SYSTEMATIC REVIEWS

Smart scaffolds in bone tissue engineering: A systematic review of literature

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Abstract

AIM: To improve osteogenic differentiation and attachment of cells.

METHODS: An electronic search was conducted in PubMed from January 2004 to December 2013. Studies which performed smart modifications on conventional bone scaffold materials were included. Scaffolds with controlled release or encapsulation of bioactive molecules were not included. Experiments which did not investigate response of cells toward the scaffold (cell attachment, proliferation or osteoblastic differentiation) were excluded.

RESULTS: Among 1458 studies, 38 met the inclusion and exclusion criteria. The main scaffold varied extensively among the included studies. Smart modifications included addition of growth factors (group Ⅰ-11 studies), extracellular matrix-like molecules (group Ⅱ-13 studies) and nanoparticles (nano-HA) (group Ⅲ-17 studies). In all groups, surface coating was the most commonly applied approach for smart modification of scaffolds. In group Ⅰ, bone morphogenetic proteins were mainly used as growth factor stabilized on polycaprolactone (PCL). In group Ⅱ, collagen 1 in combination with PCL, hydroxyapatite (HA) and tricalcium phosphate were the most frequent scaffolds used. In the third group, nano-HA with PCL and chitosan were used the most. As variable methods were used, a thorough and comprehensible compare between the results and approaches was unattainable.

CONCLUSION: Regarding the variability in methodology of these *in vitro* studies it was demonstrated that smart modification of scaffolds can improve tissue properties.

Key words: Bone tissue engineering; Scaffold; Growth factor; Nanoparticle; Extracellular matrix

Core tip: Currently, special attention has been directed to the design of new scaffolds by adding bioactive molecules and nanoparticles. "Smart scaffolds" in bone tissue engineering not only act as cell delivery materials, but they are also responsive to their environment and therefore

stem cells are more likely to attach, proliferate and differentiate on them. These scaffolds can be fabricated by adding either of growth factors, extracellular matrix proteins or nanoparticles to the bone substitutes using various techniques. These modifications can enhance the *in vitro* response of bone scaffolds toward cells.

INTRODUCTION

Tissue engineering is a helpful alternative strategy for conventional treatments in medicine. It was defined by Langer and Vacant[1] as "an interdisciplinary field of research that applies the principals of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ". Bone tissue engineering uses life sciences and engineering to aid the function of injured bone tissue *via* a triad of artificial extracellular matrix (ECM) scaffold, stem cells that can become osteoblasts and growth factors[2,3]. The efficacy of this combination has been studied in animals[4-6] and humans[7-9]. Mesenchymal stem cells, the most common source of osteoprogenitor cells, can be derived from bone marrow[10-13], adipose tissue[14-16], and dental and periodontal tissues[17-19]. Growth factors, such as bone morphogenic proteins (BMPs), have the capability to modulate stem cell activity towards bone regeneration[20-24].

Scaffolds play a key role in bone tissue engineering providing a 3-dimensional environment for cell seeding and proliferation as well as filling bone defects while providing mechanical competence during bone regeneration[25-27]. Osteoconductivity, porosity and biodegradation are the required properties for a scaffold to be successful in bone tissue engineering, enhancing bone formation and angiogenesis and supporting attachment and proliferation of osteoblasts[28]. In this context, various scaffolds such as hydroxyapatite HA, tricalcium phosphate (TCP), collagen, chitosan, polycaprolactone (PCL), and poly(lactic-co-glycolic acid) (PLGA), have been used[2,3].

Recently, tissue engineers have focused on structural design and surface properties of scaffolds. Various modifications such as addition of bioactive molecules or nanoparticles can enhance attachment and proliferation of stem cells on the scaffold[29-31]. These "smart scaffolds" improve osteogenic differentiation of stem cells leading to a more responsive reaction to changes in their surrounding environment[32]. The current study reviews newly developed scaffolds and their smart modifications.

MATERIALS AND METHODS

Study selection

Papers relevant to *in-vitro* experiments on newly fabricated smart scaffolds were reviewed. Three modifications were considered as smart namely: (1) addition of nanoparticles; (2) addition of ECM-like molecules; and (3) addition of growth factors. Studies which performed these smart modifications on conventional bone scaffold materials were included. Scaffolds with controlled release or encapsulation of bioactive molecules were not included. Experiments which did not investigate response of cells toward the scaffold (cell attachment, proliferation or osteoblastic differentiation) were also excluded.

Search strategy

An electronic search was conducted in PubMed limited to English language, relevant publications from January 2004 to December 2013 with available full texts. Published papers on smart scaffolds were found using the following keywords alone or in combination: bone tissue engineering, smart scaffold, xenograft, xenogenous bone, allograft, allogenous bone, bone substitute, bovine bone matrix, allogenic bone, xenogenic bone, coat*, surface modification, surface enhancement, biofunctional*, bioact*, biomimet*, nano*, extracellular matrix, collagen and growth factor.

Initial paper selection was done by reviewing titles and abstracts of all selected papers. The full texts of potentially suitable articles were obtained for final assessment according to the exclusion and inclusion criteria. The reports of the most relevant data were included and analyzed in a qualitative manner.

Statistical analysis

This study was a literature review and no statistical method was used in this study.

RESULTS

Figure 1 demonstrates the design of the current review. The initial search resulted in 1458 articles. Following the screening of titles, abstracts and full texts, 38 papers had the criteria for review.

