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BASIC RESEARCH

Vancomycin Containing PLLA/β-TCP Controls MRSA In Vitro

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Abstract

Background Osteomyelitis caused by Methicillin-resistant Staphylococcus aureus (MRSA) often requires surgery and prolonged systemic antibiotic treatment. Local antibiotic delivery systems of bioceramics or polymers have been developed to treat osteomyelitis. A disadvantage of biodegradable polymers is the initial burst of antibiotics into the environment; one advantage of bioceramics is its osteoconductivity. We therefore developed a vancomycin-containing poly-l-lactic acid/β-tricalcium phosphate (PLLA/β-TCP) composite to control antibiotic release and stimulate bone formation.

Questions/purposes We (1) characterized these composites, (2) assessed vancomycin release in inhibitory doses, and (3) determined whether they would permit cell adhesion, proliferation, and mineralization in vitro.

Methods We molded 250 vancomycin-containing (VC) and 125 vancomycin-free (VUC) composites using PLLA, β-TCP, and chloroform. One hundred twenty-five VC composites were further dip-coated with PLLA (CVC) to delay antibiotic release. Composites were characterized according to their pore structure, size, volume, density, and surface area. Vancomycin release and bioactivity were determined. Adhesion, proliferation, and mineralization were assessed for two and three replicates on Days 3 and 7 with mesenchymal stem (MSC) and Saos type 2 cells.

Results Pore size, volume, apparent density, and surface area of the CVC were 3.5 ± 1.9 μm, 0.005 ± 0.002 cm^3/g, 1.18 g/cm^3 and 3.68 m^2/g, respectively. CVC released 1.71 ± 0.13 mg (63.1%) and 2.49 ± 0.64 mg (91.9%) of its vancomycin on Day 1 and Week 6, respectively.

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Each author certifies that his or her institution waived approval for the reporting of this investigation and that all investigations were conducted in conformity with ethical principles of research.
This study was performed at Middle East Technical and Hacettepe Universities of Ankara, Turkey.

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MSC and Saos type 2 cells attached and proliferated on composites on Days 3 and 7.

Conclusions Vancomycin-containing PLLA/β-TCP composites release antibiotics in inhibitory doses after dip coating and appeared biocompatible based on adhesion, proliferation, and mineralization.

Clinical Relevance Vancomycin-containing PLLA/β-TCP composites may be useful for controlling MRSA but will require in vivo confirmation.

Introduction

Implant-related osteomyelitis (IRO) is a complex condition that frequently leads to bone and joint destruction [24] and often is challenging to treat [44]. Staphylococcus aureus [27] and epidermidis [2] are among the most common bacteria and frequently form biofilms resistant to systemic antibiotic therapy [6]. Although the incidence of IRO ranges from 0.5% to 1% in patients having primary elective joint replacements [40, 42], it ranges from 10% to 20% in patients who have diabetes [18, 26] and/or vascular deprivation [3], and from 2% to 20% in patients having revision surgery or trauma [14, 49]. Multiple surgical interventions [17] after the recurrence of IRO decrease the quality of life [10] and increase morbidity and mortality [3] of patients.

Systemic antibiotic administration combined with surgical removal of infected and necrotic tissues is used conventionally to treat IRO [45]. Various local methods have been developed to prevent [30, 44] and treat [25] IRO. These include antibiotic-containing polymethylmethacrylate (PMMA) [19] and biodegradable polymers [15]. PLLA is the copolymer of polylactic acid [21] and can be used as an antibiotic carrier owing to its biocompatibility [33]. Ceramics also have been used as carriers of antibiotics [16]. TCP is a biodegradable and biocompatible ceramic that can be used for bone conduction and controlled antibiotic release [11]. Unfortunately, TCP degrades rapidly over 4 weeks to 86 weeks [32] depending on its porosity and is unable to maintain appropriate antibiotic tissue levels [12]. PLLA and TCP have been combined [4] as a bone substitute with longer degradation times ranging from 3 to 6 months [4], although it has not been used as a depot for antibiotics. Vancomycin is an active glycopeptide against MRSA [22, 39] and given the longer degradation times of PLLA-TCP composites, we reasoned these composites could be combined with vancomycin to provide longer antibiotic delivery.

The aims of the study therefore were to (1) develop and characterize a VC PLLA/β-TCP composite for its pore structure, size, and volume, apparent density, and surface area, (2) release active antibiotic for 6 weeks at levels to inhibit MRSA proliferation, and (3) evaluate whether composites will allow adhesion, proliferation, and mineralization of MSC and Saos type 2 cells in vitro.

Materials and Methods

We designed an in vitro study to assess the biocompatibility of three composite types in cell culture: (1) vancomycin-containing PLLA/β-TCP (VC), (2) dip-coated vancomycin-containing PLLA/β-TCP (CVC), and (3) vancomycin-free PLLA/β-TCP (VUC) in duplicate for cell adhesion and in triplicate for cell proliferation on Days 3 and 7 with MSC and Saos type 2 cells. The same cells without the composites (4) served as the control group. All groups were evaluated on Days 10 and 21 for mineralization rates [43] (Fig. 1).

