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Review Article

Influence of Chitosan or Keratin on Titanium Implant Surface - A Systematic Review

Eliza Ranjit[1], Ajay Sharma[2], Stephen Hamlet[3], Roy George[4] and Robert Love[5*]

[1]PhD student, School of Dentistry and Oral Health, Griffith University, Australia
[2]Senior Lecturer in Periodontology, School of Dentistry and Oral Health, Griffith University, Australia
[3]Senior Research Fellow, School of Dentistry and Oral Health, Griffith University, Australia
[4]Associate Professor in Endodontics, School of Dentistry and Oral Health, Griffith University, Australia
[5]Head of School, Professor of Endodontics, School of Dentistry and Oral Health, Griffith University, Australia

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ABSTRACT

Purpose: This systematic review was carried out to investigate the effects of keratin and chitosan hydrogel preparations on dental implant osseointegration.

Materials and Methods: The electronic search was conducted on five databases: Scopus, EBSCOhost MEDLINE, EBSCOhost Dentistry and Oral Science, PubMed, and Web of Science. Studies that determined the *in vitro* or *in vivo* efficacy of keratin and chitosan hydrogel on osseointegration were included in the review.

Results: Of the 760 studies initially gathered, nine met the inclusion criteria. These studies demonstrated that dental implants coated with keratin and chitosan hydrogels resulted in improved biological properties. It was also concluded that the inclusion of chitosan in keratin hydrogels improves the mechanical strength and helps increase durability through ameliorating degradation and swelling characters. Both the polymers increased bone-implant contact and new bone formation in animal models.

Conclusion: This systematic review demonstrates that keratin and chitosan hydrogel, is effective in initiating osteogenesis, reinforcing the currently available evidence that these polymers could be a substrate in dental implant treatment.

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Introduction

Osseointegration is defined as a stable anchorage of bone tissue on an implant surface. It is a cascade of four processes namely hemostasis, inflammation, proliferation, and bone remodeling [1]. Dental implants have brought a revolution in modern dentistry [2]. The concept of integration of bone around the implant was described first by Brandmark more than 45 years ago [3]. The formation of bone to implant contact (known as %BIC) is a hallmark for successful osseointegration [4]. The degree of osseointegration depends on characteristics such as alloy type, its design, size, the surgical technique, bone quality/quantity and occlusal loading [5, 6]. All the above-mentioned attributes are needed for the long-term success and survival of the implant. Commercially pure titanium (Ti) and its alloys were widely used in orthopedic and dental implants due to its excellent parameters such as biocompatibility, good mechanical strength, and corrosion resistance [7, 8]. Although the dental implant success rate can be higher than 90%, there is still a numerically low chance of implant failure i.e., five-ten percent due to poor osseointegration, mechanical problems, immobilization, and infection [9]. Bioinert Ti lacks the properties of osteoconductivity or osteoinductivity in itself and it is susceptible to bacterial growth [10-12]. Even though Ti implants have been consistently used with a high success rate in clinics, utilization of various biomaterials has been recommended to achieve functions of bioactivity and antibacterial property [13].

There are still a number of challenges that lie in developing a dental implant possessing both enhanced osteogenic behavior and antibacterial properties. To address the aforementioned problems, various surface

*Correspondence to: Professor Robert Love, Head of School, Professor of Endodontics, School of Dentistry and Oral Health, Griffith University, Australia

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modifications such as roughness (macro, micro, nano, mixed), biofunctionalization, and texture fabrication along with a combination of various antibacterial nanoparticles (e.g. zinc oxide, silver) have been performed [7, 12, 14, 15]. Attempts using natural/synthetic polymers to mimic the extracellular matrix (ECM) of bone tissue both physic-chemically and biologically to enhance osteoconductive and/or osteoinductive effect has been investigated [16-18]. The natural polymers such as starch, collagen, gelatin, alginate, cellulose, elastin, silk fibroin, keratin, chitin etc. either alone or in combination, in different forms like gel, film, sponge, fibers, and foams have been developed. These biomaterials emulate the complex physiologic functions of the dynamic native tissue that facilitates cell-cell and cell-matrix interaction [8, 19, 20]. Among this group, the keratin protein and chitosan polysaccharide have emerged as effective natural biopolymers in the fabrication of orthopedic prosthesis and dental implants.

Keratin, a fibrous protein, is available in abundance as it is readily found nails, hair, wool, feathers, horns, and hooves [21]. Keratin contains two types of proteins; the intermediate filament protein (IF, alpha-keratin) and the matrix protein (gamma-keratin) bonded together with disulfide bonds that provide strength, mechanical integrity, and rigidity to keratin [22]. Keratin has the ability to self-assemble and polymerize into a porous and fibrous film, gel and scaffold [23]. Keratin possesses unique properties of bioactivity, biocompatibility, cytocompatibility, biodegradability, non-toxicity, and non-immunogenicity [24, 25]. Keratin derived from wool also contains cell adhesion motifs, namely arginine-glycine aspartic acid (RGD) and leucine-aspartic acid-valine (LDV), the site of cellular attachment which makes it osteoconductive [26]. Sierpinski *et al.* suggested that keratin contains regulatory molecules that induce mitogenic and chemotactic activities [27]. A biomimetic coating of keratin demonstrated significant potential in the area of wound healing, bone regeneration drug delivery, and nerve regeneration [15, 20, 26, 28-30].