Growth factors

Eleven experiments were studied (Table 1) for scaffold modification; in all these studies, surface coating *via* growth factors to increase scaffold activity was used (Figure 2A). In addition, modification of architecture and spatial conformation of the main scaffold was performed[38]. The main scaffold differed in each study. The most commonly used scaffold was PCL, followed by TCP, PLGA, Bio-Oss, Calcium/magnesium-doped mesoporous silica, calcium phosphate cement, collagen, carbon nanotubes, TolAIII fusion-osteopontin-switch tag and glutaraldehyde cross-linked gelatin (GTG). The most
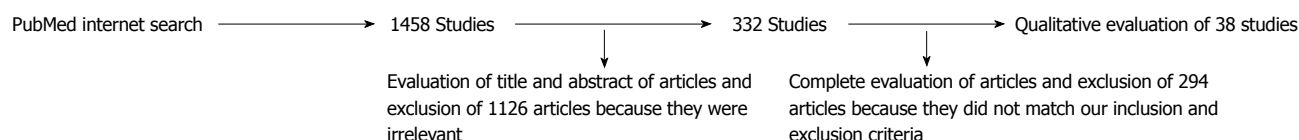
PubMed internet search $\longrightarrow$ 1458 Studies $\longrightarrow$ 332 Studies $\longrightarrow$ Qualitative evaluation of 38 studies

Evaluation of title and abstract of articles and exclusion of 1126 articles because they were irrelevant

Complete evaluation of articles and exclusion of 294 articles because they did not match our inclusion and exclusion criteria

Figure 1 Study design.

Table 1 Description of included studies which modified the scaffolds by addition of growth factors

Ref.	Scaffold	Fabrication	Type of modification	Cell type	Tests and results
Yang et al[33], 2005	GTG + BMP-4	-	Surface modification	NRCOCs	ALP activity: ALP levels in GTG + BMP-4 samples higher than GTG samples H and E staining: Greater numbers of attached cells and richer matrix deposits in the GTG + BMP-4 samples VK staining: Larger mineralizing nodules, in greater numbers
Turhani et al[34], 2007	TCP + rhBMP-2	-	Surface coating	SaOS-2Cs	Cell viability and ALP activity: TCP + BMP-2 > TCP (control) OC secretion: TCP + BMP-2 = TCP
Abarrategi et al[29], 2008	β-TCP + rhBMP-2	Homogenized + demineralized + heterogeneous deacetylation	Surface coating	C2C12Cs	No alteration in biocompatibility *In vivo*: New bone formation 3 wk after surgery, much shorter time than control β-TCP ceramics
Fei et al[35], 2008	PLGA/CPC + rhBMP-2	Solvent-extraction technique	Surface coating	BMMSCs	ELISA: OC: PLGA/CPC + rhBMP-2: 1.2 ng/mL rhBMP-2/CPC: 0.4 ng/mL ALP activity: PLGA/CPC + rhBMP-2: 0.12 μg/h rhBMP-2/CPC: 0.06 μg/h
Yilgor et al[36], 2010	PCL + BMP-2/ BMP-7	Plotting procedure Bioplotter's CAD/CAM software + wet spinning	Scaffold architecture + surface coating	BMMSCs	ALP activity: BMP-2/BMP-7 + PCL: 1.2 nmol/min
Zhang et al[37], 2010	PCL + BMP-2	Crosslinking conjugation method	Surface coating	BMMSCs	RT-PCR: (relative) p-smad: PCL + BMP-2 conjugated five times higher than adsorption and control Col 1: PCL + BMP-2 conjugated: 3 PCL + BMP-2 adsorption: 1.5 Control (PCL): 1 ALP activity: 2 times higher than adsorption and control
Mitchell et al[38], 2010	Tol-OPN-ST+BMP2	-	Spatial and conformational display + surface coating	BMMSCs	Cell attachment: Tol-OPN-ST + BMP2: 175 mm³ Tol-OPN-ST: 60 mm³ Luciferase activity: Tol-OPN-ST + BMP2: 8000 ability unit Tol-BMP-ST: 6000 ability unit
Huh et al[39], 2011	Bio-Oss® + rhBMP-2 + iH	Deep and dry methods	Surface coating	MG63Cs	Cytotoxicity and proliferation: No difference compared to control (Bio-Oss®) ALP activity: 0.2 μmol/min per μg higher than control
Dai et al[40], 2011	CMMS + rhBMP-2	Polymeric sponge method	New composition + surface coating	BMMSCs	MTT assay: More viable cells on CMMS + rhBMP-2 compared to CMMS RT-PCR: (relative) RunX2: CMMS + rhBMP-2: 32 Control (CMMS): 4 OPN: CMMS + rhBMP-2: 38 Control: 3 *In vivo*: Induced the ectopic bone formation in the thigh muscle pouches of mice
Lu et al[41], 2012	Col/PLGA + CBD-BMP4	Forming collagen microsponges	Surface coating	BMMSCs	ALP activity: No differences compared to control group Scaffold supports cell adhesion and proliferation
Li et al[42], 2013	SWNTs-COOH/ SWNTs-CH3 + BMP-2	Organic phase/aqueous phase replacement approach + sonication	Surface coating	C2C12Cs	ALP activity: (relative) SWNTs-ch3 + BMP-2: 150% SWNTs-cooh + BMP-2: 120%

