively, PCL scaffolds may be also coated either with collagen than HA, two components of natural bones. In this case, collagen, not only provides the necessary environment for cell attachment, also helps to increase the poor fracture toughness of HA [58].

Recently, there is an increasing interest in the use of collagen derived from fish to avoid the risk of transmission infectious disease from mammalian collagen, as bovine or porcine sources, to humans. Hence, tilapia fish collagen has been capable to promote cell growth and presented osteogenic properties when powder is dissolved in culture media [59,60]. Nanofibrous membranes by freeze-dried method of tilapia collagen have been developed, with favorable results in osteoblastic differentiation, and membranes by electrospinning with the inclusion of bioactive glass and chitosan, to confer a certain degree of antibacterial properties could have a potential to develop GTR/GBR membranes [61]. Porous zein fabricated by salt-leaching method, showed good mechanical properties and biocompatibility, and was demonstrated that in combination with rabbit MSCs, zein porous scaffolds could be an option to treat bone defects [62]. To improve the osteointegration of zein scaffolds, the addition of calcium phosphates as hydroxyapatite (HA) have been proposed as a biomaterial for bone regeneration improving mechanical and biological properties of scaffold [63,64]. Electrospun zein fibers have been develop for tissue regeneration, which shown

good biocompatibility and have been crosslinked with citric acid to enhance their water stability [65,66]. Co-electrospinning of two proteins as gelatin and zein showed that addition of gelatin increases the elastic modulus of the scaffold and cell adhesion, of periodontal ligaments stem cells (PDLSCs); meanwhile, zein confers stability resulting non-degradable at early stage to achieve bone regeneration in the recovery of periodontitis [67]. Bone defect filling in dentistry is challenge in GBR because the defect is surrounded by connective tissue which could migrate into defect area. Silk fibroin has demonstrated to be a biocompatible biomaterial and supports cell attachment for bone regeneration. Studies on the efficacy of silk fibroin fibres showed that they are capable to enhance new bone formation, similar to collagen membranes currently used [68]. Silk scaffolds for GBR have been prepared with various casting temperatures and concentrations, showing bone regeneration at 4 and 8 weeks on rabbit calvaria defect model [69]. Porous silk fibroin films fabricated via lyophilization and densification have been recently presented as an alternative option to commercial collagen systems due to their osteoconductive properties, adjustable mechanical properties and degradability [70]. Electrospun silk fibroin nanofibers may be fabricated by electrospinning with the advantage to provide a biomimetic ECM environment, improving the biological performance of protein [71]. Reinforced silk fibroin with hydroxyapatite is used to fabricate devices with improved mechanical response, but preserving instructive properties of silk, i.e, cytocompatibility—and ceramic phase, i.e., osteoconductivity—to more efficiently guide bone regeneration [72]. The fabrication of bilayer systems with controlled silk fibroin content may contribute to decrease the contact angle value due to its hydrophilic amino groups and carboxylic groups, improving cell attachment [73]. FN has been used to biochemically modify scaffolds, improving the interactions with integrin involved in the early stages of bone formation [74]. In particular, composite nanofibers made of PCL/nHA have been coated by FN. In this case, FN and nHA have a synergistic effect to promote increasing calcium deposition and osteogenic differentiation [75]. Moreover, silk fibroin scaffolds have been modified based on decellularized pulp and FN for maxillofacial bone defects, where FN induced cell adhesion, proliferation and migration of osteoblasts due to its capability to interact with other microenvironment components [76]. Poly-(L-Lysine) (PLL) has been used to improve the compatibility of chitosan membranes in the presence of osteoblasts, improving adhesion, proliferation and differentiation [77]. Indeed, it has been proved that PLL is also able to enhance calcium deposition, so that it has been proposed as a bioactive coating for titanium dental implants to reduce healing time and enhance osseointegration [78]. Improvements of the osteoinduction of titanium surfaces mediated via ECM proteins such as FN and VN confirmed that protein adsorption may trigger adhesion and proliferation of human osteoblasts [41].

5.2. Soft tissues

In oral reconstructive surgical treatments, autologous and allogenic grafts or synthetic materials are commonly used for GTR for soft-tissues regeneration, i.e., soft tissue augmentation, defects associated with prosthetic restorations, gingivitis and periodontitis [79]. Even in these cases, collagen is the most clinically used to mimic the natural environment of cells, despite materials post treatments are often mandatory to increase some specific properties of the protein [80,81]. Collagen and fibroin deposited on gas-brushing PLGA nanofibers, are capable to attract and stimulate gingival fibroblasts for engineering oral mucosa because collagen improves cell attachment and meanwhile

fibrin participates in the initial stage of wound healing process [82]. The crosslinking of collagen chains may increase the stability of proteins for the fabrication of instructive scaffolds under physiological conditions. Alternatively, it may be combined with other natural or synthetic polymers and/or inorganic components [83]. Collagen from salmon has been extracted and prepared as a gel to its potential use for periodontal ligament regeneration and its stimulatory effect over human periodontal ligament fibroblasts [84]. Also, for GTR, the addition of keratin may be used to enhance mechanical properties of GTR scaffolds [85]. To improve the physical and biological properties, gelatin and elastin proteins have been frequently chosen for their biocompatibility, long-term stability and capability to promote fibroblast-attraction [86]. These proteins, in the hydrogel form, have showed a comparable cytocompatibility to collagen ones, selectively supporting odontoblast-like cells for pulp-dentine regeneration [87]. Keratin, has been also used as a coating for titanium surfaces of transmucosal implants, to drive adhesion and proliferation of gingival fibroblasts at the interface between dental implant and adjacent soft tissues [88]. Electrospun silk fibroin matrix was evaluated successfully as buccal mucosa matrix, avoiding the scar formation and presented lower inflammatory reaction compared with commercial matrices and also the content of silk could induce vascularization and accelerate the wound healing process of mucosa repair without using autografts [89]. Modification of electrospun PLGA scaffolds by FN has been proposed for periodontal regeneration, resulting in decreased hydrophobicity and increased biocompatibility of PDL cells which showed cell adhesion structures with spread morphology [90]. The main cause of damage in oral tissues is the presence of microorganisms which leave to destruction of soft and hard tissues, namely gingivitis and periodontitis. During or after conventional treatments, bacterial colonization is a relevant problem so that, antimicrobial strategies have to be explored to design antibacterial systems with local drug delivery capabilities [79,91]. For example, Minocycline loaded keratin has been used in periodontal tissue regeneration, confirming a suppressing effect on bacterial growth, without negative effects on biocompatibility [92].

For endodontic approach, biocompatibility of silk fibroin sponges on stem cells from human exfoliated deciduous teeth (SHEDs) has been investigated with success, highlighting a favorable contribution of proteins on cellular attachment and spreading [93]. In this context, FN molecules may be also immobilized on the scaffold surface, improving cell (i.e., hDPSCs) attachment and proliferation for the fabrication of substrates for dental pulp and periodontal regeneration [37,94].

6. Conclusions

The use of instructive devices currently represents a consolidated practice for treatment of hard and soft tissues in oral compartment [95,96]. In the last years, biological approaches based on the use of biomolecules inspired by tissue engineering principles is tracing new routes for the restoration/regeneration of natural tissues located in the oral cavity, from hard (i.e., bone, dentin) to softer ones (i.e., pulp, gum). Several studies have variously confirmed that proteins from natural source may be candidate as gold standard biomaterials with unmatchable regenerative properties. Hence, several biological effects need to be more deeply explored to validate their clinical use, and ultimately, to identify the most effective and safe treatment for personalized therapies of patients.

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Conflict of interest

No conflicts of interest.

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