Chitosan is a chitin-derived natural polysaccharide produced by a deacetylation reaction commonly from the shells of marine crustaceans such as shrimps, lobsters, crabs, and prawns or in the cell walls of fungi and yeasts and the pens of squids [31, 32]. Currently, marine-derived biopolymers like chitosan have shown effective applications in reconstructive plastic surgery, surgical, dental and orthopedic materials [32]. Chitosan has been used in different biomedical applications, including wound healing, soft tissue regeneration, drug delivery, bone regeneration, and infection, etc. [33]. Furthermore, chitosan is widely acknowledged for being biocompatible, biodegradable, cost-effective and having antimicrobial properties.

The combined effect of surface chemistry, topography, and bioactivity of a biomaterial determines its ultimate bonding with the surrounding tissue [28]. Other features such as a material being user-friendly and eco-friendly have increased the use of polymer-based materials. Their desirable biological properties have made them a suitable substrate for bone regeneration. Keratin and chitosan as a composite have shown a synergistic effect when applied in combination [34-36]. Although many studies have been reported on their use as a scaffold, their role in enhancing osseointegration for dental implants in the form of hydrogel is still limited. This systematic review, therefore, epitomizes the efficacy of keratin and chitosan hydrogel in accelerating and improving bone regeneration around an implant.

Table 1: Review protocol.

Review question	What is the effect on osseointegration of biomimetic coating of keratin and chitosan hydrogel on an implant?
Inclusion criteria	Paper focused on keratin and chitosan hydrogel in implantation Research mainly done for enhancement of osseointegration Research focused only in the implant Research done till cell differentiation in *in vitro* and *in vivo* Publication between 1980 and April 2019
Exclusive criteria	Presentations, book reviews, and all studies reported in non- English publication.
	Keratin/chitosan used in soft tissue engineering, drug release and antimicrobial activity
	Fabrication and characterization of the composite without titanium implant
Literature search	**Methods:** database searching, reference list checking, citation searching and consultation with an expert
	Databases searched: Scopus, Web of science, Pubmed, EBSCOhost dentistry and oral science source and EBSCOhost Medline
	Key words for database searching: 'keratin', 'keratin hydrogel', 'chitosan', 'chitosan hydrogel', keratin chitosan hydrogel',' keratin chitosan composite', 'titanium', 'implant', MeSH term-'dental implant' or 'tooth device' or 'implantation', 'bone tissue engineering', 'osseointegration', 'integration'
Quality assessment	**Methods**: *in vivo* and *in vitro* experiments
Data extraction	**Following information were extracted from relevant studies**: reference, place of study, study type, aim/objectives, brief description of methodology, result include physicochemical characteristics, mechanical properties, degradation properties, swelling properties, contact angel, bioactivity, bone to implant contact, resonance frequency analysis, conclusion. Software used for extraction data: Microsoft Excel

Materials and Method

I Protocol Development/ Focused Question

A protocol designed for the review process, including undertaken steps and criteria has shown in (Table 1) [37]. PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) was followed for the review (Figure 1) [38]. A specific research question was established according to the Participants, Interventions, Control, and Outcomes (PICO) principle: "What is the effect on osseointegration of biomimetic coating of keratin and chitosan hydrogel on an implant?"

Participants: the use of the dental implant in the study samples.
Interventions: the effect of the keratin and chitosan hydrogel on osseointegration.
Control: Comparison without keratin and chitosan hydrogel incorporation.