TCP: Tricalcium phosphate; PCL: Poly(e-caprolactone); CMMS: Calcium/magnesium-doped mesoporous silica; CPC: Calcium phosphate cement; GTG: Glutaraldehydecrosslinked gelatin; PLGA: Poly(lactic-co-glycolic acid); col: Collagen; SWNTs-COOH and SWNTs-CH3: Hydrophilic COOH- and hydrophobic CH3-terminated single-walled carbon nanotubes; Tol-OPN-ST: TolAIII fusion-osteopontin-switch tag; rh: Recombinant human; BMP: Bone morphogenetic protein; CBD: Collagen-binding domain; BMMSCs: Bone marrow mesenchymal stem cells; NRCOCs: Neonatal rat calvaria osteoblast cells; MG63Cs: Human osteosarcoma cells; SaOS-2 Cs: Sarcoma osteogenic cells; C2C12C: Immortalized mouse myoblast cells; ALP: Alkaline phosphatase; H and E: Hematoxylin and eosin; VK: Von Kossa; RT-PCR: Real time polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; XTT: Sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt; RunX2: Runt-related transcription factor 2; OPN: Osteopontin.
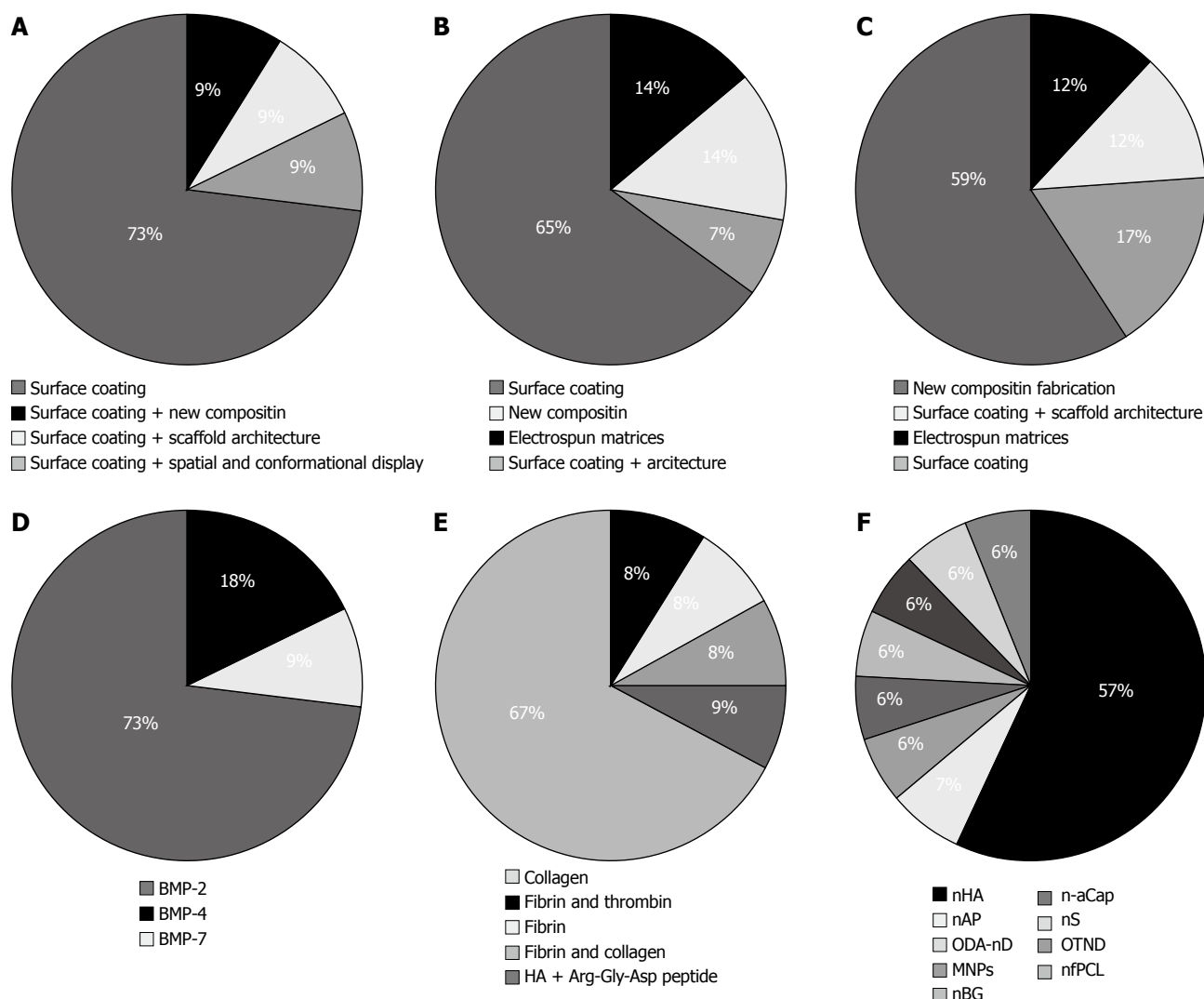
Figure 2 Type of modification method and smart materials in each category. A: Methods for growth factor addition; B: Methods for ECM molecules addition; C: Methods for nanoparticles addition; D: Growth factors added; E: ECM molecules added; F: Nanoparticles added. ECM: Extracellular matrix.

commonly applied growth factor was BMPs including BMP-2, BMP-4 and BMP-7 (Figure 2D). Different methods were used to add BMPs to the main scaffold: attachment of BMP-4 to scaffolds by cross linking conjugating techniques, deep and dry methods for addition of BMP-2 to Bio-Oss and using a set of nanoparticles providing the release of BMP-2. Scaffold fabrication methods varied vastly among studies.

Regarding the study methods, bone marrow mesenchymal stem cells (BMMSCs) were used in 6 experiments, while different type of cells such as neonatal rat calvarial osteoblast cells, Human osteosarcoma cells (MG63Cs), sarcoma osteogenic cells (SaOS-2Cs) and immortalized mouse myoblast *cell*s (C2C12Cs) were used in other studies. In almost all studies, cell viability was assessed with alkaline phosphatase (ALP) activity assay and the best results were obtained using new compositions compared to control groups. In just one study, no meaningful changes were observed[41]. One study used luciferase activity as their major assay[38]. In 2 studies, real time

polymerase reaction assay was used with different biomarkers such as: BMP-2, Runt-related transcription factor 2 (Runx-2), Osteopontin, P-smad and collagen 1.