We mixed 80 mg of vancomycin hydrochloride (Abbott, Wiesbaden, Germany) with 920 mg deionized water to prepare the 8% vancomycin solution to create the composites. Seven hundred twenty milligrams β-TCP (produced at Middle East Technical University, Ankara, Turkey [23]) was added to this solution and dried at 37°C for 12 hours. The solid mixture was added to 2.67 g of chloroform (J.T. Baker, Phillipsburg, NJ, USA) solution containing 3% PLLA (Boehringer-Ingelheim, Ingelheim am Rhein, Germany). Two hundred fifty spherical VC beads were obtained from the paste by putting it into a mold with a 1.5-mm circumferential diameter and dried at room temperature for 24 hours to evaporate the chloroform. One hundred twenty-five VC beads were further coated with a 15-μm thin PLLA film by dipping them into 3% (w/w) PLLA solution for 10 seconds. Composites were dried at room temperature for 24 hours [38]. One hundred

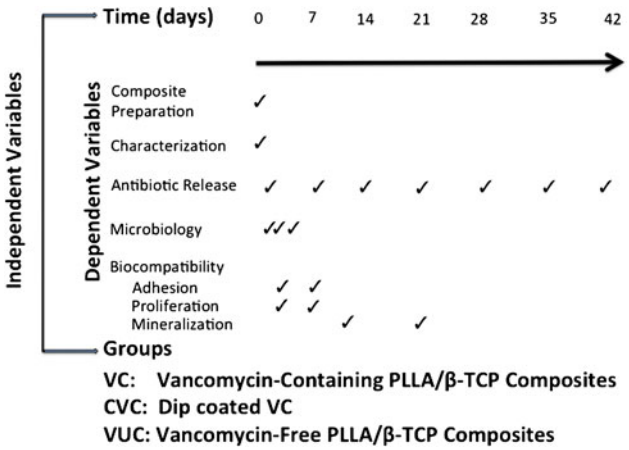

Fig. 1 The experimental design is shown.

twenty-five VUC beads were produced with the same method for VC without using vancomycin.

We assessed the chemical composition of the β-TCP composites using the JSM 6400 (Jeol Ltd, Akishima, Japan) scanning electron microscope. Composites were coated with gold to obtain a conducting surface and pictures were obtained at ×2000 magnification.

Pore-size distribution and apparent density of the composites were analyzed with a mercury porosimeter (Polemaster 60; Quantachrome Instruments, Boynton Beach, FL, USA) at 50 psi.

Composites were preheated to 30°C and surface area was determined by nitrogen adsorption in a single-beam surface characterization device (Quantachrome Autosorb 6, Quantachrome Instruments).

A calibration curve was obtained by dissolving 1, 2, 4, 6, 8, 10, 12, and 14 mg vancomycin in 20 mL deionized water. Solutions were analyzed using a single-beam diode array spectrometer (Hewlett Packard 8452A; Hewlett Packard, Palo Alto, CA, USA) at 280 nm UV. The weight and amount of vancomycin in the composites were calculated (Table 1). VC and CVC composites were immersed in 20 mL deionized water at pH 7.0. On the first day and at 1, 2, 3, 4, 5, and 6 weeks, the 20 mL of deionized water was removed to spectrophotometrically (280 nm) determine the amount of vancomycin release and the removed water was replaced with 20 mL fresh deionized water.

We assessed antimicrobial activity of VC and CVC composites on Days 1, 2, and 4 against MRSA (strain, ATCC 2592) grown on agar plates (10^6 CFU/cm^3) at 37°C. Antimicrobial Susceptibility Test Discs (BD BBL Sensi-Disc; Becton Dickinson, Franklin Lakes, NJ, USA) were used as the control. Inhibition zone diameters were measured and scaled. Zones that were 9 mm or less were considered resistant. Zones between 10 to 11 mm were considered intermediate and those that were 12 mm or greater were considered sensitive to MRSA.