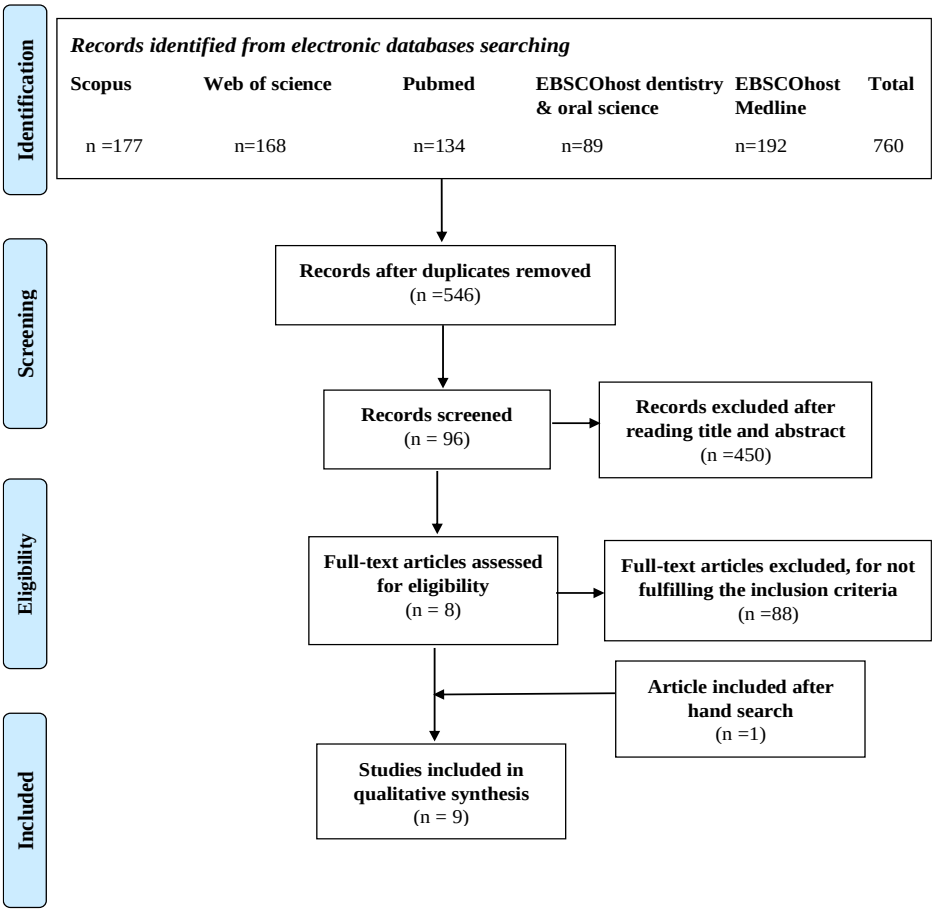

Figure 1: PRISMA flow-chart of the systematic literature review [38].

II Outcome Measure

i. ***In vitro* outcomes:** The primary outcome was the physicochemical, mechanical properties and the bioactivity of the polymers, including cell morphology, cell viability, cell proliferation, and differentiation.

ii. ***In vivo* outcomes:** The primary outcome was the BIC without an intervening connective tissue layer, new bone formation (NBF), and bone volume to total volume (BT/TV). Resonance frequency analysis (RFA), micro computed tomography (micro-CT), and radiographs come under secondary outcomes.

III Inclusion Criteria

i. Studies focusing on keratin and chitosan hydrogel for enhancement of osseointegration.
ii. Studies mainly reporting a coating on the implant.
iii. Both *in vitro* and *in vivo* studies were comprehended. Studies reporting in vitro (cell morphology, cell viability, cell proliferation, cell differentiation, and gene expression) and in vivo (%BIC) with a histological measurement of implant osseointegration and stability by resonance frequency analysis.
iv. Only publications in a peer-reviewed journal written in English were considered.

IV Exclusion Criteria

i. Presentations, book reviews, and all studies reported in non-English publications.
ii. Keratin/chitosan used in other applications such as soft tissue engineering, drug release, and antimicrobial activity.
iii. Fabrication and characterization of scaffold without titanium.

V Search Strategy

Five databases i.e., PubMed, Scopus, Web of Science, EBSCO host dentistry & oral science and EBSCO host Medline were searched electronically to extract all relevant papers for conducting a literature search until April 2019. The manual hand search of the reference lists of the selected studies was performed. To achieve a significant view on the effect of polymers on the implant, both *in vitro* and *in vivo* studies were included in this review. The keywords were searched in databases either separately or combined with AND or OR. The medical subject headings (MeSH) terms with the following terms: 'keratin', 'keratin hydrogel', 'chitosan', 'chitosan hydrogel', keratin chitosan hydrogel',' keratin

chitosan composite', 'titanium', 'implant', 'dental implant', 'tooth device', 'bone tissue engineering', 'osseointegration' were applied to fulfill the search strategy.

VI Screening Methods and Data Extraction

The studies with titles and abstracts that met inclusion criteria were screened and assessed. Data that was retrieved from the relevant studies included the following parameters: reference, place of study, study type, aim/objectives, a brief description of the materials and methodology, results, including physicochemical characteristics, mechanical properties, degradation properties, swelling properties, contact angel, bioactivity, and the conclusion. The screening process and data extraction was checked and approved by two independent examiners.

VII Data Synthesis and Statistical Analysis

Due to the significant heterogeneity in variables in the methodology, for instance, different concentrations and composition of the biopolymers used, implant types, its modifications method, different properties studied, time points, its coating process, drying, sterilization, animals used and cell types, meta-analysis could not be performed.

Results

Initially, 760 studies were identified through five electronic databases. After removing duplicates (n=214) and screening the titles and abstracts, 96 studies were selected and 450 were excluded for not relating to the topic. The full-text evaluation of screened studies was performed, 88 studies were omitted because of the exclusion criteria listed in the figure. Only 8 studies were finally selected, which satisfied the inclusion criteria [39-47]. Furthermore, an additional paper was found whilst hand searching the reference list of the selected studies thoroughly. Out of the nine experimental studies, four were *in vitro* studies, four were *in vivo* studies and one was an *in vitro*/*in vivo* study [39-47]. Among these studies, only two of the *in vivo* studies were performed in keratin and the rest of them, including *in vitro* and *in vivo* were conducted in chitosan [39-47]. Most of the papers were studied in China followed by New Zealand, USA, and Venezuela.