ECM-like molecules

Among 67 articles, which used ECM-like molecules to enhance scaffold properties, 13 experiments were studied (Table 2). Type of scaffold modification in 10 studies was surface coating. In 1 study, architectural and spatial conformation was performed on the scaffolds[48], and in 2 experiments collagen fibers were elecrospun in conjugation with polymer and ceramic components to form a fibrous scaffold (Figure 2B)[45,52]. Xu *et al*[30] produced a new composite scaffold by combining collagen, bioglass, hyaluronic acid and phosphatidylserine. In a study by Lechner *et al*[44], fibrinogen was added to bone tissue core. In their study, CD31 was assessed before and after treatment with newly developed scaffold by fluorescent-activated cell scan techniques and their results showed 100-fold increase in expression of CD31 marker[44]. The most

Table 2 Description of included studies which modified the scaffolds by addition of extracellular matrix molecules

Ref.	Scaffold	Fabrication	Type of modification	Cell type	Tests and results
Kim *et al*[43], 2006	BGNF + Col 1	Electrospinning process	Surface coating	MG63Cs	ALP activity: BGNF + Col 1 > Col 1
Lechner *et al*[44], 2006	β-TCP + F + T	-	New composition	BMMSCs	ALP activity: Increased during 28 d of culture FACS: CD 31: Increased expression by 100 folds
Turhani *et al*[34], 2007	HA + Col 1 + rhBMP-2	-	Surface coating	SaOS-2Cs	XTT proliferation assay: (relative) HA + col 1 + rhBMP-2: 1.5 Control: 0.5 ALP activity: HA + col 1 + rhBMP-2: 50 U/μg Control: 25 U/μg
Srouji *et al*[45], 2008	PCL + Col	Electrospun meshed scaffold	Electrospun nanofiber membrane	BMMSCs	Alamar Blue assay: PCL + Col = PCL [Data as mean ± SD (P < 0.05)] *In vivo*: Good integration after subcutaneous implantation
Hao *et al*[46], 2010	A: ADSCs-Col/PLGA-β-TCP B: Acellular Col/PLGA-β-TCP	Low- temperature deposition manufacturing (LDM) based on the layer-by-layer manufacturing principle of solid free-form fabrication	Surface coating	ADSCs	ALP activity: ECM mineralization in group A > group B Evident calcification level in group A, no apparent calcification in group B *In vivo*: Woven bone with a trabecular structure in group A No bone formation in group B
Xu *et al*[30], 2009	BG + Col-HYA-PS	Sol–gel method	New composite fabrication	BMMSCs	Cell attachment: Number of attached cells on BG + Col - HYA - PS was the highest Cell proliferation: BG + Col - HYA - PS > BG + Col and BG + Col - HYA > BG ALP activity: BG + Col - HYA - PS > BG + Col and BG + Col - HYA > BG
Kawai *et al*[47], 2009	OCP + Col	Mixing + lyophilization	Surface coating	MSST-2 Cs	Proliferation and attachment : OCP + Col > OCP (control) *In vivo*: OCP + Col: Enhanced bone regeneration ratio 83:17 generated maximum repair level of approximately 64% of the defect at 12 wk
Zhang *et al*[48], 2010	β-TCP + Col	Electrospun + impregnating methods	Architecture + surface coating	MG63Cs	MTT assay: (relative) β-TCP + Col: 0.30 Control (β-TCP): 0.25
Qu *et al*[49], 2010	C + HA + RGD peptide	*In situ* compositing hybridization + lyophilization	Surface coating	BMMSCs	Fluorescence microscopy: Cell adhesion rate: CS + HA: 54.7% C + HA + RGD peptide: 71.6% and 80.7% ALP activity: C + HA + RGD peptide: 0.00596 ± 0.00081 U/L per ng C + HA: 0.00283 ± 0.00025U/L per ng
Kang *et al*[50], 2011	Fi + HAH	The multi-head deposition system	Surface coating	ADSCs	ALP activity: Single day treatment: 0.5 mmol/mg No treatment: 0.5 mmol/mg Daily treatment: 3 mmol/mg
Marelli *et al*[51], 2011	nBG + DC	Plastic compression technique	Surface coating	MC3T3-E1 CS	Confocal microscopy of fluorescently: Attachment no difference ALP activity: nBG + DC: 3.5×10^5 Control (nBG): 2.5×10^5 Alamar blue assay: nBG + DC: 6.5×10^5 Control: 8×10^5
Phipps *et al*[52], 2011	PCL + Col I + nHA	Electrospun	Bone-mimetic electrospun matrices	BMMSCs	Activation of focal adhesion kinase: Cells seeded onto PCL/Col/nHA scaffolds were better spread, and exhibited greater amounts MTS assay: (relative) PCL + Col + nHA: 5 PCL + nHA: 2.5 PCL: 1
Weeks *et al*[53], 2012	PLLA + CXCL12, 13 + F + Col IV	-	Surface coating	BMMSCs	Antibody-blocking studies: Anti-α5β1 inhibits MSC attachment: PLLA + CXCL12, 13 + F + Col IV: 500 cells/mm² PLLA + F + Col IV: 400 cells/mm²

BG: Bioglass; PLLA: Poly(l-lactic acid); Col: Collagen; C: Chitosan; PLGA: Poly(D,L-lactic-co-glycolic acid); PCL: Poly(e-caprolactone); HA: Hydroxy apatite; TCP: Tricalcium phosphate; HAH: Hyaluronic acid hydrogel; nBG: Nano-sized bioactive glass; BGNF: Bioactive glass nanofiber; OCP: Octacalcium phosphate; F: Fibronectin; Fi: Fibrin; nHA: Hydroxy apatite nano particles; CXCL: Chemokine ligand; T: Thrombin; rh: Recombinant human; BMP: Bone morphogenetic protein; HYA: Hyaluronic acid; PS: Phosphatidylserine; DC: Dense collagen; iH: Immobilized heparin; RGD: Arg-Gly-Asp; BMMSCs: Bone marrow mesenchymal stem cells; MG63Cs: Human osteosarcoma cells; SaOS-2 Cs: Sarcoma osteogenic cells; MC3T3-E1: Osteoblast precursor cells; ADSCs: Adipose-derived stem cells; MSST-2 Cs: Mouse bone marrow cells; ALP: Alkaline phosphatase; FACS: Fluorescent activated cell scan; XTT: Sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt; MTT: 2-(4,5-dimethyl-2 thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; MTS: 5-[3-(carboxymethoxy)phenyl]-3-(4,5 dimethyl-2-thiazolyl)-2-(4-sulfophenyl)- 2H-tetrazolium inner salt; CD: Cluster of differentiation.

commonly used ECM-like molecule was collagen 1. Other molecules were fibrin, collagen IV, and hyaluronic acid (Figure 2E). The main scaffolds used were TCP, HA, PCL, bioactive glass nanofiber, octacalcium phosphate, Bio-Oss and Bioglass. Scaffold fabrication methods varied vastly among included studies.

Regarding the study method, in the majority of studies cell differentiation was assessed with ALP activity assay and higher activity was reported for experimental scaffolds compared to control groups. In addition, various methods were applied to assess cell attachment, proliferation and differentiation. In 6 studies, the cell type was BMMSCs; whereas in other studies MG63Cs, SaOS-2Cs, osteoblast precursor cells (MC3T3-E1Cs), adipose-derived stem cells (ADSCs), and mouse bone marrow cells (MSST-2 Cs) were used.

Nano particles
Among 85 articles, which used nano particles to enhance scaffold properties, 17 were studied (Table 3). Type of modification was surface modification and scaffold architecture in 5 studies; in another 6 experiments, spatial conformation was performed. Others fabricated new scaffolds by adding nanoparticles to the scaffold composition (Figure 2C). The main scaffold on which modifications were made varied in the studies. These scaffolds included PCL, chitosan, PLGA, gelatin, TCP, poly l-lactic acid (PLLA), single-walled carbon nanotubes, polyamide and HA. Nanoparticles of HA were the most commonly added particles followed by nano fibrous PCL, nano diamond, amorphous calcium phosphate nanoparticles, nano silica, nano apatite, magnetic nanoparticles and nano-sized bioactive glass (Figure 2F). Different fabrication techniques were applied: flame spray pyrolysis, sonication, thermally induced phase, electrospun, blunt-end needle tip and gamma high voltage. Scaffold fabrication method varied vastly among the included studies.

Regarding the study method, various tests were performed to assess cell viability, attachment, proliferation and differentiation. ALP activity assay, 3-(4,5-dim-ethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)- 2H-tetrazolium inner salt assay, bicinchoninic acid assay, bone Gla protein activity and Alamar blue assay were among these tests. In 5 studies, PCR test was used to assess level of gene expression. Various biomarkers were used in each study such as ALP, OCN, Runx-2, bone sialoprotein, TGF-β1, BMP-2, collagen 1 and integrin subunits together with myosins. In 10 cases, cell type used in the study was BMMSCs. Other cell types used in the studies were: Mouse bone marrow cells (7F2Cs), MC3T3-E1Cs, osteoblastic cells (ROS 17/1.8 Cs), human airway fibroblast cells, ADSCs, MG63Cs, primary human osteoblasts-like cells and rat calvarial osteoblast cells.

DISCUSSION
This study reviewed current trends in smart scaffolds for

bone regeneration. The most relevant applied methods to design smart scaffolds are surface modification by adding nano particles such as nHA and adding an ECM-like molecule such as collagen or growth factor like BMP-2 to the scaffold. Factors such as scaffold material, fabrication method and type of modification defining the physical, chemical and mechanical properties of scaffolds need assessment. Due to the variability of influencing factors including methodology and scaffold properties, the results were not comparable to determine the most successful design. *In vitro* tests for analyzing the behavior of the fabricated scaffolds vary among the studies. The source of cells also influences the results, and qualitative report of cell migration, attachment, infiltration and differentiation must be similar to compare the results. The results of included *in vitro* experiments demonstrated that smart design of scaffolds supported cell attachment, proliferation and differentiation.

Modification
Surface modification of scaffold materials was the most commonly used design. However, when implanting the graft materials, their surfaces are covered with ECM proteins and molecules and thermodynamic forces cause surface absorption. This issue is commonly overlooked. Alteration of surface properties influences cell adhesion. In addition, surface plays a role in cell migration towards the scaffold and stem cell differentiation. Addition of either HA or collagen to the outer surface of polyethylene terephthalate (PET) caused greater attachment of rat bone marrow cells to the surface and deposition of an HA layer on the surface[69]. The morphology of attached cells mimics that of more differentiated cells. Although chemical modification of scaffold surface affects cell to scaffold response, effect of material design on cell behavior should also be considered[70]. Hatano *et al*[71] demonstrated that cell differentiation is affected by surface roughness. In the cited study, rougher surfaces caused expression of osteoblastic markers.