I Study Characteristics

The polymers characteristics, implant characteristics, physicochemical properties and bioactivity of the polymers of the final selected nine studies were depicted in Table 2, Table 3, Table 4 and Table 5 (*in vitro and in vivo*), respectively.

II *In vitro* Studies

All the studies identified were performed to assess the effect of chitosan on implants, i.e., no keratin studies where identified [39, 41, 44, 46, 47]. The degree of deacetylation of chitosan used ranged between 80% and > 95% and molecular weight 4.69 x 10^5 Da and 200 KDa [41, 44, 47]. In the studies, the follow-up period ranged between 1 day and 28 days. In three of the studies, composite such as chitosan + CaP, chitosan + strontium ranelate and chitosan + gelatin was used [39, 44, 46]. Photo-crosslinked chitosan with 4-azidobenzoic acid (AZ) and lactobionic acid (LA) was used in one study, whereas in one of the studies, the only chitosan was used [41, 47].

III Implant Characteristics

Pure Ti implants were used in three studies, while two studies used Ti alloy (Ti6Al4V). Different implant sizes and shapes were reported by different studies [39, 41, 44, 46, 47]. The dimension (diameter × length mm) of the implants used ranged between 10 mm x 1 mm and l4 mm x 1 mm [39, 46, 47]. Ti coupons of 5 x 1.5 x 0.2 cm and 1.5 x 1.5 x 0.2 cm were used in one study, while plates of size 20 x 20 x 1 mm were used in the other [41, 44]. Cylindrical type implant was placed in two studies, whereas coupons and plates were placed in two studies, respectively [39, 41, 44, 47]. Different types of methods to modified surface roughness was reported in three studies, which include 80 grit SiC paper, sandblasting with alumina particles, and coarse grit blasting [41, 46, 47]. However, in one study, grit blasting and acid etching were used together [39]. Only one study was found using a readymade implant with a roughness of 4 µm [44].

IV Fabrication of Polymers on Titanium Implants

Bumgardner *et al.*, fabricated chitosan on Ti by silanization followed by solvent casting to study the physicochemical properties and biocompatibility of chitosan [41]. Electromechanical deposition was used to modify Ti implant surfaces to study the potential of chitosan composite for bioactivity improvement [44, 46]. In two studies, dip coating and solvent casting were used for fabrication [41, 47]. Out of the five studies, three studies used a sterilization process, namely, ethylene oxide gas, gamma-ray and autoclave [39, 41, 46]. Four of the included studies employed an air-drying process [39, 41, 44, 46].

V Physicochemical Properties

Tensile strength was calculated in only one study [41]. Two studies evaluated the degradation rate and showed their gradual degradation after 28 days and 8 weeks [41, 46]. Out of the five studies, two studies discussed swelling properties which became stable between 50-120 minutes and only one study reported the contact angle [46, 47].

VI Assessment of Bioactivity

A variety of cells have been used to determine cellular affects, including primary cells and cell lines i.e., UMR-106 osteosarcoma cells, MC3T3-E1 cells, mesenchymal stem cells, primary osteoblasts and mouse MC3T3-E1 osteoblast [39, 41, 44, 46, 47]. Cell adhesion was determined as F actins, filopodia, spindle, and triangle or polygonal in three studies [39, 46, 47]. Only two studies showed data regarding cell viability [40, 46]. Out of the five studies, four reported cell proliferation [39, 41, 44, 47]. Cell differentiation was explained in two studies where alkaline phosphatase and collagen production were increased [44, 46]. Gene expression was analyzed in two studies and showed upregulation of gene expression of bone sialoprotein and osteocalcin [39, 44].

VII Main Outcomes

The results from all the studies showed that a coating of chitosan and its composite onto implant surfaces enhanced osseointegration, except in

the study carried out by Ma *et al.*, in which MC3T3-E1 cell proliferation and the ALP activity was not affected by a coating [46].

VIII *In vivo* Studies

Three chitosan studies were performed in male rabbits [40, 45, 46]. The different time points from 2 to 52 weeks were followed up to assess BIC value [40, 45, 46]. Chitosan alone was used in one of the studies, whereas in the remaining two, its composite with calcium phosphate (CaP) or gelatin was studied respectively [40, 45, 46]. The number of animals used in the studies was heterogeneous, ranging from 16 to 36 [40, 45, 46]. The concentration of chitosan used ranged from 92% to >95% degree of deacetylation with a molecular weight from 105KDa to 4.69 x 10^5 Da [40, 46]. Bumgardner *et al.* studied the influence of chitosan on osseointegration in trabecular bone [40]. Jiawei *et al.* studied the effect of chitosan for early bone formation and Ma *et al.* assessed the osteogenic behavior of coated chitosan [45, 46]. Two keratin studies were carried out in mixed breed sheep with different time points from 2 to 16 weeks. The number of animals used in both studies was 6 to 12 respectively [42, 43].

IX Implant Characteristics

In all studies, between twenty and seventy-two Ti implants were used [40, 42, 43, 45, 46]. The dimension (diameter x length mm) of the implants used ranged between 4 mm x 2 mm to l3.5 mm x 7 mm [40, 42, 43, 45, 46]. Rough surface implants were used in all studies [40, 42, 43, 45, 46]. Neoss Ti implants were used in keratin experiments. Both keratins treated implants were placed in femur condyles. Screw type implant and bimodal surface was placed in two studies [42, 43].