Growth factors and bioactive molecules
Understanding the physiological processes of bone regeneration and the involved regulating molecules is necessary to enhance bone repair. Several attempts at adding these molecules to scaffolds were demonstrated (Tables 1 and 2). Regulating molecules include growth factors and other proteins found in bone ECM.

In the reviewed studies, BMPs seemed to be more frequently used in the scaffolds compared to other growth factors. BMPs induce osteogenic differentiation of stem cells and osteoprogenitor cells, and their efficient and safe use in human was licensed in 2001. In addition, growth factors like BMPs and vascular endothelial growth factors can accelerate healing processes[72,73]. Chemically conjugated BMP-2 on PCL scaffolds caused significantly greater *ALP* gene expression compared to un-treated PCL scaffolds[37]. In a study by Lu *et*

Table 3 Description of included studies which modified the scaffolds by addition of nanoparticles

Ref.	Scaffold	Fabrication	Type of modification	Cell type	Tests and results
Kim *et al*[54], 2006	PLGA + nHA	SC/PL and GF/PL	New composition fabrication	RCOCs	Average cell density: GF/PL = 2.4×10^6 cells/scaffold (86.5% increase) SC/PL = 2.1×10^6 cells/scaffold (69.7% increase) ALP activity: GF/PL= 0.6 mol/min per 10^6 SC/PL= 0.5 mol/min per 10^6
Wang *et al*[55], 2007	PA + nHA	Thermally induced phase inversion	Surface coating	BMMSCs	MTT assay and ALP activity: No negative effects on the BMMSCs *in vitro* *In vivo*: Good biocompatibility and extensive osteoconductivity with host bone *in vivo*
Lv *et al*[56], 2009	PLGA + nHA	Microsphere sintering method (modification of the emulsion and solvent evaporation method)	New composition fabrication	BMMSCs	MTS assay: (cell number) PLGA + nHA : 1.2 million/mm^2 PLAGA: 0.06 million/mm^2 ALP activity: PLGA + nHA: 0.10 mL/μg PLAGA: 0 mL/μg
Roohani-Esfahani *et al*[57], 2010	BCP/PCL + nHA	Sonication	Surface coating	PHOLCs	ALP activity: BCP/PCL + nHA: 2 mmol/h per mg BCP: 0.5 mmol/h per mg RT-PCR: (relative) BSP: BCP/PCL + nHA: 0.4, PCL: 0 Runx2: BCP/PCL + nHA: 8, PCL: 5 OCN: BCP/PCL + nHA: 1.2, PCL: 0.8 Col I: BCP/PCL + nHA: 4.5, PCL: 2.5
Ye *et al*[58], 2010	PCL + nAP	Freeze-dried	Scaffold architecture	MG63Cs	Cell proliferation on composite scaffolds porosity of 76% > porosity of 53%
Phipps *et al*[52], 2011	PCL+ Col I + nHA	Electrospun	Bone-mimetic electrospun matrices	BMMSCs	Activation of focal adhesion kinase: Cells seeded onto PCL + col I + nHA scaffolds were better spread, and exhibited greater amounts MTS assay: (relative) PCL + Col + nHA: 5 PCL + nHA: 2.5 PCL: 1
Zhang *et al*[59], 2011	PLLA + ODA-nD	Sonication	Surface coating	7F2CS	Alamar Blue assay: A slight reduction in cell viability compared to control RT-PCR: (relative) ALP: PLLA + ND-ODA: 1, PLLA: 1 OCN: PLLA + ND-ODA: 3, PLLA: 2.8
Zeng *et al*[31], 2012	HA + MNPs	Tuning	New composition fabrication	MC3T3-E1Cs ROS 17/1.8Cs	MTT assay: HA + MNPs: MC3T3-E1: 0.2 OD value, ROS 17/1.8: 1.8 OD value HA: MC3T3-E1: 0.9, ROS 17/1.8: 0.25 ALP activity: HA + MNPs: MC3T3-E1: 0.75 U/mg, ROS 17/1.8: 4.5 U/mg HA: MC3T3-E1: 0.5 U/mg, ROS 17/1.8: 3.5 U/mg BGP activity: HA + MNPs: MC3T3-E1: 350 ng/L, ROS 17/1.8: 4000 ng/L HA: MC3T3-E1: 300 ng/L, ROS 17/1.8: 3500 ng/L
Hafezi *et al*[60], 2012	G + nBG	Homogenization through stirring	New composition fabrication	hAFCs	MTT assay: No difference compared to control *In vivo*: Radiographic evaluation: Improved the speed of the bone healing process
Buschmann *et al*[61], 2012	PLGA + n-aCaP	Electrospun	Electrospun PLGA/a-CaP scaffold architecture	ADSCs	MGTS: Extracellular matrix production was significantly higher FACS: CD13, CD29, CD44 and CD105 were expressed on PLGA + n-aCaP
Ganesh *et al*[62], 2012	nfPCL + nS	Electrospun	New composition fabrication	BMMSCs	FACS: CD29 = 3.3%, CD44 = 77.1%, CD73 = 94%, CD31, 34, 45 = 0% Cell viability: No difference compared to control BCA assay: NS + PCLN: 250 Ug/mg, PCLN: 100
Im *et al*[63], 2012	SWCNT + C + nHA	Lyophilization procedure	Scaffold architecture + new composition	BMMSCs	Cell adhesion and proliferation: SWCNT + C + nHA > SWCNT + C
Rodrigues *et al*[64], 2012	TCP + nfPCL	Electrospun + dynamic culturing environment	Scaffold architecture + new composition	BMMSCs	ELISA: TCP + nfPCL: 0.8 μg/mL TCP: 0.2 μg/mL ALP assay: TCP + nfPCL: 100 mol/h TCP: 20 mol/h
Panzavolta *et al*[65], 2013	G + nHA	Foaming + freeze-drying method	Surface coating+ scaffold architecture	BMMSCs	ALP activity: No difference compared to control RT-PCR: (Relative) ALP: G + nHA: 3.2, G: 1.8 Col I: G + nHA: 0.25, G: 0.25 Runx2: G + nHA: 0.5, G: 0.4 TGF-b1: G + nHA: 0.6, G: 0.55