For chitosan, two studies used a pure Ti implant and only one study was found that used Ti alloy - Ti6Al4V [40, 45, 46]. Two studies had placed the implant in femur condyles whilst one study experimented with the tibia as an anatomical position [40, 45, 46].Various implant shapes were used in experiments, including pin-shaped, cylindrical-shaped and a special design, including one gap (1.6 mm width and 0.3 mm depth) implant [40, 45, 46]. The grit blasting Ti implant, Ti grit with 80 silica carbide paper and a sandblasted Al2O3 implant were placed respectively [40, 45, 46].

X Fabrication of Polymers on the Implants

Bumgardner *et al.*, used the salinization process to coat chitosan on the implant surface followed by a solvent casting method [40]. Jiawei *et al.*, and Ma *et al.*, fabricated chitosan composite onto implants using an electrodeposition process [45, 46]. Only in two studies, application of air-drying process was noted after coating [40, 46]. Two studies used sterilization method before inserting implant into animals i.e., ethylene gas and autoclave [40, 46]. However, the keratin coating method was not mentioned in either of the studies, while Gamma radiation was used to sterilize the coated implant [42, 43].

XI Physicochemical Characteristics

The thickness of the coating was measured only in one chitosan study [45]. In two studies of chitosan composite, the degradation rate was measured where no coating was noted after 26 weeks and 12 weeks [45, 46]. None of the included studies reported on the mechanical properties of polymers after coating on the implant.

XII Assessment of Osseointegration

In all studies, osseointegration was determined by using a histologic analysis to evaluate the efficacy of chitosan and keratin [40, 42, 43, 45, 46]. For chitosan, two studies used histomorphometric analysis and BIC was calculated to determine the rate of osseointegration [45, 46]. Micro-CT, BT/TV, and radiographs were used to assess new bone [40, 46]. Keratin studies used histomorphometric analysis and calculated BIC to determine the rate of osseointegration [42, 43].

XII Main Outcomes

The outcomes of in vivo studies are detailed in (Table 6).

Table 2: Polymer characteristics and fabrication methods applied in *in vitro* and *in vivo* studies.

S. No	Ref.	Place	Polymers	MW &DDA	Source	Coating method	Drying	Sterilization
		In vitro						
1	(41)	USA	Chitosan	91.2%/200KDa	-	Silanization by IPTS & solvent casting	7–10 days at 21°C	Ethylene oxide gas
2	(44)	China	Chitosan+CAP	85%/ -	-	Electrodeposition	50°C overnight	-
3	(47)	Venezuela	Photocrosslinked chitosan	80%/ 4.3x105 Da	-	Dip coating	-	-
4	(39)	China	Chitosan+Strontium Ranelate	-	-	Solvent casting	50°C	Gamma radiation
		In vivo						
1	(42)	New Zealand	Keratin	-	Wool	-	-	Gamma radiation
2	(43)	New Zealand	Keratin	-	Wool	-	-	Gamma radiation

3	(40)	USA	Chitosan	92.3%/4.69x105 Da	-	Silanization by APTES & solvent casting	Drying for 7 days	Ethylene oxide gas
4	(45)	China	Chitosan+CAP	-	-	Electrodeposition	-	-
5	(46)	China	Chitosan+gelatin	<95%/100KDa	-	Electrodeposition	Air drying	Steam autoclaving machine

DDA: Degree of deacetylation; MW: Molecular weight; APTES: Aminoprophyltriethoxysilane; IPTS- 3: Isocyanatopropyltriethoxysilane.

Table 3: Implant Characteristics used in *in vitro* and *in vivo* studies.

S No	Ref.	Place	Polymers	Animal	No. of animal used	Implant type	Implant No.	Implant size	Implant character	Implant shape	Animal anatomical
In vitro											
1	(41)	USA	Chitosan	-	-	Titanium coupons	-	26 coupons: 5 x1.5x0.2 cm; 58 coupons: 1.5x 1.5x 0.2 cm	Wet ground with 80 grit SiC paper	Coupons	-
2	(44)	China	Chitosan+ CAP	-	-	Ti6Al4V plates	-	20 x 20 x 1 mm	Readymade roughness of 4.0 um	Plates	-
3	(47)	Venezuela	Photocrosslinked chitosan	-	-	Ti6Al4V implants	-	10 mm x 1 mm	Sandblasting using alumina particles, 4.1 ± 0.9 µm	Cylindrical	-
4	(39)	China	Chitosan+Strontium Ranelate	-	-	Pure titanium	-	14 mm x 1 mm	Grit-blasting and acid etching	Cylindrical	-
In vivo											
1	(42)	New Zealand	Keratin	Sheep	6	Neoss titanium	24	7 mm x 3.5 mm	Bimodal	Screw	Femoral condyle
2	(43)	New Zealand	Keratin	Sheep	10	Neoss titanium	20	l 3.5 mm x 7 mm	Bimodal rough surface, double particle blasting with two grades of ceramic particles	Screw	Femur
3	(40)	USA	Chitosan	Rabbit	16	Titanium pins	64	4 mm x 2 mm	Wet ground with 80- grit SiC paper	Pins	Tibia
4	(45)	China	Chitosan+CAP	Rabbit	36	Ti6Al4V rods	72	8 mm x 3 mm	Sandblasted with Al2O3	Cylindrical	Femoral condyle
5	(46)	China	Chitosan+gelatin	Rabbit	16	pure titanium rods	32	8 mm x 3.3 mm	Coarse grit blasted with 0.25–0.05 mm corundum grit	implants with one gap	Femoral condyle