Xing *et al*[66], 2013	PLLA + OTND	Manual perfusion technique under pressure	New composition fabrication	BMMSCs	BCA assay: No difference compared to control RT-PCR: (relative) OPN: PLLA + OTND: 3.5, PLL: 1 BSP: PLLA + OTND: 3, PLL: 1 BMP-2: PLLA + OTND: 4, PLL: 1 *In vivo*: New bone formation: PLLA + OTND: 50%, PLL: 10%
Liu *et al*[67], 2013	C + nHA	Electrospun	New composition fabrication	BMMSCs	Cell attachment: C + nHA: 1100 μm^2 C: 250 RT-PCR: (Relative) BMP-2: C + nHA: 1.5, C: 1 BMP-4: C + nHA: 25, C: 0 Smad1: C + nHA: 9, C: 1 ALP: C + nHA: 1.2, C: 1 Runx2: C + nHA: 22, C: 1 Itga1: C + nHA: 17, C: 1 Itgb1: C + nHA: 7, C: 1 Itgb3: C + nHA: 8, C: 1 Myh9: C + nHA: 7, C: 1 Myh10: C + nHA: 3.5, C: 1 Col 1: C + nHA: 4.5, C: 1 *In vivo*: Superior ability of bone reconstruction
Wang *et al*[68], 2014	C + nHA	Lyophilization procedure + cold atmospheric plasma (CAP) treatment	Scaffold architecture + surface coating	BMMSCs	SEM: MSCs adhesion and infiltration were enhanced ELISA: (Relative) Fibronectin: C + nHA 0.8 compared to control (C) Vitronectin: C + nHA 1.1 compared to control

TCP: Tricalcium phosphate; PLLA: Poly(l-lactic acid); Col: Collagen; C: Chitosan; PCL: Poly(e-caprolectone); HA: Hydroxy apatite; G: Gelatin; PLGA: Poly(D,L-lactic-co-glycolic acid); GF/PL: Gas forming and particulate leaching; SC/PL: Solvent casting and particulate leaching; BCP: Biphasic calcium phosphate; SWNT: Single-walled carbon nanotubes; PA: Polyamide; nf: Nano fibrous; NHA: Hydroxy apatite nano particles; nAP: Nano apatite; OTND: Oxygen-terminated nanodiamond particles; nS: Nanoparticles of silica; n-aCaP: Amorphous calcium phosphate nanoparticles; MNPs: Magnetic nanoparticles; ODA-ND: Octadecylamine-functionalized nanodiamond; nBG: Nano-sized bioactive glass; BMMSCs: Bone marrow mesenchymal stem cells; MG63Cs: Human osteosarcoma cell line; MC3T3-E1: Osteoblast precursor cells; ADSCs: Adipose-derived stem cells; RCOCs: Rat calvarial osteoblasts cells; hAFCs: Human airway fibroblast cells; ROS 17/1.8 Cs: Osteoblastic cells; 7F2CS: Mouse bone marrow cells; PHOLCs: Primary human osteoblasts-like cells; ALP: Alkaline phosphatase; MTT: 2-(4,5-dimethyl-2 thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; MTS: 5-[3-(carboxymethoxy)phenyl]-3-(4,5 dimethyl-2-thiazolyl)-2-(4-sulfophenyl)- 2H-tetrazolium inner salt; FACS: Fluorescent activated cell scan; RT-PCR: Reverse transcriptase polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; SEM: Scanning electron microscope; BGP: Bone Gla protein; BCA: Bicinchoninic acid; MGTS: Masson Goldner Trichrome staining; CD: Cluster of differentiation; Itga1: Integrin a1; Itgb1: Integrin b1; Itgb3: Integrin b3; Myh9: Non-muscle myosin 9; Myh10: Myosin 10.

al[41], results indicated that spatial immobilization of BMP-4 in a collagen-PLGA hybrid scaffold caused high expression of osteogenic genes and supported cell adhesion and proliferation[41]. In 2005, Yang *et al*[33] evaluated the effect of BMP-4 immobilization on GTG scaffolds on NRCOC activity. A significant effect of BMP-4 was reported after 4 wk. The cited study also showed greater cell attachment and osteoblast differentiation, higher Gla-type osteocalcin (Gla-OC) activity and larger mineralizing nodules following co-culture of osteoblasts with BMP-4 immobilized scaffold. The same results were achieved using BMP-2. Dai *et al*[40] reported that rhBMP-2 significantly promoted the osteogenic differentiation of BMMSCs, by enhanced expression of Runx-2, osteopontin, osteocalcin and bone sialoprotein. On the other hand, combination of BMP-4 and PLGA scaffold resulted in a reduction in ALP activity[41]. Although BMPs seem to have positive effects on the function of scaffolds and cells, their regularity and efficacy constraints limit their clinical application.

Surface of scaffolds can be modified by proteins and plasma treatment. Immobilization of proteins like integrin and laminin on the scaffold surface not only facilitates cell adhesion but also increases the surface wettability[74]. To add a mixture of fibrin and hyaluronic acid, multihead deposition system and fibrin-thrombin shell formation were used[50]. Using immobilized RGD peptide (Arg-Gly-Asp) improved cell adhesion from 71.6% to 80.7% and increased ALP activity[49]. Similarly, high cell proliferation, differentiation and viability were reported following addition of other ECM molecules to scaffold surfaces[34,47].