Table 4: Physicochemical properties of the polymers in *in vitro* and *in vivo* studies.

S. No	Ref.	Polymers	Thickness	Contact angel	Mechanical properties	Degradation	Swelling ratio
In vitro							
1	(41)	Chitosan	-	-	1.5–1.8 Mpa, three times more than non-coated	Stable and minimal degradation after 8 weeks	-
2	(44)	Chitosan+CAP	-	-	-	-	-
3	(47)	Photocrosslinked chitosan	-	67.5° ± 0.9°	-	-	Rapid till 60 mins

4	(39)	Chitosan+Strontium Ranelate	-	-	-	-	-
5	(46)	Chitosan+gelatin	-	-	-	Significant degradation after 28 days	Stable after 50 -120 min
In vivo							
1	(42)	Keratin	-	-	-	-	-
2	(43)	Keratin	-	-	-	-	-
3	(40)	Chitosan	-	-	-	-	-
4	(45)	Chitosan+CAP	30–40 mm	-	-	No coating present after 26 and 52week	-
5	(46)	Chitosan+gelatin	-	-	-	Coating found hardly till 12 weeks	-

Table 5: Effect of polymers on the biological behavior in *in vitro* and *in vivo* studies.

S. No	Ref.	Polymers	Cell types	Time points (Days)	Cell adhesion	Cell viability	Cell proliferation	Cell differentiation	Gene expression	Remarks
In vitro										
1	(41)	Chitosan	UMR-106 osteosarcoma cells	1,2,3 (Viability)	-	↑	↑ than uncoated Ti	-	-	Supported and facilitated osseointegration
2	(44)	Chitosan+CAP	MC3T3-E1 Subclone 4	3,5,7,9 (Proliferation) /7,10,14 days (differentiation)	-	-	↑ than CAP coated	↑ALP/Collagen 14 days	↑sialoprotein and osteocalcin	Favored proliferation and differentiation of MC3T3-E1 cells
3	(47)	Photocrosslinked chitosan	Mesenchymal stem cells (MSC) from male Sprague Dawley rats	4,10 and 16 (proliferation)	Filopodia	-	↑ in 16 days	-	-	Improved osteogenic potential at the tissue–implant interface
4	(39)	Chitosan+Strontium Ranelate	Primary osteoblasts (the calvaria of 1- to 3-day-old neonatal mice)	1,3,5, and 7 (Proliferation, differentiation and gene expression)	Spindle/ triangle /polygonal	-	↑ than uncoated Ti	-	↑ Runx2, ALP, BMP, OCN	Promotes osteoblast proliferation and differentiation in a dose-dependent manner
5	(46)	Chitosan gelatin	Mouse MC3T3-E1 osteoblast cell line	1,3,7 (Proliferation) 7,14,28 (Differentiation)	F actins in 2 day	↑ 7 days	-	↓ from 7 to 28 days	-	Coating did not affect the MC3T3-E1 cell proliferation and the ALP activity

In vivo					
S. No	**Ref.**	**Polymers**	**Implantation period (weeks)**	**Analysis Methods**	**Remarks**
1	(42)	Keratin	2, 4, 8, 12 and 16	Histologic, Histomorphometric analysis	Improved the % BIC of titanium implants after 2, 4, 8, 12, and 16 weeks with higher improvement at 4 weeks
2	(43)	Keratin	4	Histologic, Histomorphometric, Resonance frequency analysis (RFA)	↑ %BIC of implants by 169% compared to control implants,

					RFA value higher on test implant than control
3	(40)	Chitosan	2, 4, 8, and 12	Histologic, Radiographs	Develop new bone in 2 weeks, and higher rate found in 12 weeks
4	(45)	Chitosan +CAP	2,4,26, and 52	Histologic, Histomorphometric analysis	Bone apposition is different at early time but almost the same after 52 weeks
5	(46)	Chitosan +gelatin	2, 4, 8, and 12	Histologic, Histomorphometric, Micro-CT, Bone volume to total volume (BV/TV)	↑ newly formed bone in the region of interest from 2 to 12 weeks, Improved BIC after 2, 4, 8,12 weeks. Macroporous structure facilitated osteogenesis in vivo

Table 6: Outcome of chitosan and keratin hydrogel on implant.

Polymers	Outcomes	Reference
Chitosan	Chitosan-CAP composite reported greater bone apposition than chitosan coating	(45)
	Chitosan-gelatin composite showed significant new bone formation on Ti implant than sandblasted implant	(46)
	Chitosan with silanized implant showed successful osseointegration	(40)
Keratin	Keratin enhanced early osseointegration around titanium implant BIC% was higher than test samples.	(42)
	Keratin coated implant promoted osseointegration in poor bone quality BIC% was increased significantly Higher implant stability quotient values (ISQ)	(43)

Discussion

To our knowledge, this is the first systematic review that investigated the efficacy of chitosan and keratin polymers on enhancement of dental implant osseointegration. Overall, all studies showed a positive effect in promoting osseointegration regardless of the types and shapes of disk used, roughness process, sterilization methods, and animals used.

Different concentrations of chitosan were used among the studies conducted. Notwithstanding, 2% of chitosan concentration was used in three studies and has shown good cellular behavior with osteoblastic cells. Thus, it may be proposed that 2 % of chitosan concentration is suitable for enhancing cellular activity in all aspects, including cell recruitment, cell proliferation, and cell differentiation.

According to Xuereb *et al.*, two criteria determine the function and durability of the coating material: the ability to resist the load-bearing forces developed during mastication and to maintain a strong bonding between biomaterials and implant. The coated implant should be capable of withstanding all the forces which are imposed on it. Stress generated on the bone-implant interface exceeding the bonding strength can result in delamination and cracking of the coating, this can be prevented by forming strong and stable bonding [48]. Several methods have been employed for fabricating coating materials on the Ti implant surface. Jiawei *et al*., Wang *et al.*, and Ma *et al*., immobilized chitosan on Ti implant surfaces using electromechanical depositions, whereas chemical process called silanization was used to incorporate chitosan under Ti implant surfaces in two studies [40, 41, 44-46]. Investigation of the bone-forming process with keratin is limited. The coating process of keratin on an implant was not explained clearly in the listed studies. It is important to study the mechanical and physicochemical properties of the hydrogel after coating. None of the *in vivo* studies have demonstrated physicochemical and mechanical characterization of the coated Ti implant except in one chitosan study done by Bumgardner *et al*., in which tensile strength was calculated to be three times greater than the non-coated i.e., 1.5-1.8 Mpa [41]. This shows that chitosan integration by chemical functionalization on Ti was able to establish a stable coating. Regardless of various immobilization techniques designed, there is still a need for consensus for surface modification techniques in the studies included.

While studying the coating materials and techniques, it is mandatory to study their degradation, swelling, contact angle and thickness as all these properties directly affect the implant's function both mechanically and biologically. The degradation rate for scaffold materials should match the speed of the new tissue formation. To gain mechanical stability for the long term, coating material should exhibit optimal degradation behavior i.e., slow and controlled manner [49]. In an *in vitro* study, the degradation rate was found stable after 8 weeks and 28 days [41, 46]. Degradation of chitosan is dependent on the solvent used, the pH of the solution, crystallinity, sterilization procedures and presence of enzymes. Chitosan was found degraded significantly faster in the presence of lysozyme than in phosphate-buffered saline (PBS) [50]. The *in vivo* studies conducted by Jiawei *et al*. and Ma *et al*., the degradation rate of chitosan was found stable after 26 weeks and 12 weeks, respectively. The variation in the results may be attributed to their compositional and physicochemical characteristics [45, 46]. The other reason could be because of the utilization of chitosan with different degrees of deacetylation and molecular weight. Higher the degree of deacetylation, lower the rate of degradation of the chitosan and vice-versa [41].

Another important parameter of biomaterials is the thickness of the material coating the Ti implant. In the study by Jaiwei *et al*., a coating thickness of 30-40 µm was prepared which showed better early bone apposition without any inflammation signs [45]. The surface roughness could give an unwanted irregular texture if modified by a thick or irregular coating layer so, to maintain the original surface roughness, it is necessary to obtain a thin and uniform coating. A uniform and thin hydroxyapatite coating layer on Ti implants exhibited increased bond strength, and wettability properties [51]. Similar results were demonstrated in the aspects of swelling as well. In the *in vitro* study, stable swelling after 60 mins were demonstrated [46, 47]. This result indicates that the dried scaffold of a polymer when immersed in PBS, swells over ten times more than in dry conditions [46].

Implant surface roughness has been shown to increase the surface area that ultimately enhances osseointegration. Numerous studies have demonstrated that rough implant surfaces show better bone apposition and BIC than smooth implant surfaces [52]. Hence, modification of the rough implant surfaces with biopolymers like chitosan and keratin may further enhance bone formation, thereby increasing BIC and NBF. In all the identified studies, bone formation and osseointegration was enhanced after addition of these polymers on the rough surface implants.

Keratin extracted from sheep wool was employed in the listed studies to promote the response of osteoblastic cells. However, many studies have demonstrated the use of keratin derived from human hair and its role in bone regeneration and osseointegration and thus, there is a need for further researches to exploit keratin from different sources in biomedical applications [53, 54].