On the other hand, although biomolecules like growth factors and ECM proteins can improve scaffold properties, these molecules do not tolerate severe chemical conditions or high temperature and only soft fabrication methods like sol-gel, which does not allow interconnected porous scaffold synthesis, are applicable[75].

Nano particles
Nano particle addition to the scaffold is another way to improve scaffold properties. The most commonly applied nanoparticle was nano-HA, which provides osteoconductivity while the main scaffold provides the porosity. This combination resembles organic/inorganic nature of the bone matrix and mimics the nano-sized characteristics of natural bone[26]. Addition of nano-

HA to scaffold doubled the expression of osteogenic genes[67] and increased osteoblastic attachment[63]; whereas osteocalcin gene expression level in PLLA-nano diamond composites was 3 times less than in the PLLA scaffold[59]. Ye *et al*[58] produced a composite scaffold containing nano non-stoichiometric apatite and poly-epsilon-caprolactone, which had well-interconnected pores encouraging cell proliferation, migration and stimulation.

Future prospects

In vitro studies are generally considered as primary steps for evaluating newly developed materials. These experiments evaluate cell viability, attachment, proliferation and differentiation on new scaffolds by various methods. Although some factors like the effect of recipient bed, body fluids and interaction between different cell types cannot be evaluated in *in-vitro* experiments, they are useful to analyze the behavior of materials toward cells omitting individual characteristics of animal or human models. However, animal and clinical studies are necessary to assess the regenerative ability of materials *in-vivo*. A systematic review on *in-vivo* bone tissue engineering studies by Khojasteh *et al*[2] demonstrated that only few animal experiments used smart modification of scaffolds; most of which, were addition of BMP-2. It seemed that the application of BMP-2 with stem cells such as ADSCs and BMSC in TCP scaffolds significantly enhanced osteogenesis[2]. Use of growth factors such as BMP-2 in combination with scaffold and stem cells could complete the classic tissue engineering triangle. However, the results of *in vitro* studies showed that addition of ECM-like molecules and nano-particles also induced osteoblastic differentiation. The most common composite used in bone tissue engineering was found to be polymer-ceramic and use of smartly modified scaffolds in animal models has been relatively fewer[76]. Compared to the large number of *in-vitro* studies assessing the properties of newly developed materials, *in-vivo* application of these smart scaffolds and the effect of structural features and surface modification on *in-vivo* bone regeneration have yet to be studied.

In conclusion, comparing smart scaffolds to other presently available conventional materials shows great advantages, as they have the ability of *in-situ* osteoblastic differentiation induction and interesting biological functions. The more we understand the fundamentals of cell proliferation, differentiation and mechanisms of osteoblast adhesion, the better we can design smart materials. The researches now aim to design and synthesize composite materials made from fusion of different kinds of synthetic and natural materials. Surface treatment for facilitating cell attachment and differentiation and fabrication of strong scaffolds that can tolerate physiological forces are the main challenges in this regard. *In-vitro* studies should be performed primarily using standard methods followed by *in-vivo* investigations. Combination of stem cells and smart scaffolds with a modulus of elasticity of the same magnitude as that of natural bone, and incorporating growth factors may complete the bone tissue engineering triad. Several factors including material, fabrication and modification methods as well as physical, chemical and mechanical properties may determine bone scaffold features. Considering the variability of *in-vitro* methodology of researches and variety of scaffolds, a thorough and comprehensible comparison between the results and approaches of these *in vitro* experiments was unattainable. However, the results of the included *in-vitro* studies indicate that incorporation of growth factors and other bioactive molecules as well as nano-particles as smart modifications can enhance cell differentiation, proliferation and attachment and therefore may improve new bone formation. The current study presented various types of smart modifications, which may provide an acceptable design to approach *in-vivo* and *in-vitro* hard tissue engineering.

COMMENTS

Background
Scaffold, cells and growth factors are the three main parts of bone tissue engineering. Currently, special attention has been directed to the design of new scaffolds by adding bioactive molecules and nanoparticles. Hydroxyapatite-based scaffolds carry cells to bone defects and provide an extracellular matrix (ECM). By changing the physical and chemical properties of the bone substitutes, smart interaction with seeded cells such as acceleration of differentiation, increasing in proliferation and attachment of the cells has been reported. It seems that these "smart modifications", can improve osteogenic differentiation and attachment of cells; therefore, they can better respond to their surrounding environment.

Research frontiers
Surface modification of scaffold materials was the most commonly used design. However, it is not clear how surface would influence bone regeneration *in vivo*. This study reviewed current trends in smart scaffolds for bone regeneration. Adding nano particles such as nHA and adding an ECM-like molecule such as collagen or growth factor like bone morphogenic protein (BMP)-2 to the scaffold increase bioactivity of synthetic materials.

Innovations and breakthroughs
Several factors such as scaffold material, fabrication method and type of modification defining the physical, chemical and mechanical properties could affect *in vitro* experiments. Due to the variability of influencing factors including methodology and scaffold properties, the results were not comparable to determine the most successful design. Overall conclusion shows that in most studies smart design of scaffolds supported more cell attachment, proliferation and differentiation.

Applications
Although use of growth factors such as BMP-2 in combination with scaffold and stem cells could complete the classic tissue engineering triangle and result in bone regeneration *in vivo*, *in vitro* studies suggest that addition of ECM-like molecules and nano-particles also might have same results as BMP-2. The efficacy of these modifications should be assessed *in vivo*.

Peer-review
The matter of the review is interesting.

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