Chitosan has attracted considerable attention in dentistry due to its strong mechanical properties and biocompatibility, but it lacks bioactive signaling for cellular activity. Furthermore, it has also been reported that it is not osteoconductive by itself [55]. Recently, the keratin scaffold has been intensively developed by many researchers because of its potential application in the tissue healing process. Nevertheless, in practical use, pure keratin is weak in mechanical strength due to cleavage of disulfide bonds that occur during its extraction process as it contributes to the mechanical strength of keratin [21, 56, 57]. The main downside of these coatings is the limited usability in load-bearing areas. Hence, to rectify such drawbacks, two or more polymers are cross-linked, tailored and tuned to improve mechanical and biological properties. Such composite combines the desirable attributes from each polymer, for example, cellular responses and mechanical strength. Therefore, combining the osteoconductivity and osteoinductivity of keratin and the mechanical properties of chitosan, could improve the mechanical stability of the composite and effectively fasten the rate of osseointegration [58]. Besides that, this composite also improves structural properties, slows degradation rate and increases swelling properties of the scaffold that are associated with cellular activity. Till date, keratin-chitosan hydrogel has been employed in different biomedical areas such as tissue engineering, soft tissue engineering, nerve regeneration, an infection like gingivitis, corneal treatment, wound healing, bone regeneration, drug release, and antimicrobial property [15, 25, 26, 28, 30, 35, 55, 59-68]. However, there is no evidence of this composite to have been used for a dental implant yet.

Osteoblast cell culture grown in chitosan and its composite showed increased cell proliferation, cell differentiation and an increase in different osteoblast markers gene expressions such as alkaline phosphatase (ALP), bone matrix protein (BMP), osteocalcin (OC), sialoprotein (SP), and RUNX2. This led to the conclusion that chitosan and its composite have biocompatibility for different osteoblastic cells and primary cell lines like mesenchymal stem cells (MSCs), thereby supporting and facilitating osseointegration on the peri-implant sites. However, there is no *in vitro* study done on keratin coated Ti for an acceleration of osseointegration.

Chitosan studies were primarily performed in rabbits, while sheep were used for keratin studies. International standards have stated that dogs, sheep, goats, pigs or rabbits are suitable for testing materials for bone implantation (International Standard ISO 10993-6, 1994) [69]. Rabbits were used due to the similar mechanical properties of the human bone and large animals like sheep was emphasized mainly due to its cost, ease of handling, and also due to the fact that they have shown more promise as animal models [43, 69, 70]. The *in vivo* studies are of long-term duration which is performed for a period from 2 to 52 weeks. While comparing the %BIC of keratin and chitosan, both polymers displayed similar influences after 2 weeks of implantation as compared to uncoated Ti. Keratin hydrogel has shown better osteogenic behavior in trabecular and cancellous bone.

A variety of methods were proposed in the identified studies to determine osseointegration. All the studies used histology to assess BIC around implants. Histomorphometric analysis was performed to determine BIC, except in the study by Bumgardner *et al.*, [40, 42, 43, 45, 46]. Radiographs were used in a study by Bumgardner *et al*., while Ma *et al*., used micro-CT and BV/TV to evaluate the osseointegration by measuring NBF. Resonance frequency analysis was used to test the stability of a keratin coated implant. It is a common technique that measures ISQ value which was found significantly higher in keratin-treated implants after 1-month of healing, indicating improved osseointegration [71]. However, its reliability and accuracy in determining osseointegration are still contradicting in the literature. Therefore, in experimental studies it should be done concurrently with histological and histomorphometric analysis to determine osseointegration. The histological examination continues to be taken as a gold standard, although many studies have applied different methodologies to measure BIC and NBF.

The inadequate number of studies that were included due to the unavailability of related research papers in this field of study is the limitation of the presented review. Meta-analysis was also not performed in the review due to the significant heterogeneity in the outcomes of the data presented. Lack of data on the physicochemical and mechanical properties in the included studies which were required to design an appropriate scaffold in bone tissue engineering also presented itself as one of the major limitations of this review. The use of fewer animals and lack of stability tests after implantation in the *in vivo* study is another limitation. By considering these limitations, the findings from this systematic review should be implicated with caution.

Future Direction

The utility of chitosan and keratin as bioactive molecules has been exponentially increasing in the field of tissue engineering due to their ability to mimic the native tissue/microenvironment and excellent properties such as biocompatibility, biodegradability, non-toxicity, and non-immunogenicity. However, further investigation, including physicochemical and mechanical parameters (such as surface chemistry, topography, microstructure, biodegradation, swelling property, tensile, and compression strength), and its biological function in both lab and animal should be undertaken thoroughly to elucidate the role of these biomaterials on the implant surface. In addition, feasible and reproducible grafting techniques for forming stable and strong bonding between two dissimilar materials need to be studied and implemented for the scientific and clinical uses.

Well-designed research studies are required to assess if the modification on the implant surface with chitosan and keratin hydrogel in a clinical

context would facilitate osseointegration in patients with different conditions like poor bone quality and quantity, immunocompromised and diseased case along considering their behavior characteristics. Finally, to improve the mechanical strength of chitosan and biological function of keratin, the composite on the implant need to be focused, as both biopolymers may work synergistically to facilitate osseointegration.

Conflicts of Interest

None.

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