We sterilized composites with 25 kGy gamma radiation for 10 minutes. We performed cell adhesion, proliferation MTT (thiazolyl blue tetrazolium bromide), and mineralization assays using human bone marrow-originating MSC [5] and Saos-2 type cells (European Collection of Cell Cultures [ECACC], Salisbury, Wiltshire, UK). Human MSCs were obtained from healthy bone marrow transplantation donors and characterized according to the International Society of Cellular Therapy (ISCT) mandatory criteria [13]. We incubated the cells with the composites in growth medium (DMF10) composed of 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 1% penicillin-streptomycin (Biochrom AG, Berlin, Germany) and DMEM-LG (Biological Industries, Ashrat, Israel) containing 1% L-glutamine on Day 1. The medium was replaced every 3 to 4 days. A total of 462,000 cells (4000 cells × 9.6 cm^2 × 12 wells) and 12 wells were used for the adhesion study. We washed the wells with PBS and 1 mL Trypsin/EDTA on Days 3 and 7. A total of 27,000 cells (1500 cells/well) and 192 wells (two 96-well plates) were used for the MTT cell proliferation assay. MTT (Sigma-Aldrich, St Louis, MO, USA) was added to the wells on Days 3 and 7 of culture and read with the ELISA device (Tecan Trading AG, Mannedorf, Switzerland) at 620 nm after 4 hours of incubation. We used a total of 375,000 cells (4000 cells × 3.9 cm^2 × 24 wells) and 24 wells to assess mineralization. The differentiation medium containing DMEM-LG, 10% FBS, 100 nmol/L dexamethasone (Sigma-Aldrich), 10 mmol/L beta glycerophosphate (Sigma-Aldrich), and 0.2 mmol/L ascorbic acid (Sigma-Aldrich) was added when the cells reached 50% to 60% confluence. A well was dyed with Alizarin Red whereas the other two wells were analyzed with the QuantiChrom™ Calcium Assay Kit (DICA-500; BioAssay Systems, Hayward, CA, USA) at 620 nm on Days 10 and 21.

Results

The VC and CVC had porous structures. The rough surface of the VC was of β-TCP and vancomycin powder (Fig. 2). The CVC had a network-like structure covering the composite structure (Fig. 3). The pore size of the PLLA network covering the CVC was 3.5 ± 1.9 μm (range, 0.8–4.8 μm). The normalized internal volume of the pores of

Table 1. Weight and the amount of vancomycin in the PLLA/β-TCP composites

Composites	Weight of the composites (mg) (average ± SD)	Vancomycin (mg) entrapped in the composites (average ± SD)	Vancomycin (mg) released from the composites on Week 1 (average ± SD [percentage])	Vancomycin (mg) released from the composites on Week 6 (average ± SD [percentage])
VC	29.32 ± 3.48	2.67 ± 0.31	2.67 ± 0.31 (100%)	–
CVC	32.43 ± 4.93	2.71 ± 0.44	1.71 ± 0.13 (63.1%)	2.49 ± 0.64 (91.9%)
VUC	24.73 ± 6.88	–	–	–

VC = vancomycin-containing PLLA/β-TCP; CVC = dip-coated vancomycin containing PLLA/β-TCP; VUC = vancomycin-free PLLA/β-TCP.

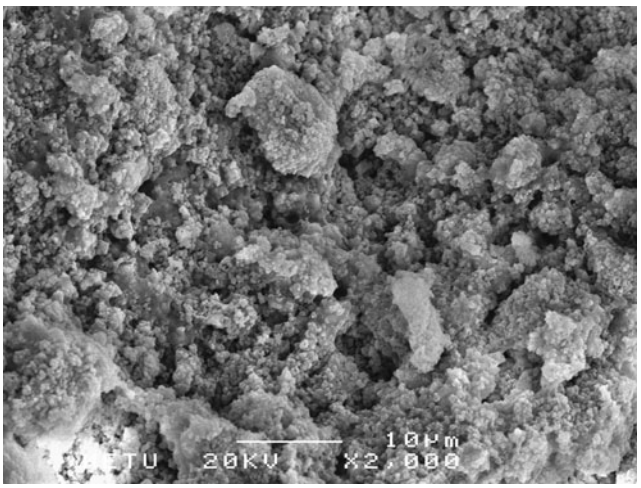

Fig. 2 Scanning electron microscopy of the vancomycin-containing PLLA/β-TCP (VC) composite showed the porous structure of the surface that is composed of β-TCP and vancomycin powder.

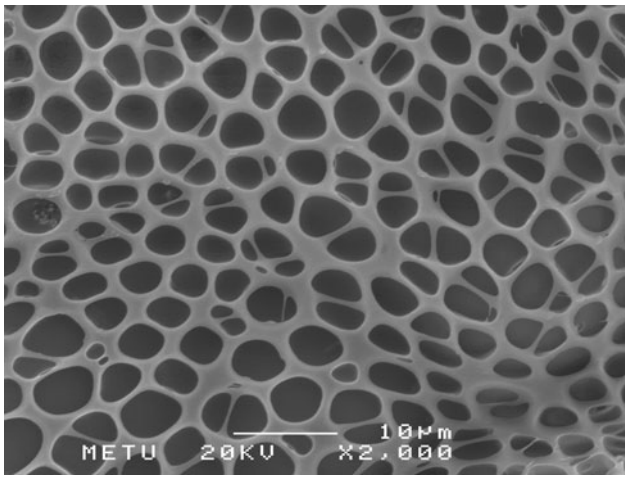

Fig. 3 Scanning electron microscopy of the dip-coated vancomycin-containing PLLA/β-TCP (CVC) composite showed the PLLA network of the surface. The pores were 3.5 ± 1.9 µm (range, 0.8–4.8 µm).

the composites was 0.005 ± 0.002 cm^3/g (range, 0.0003–0.009 cm^3/g) and the apparent densities of the CVC, VC, and VUC were 1.18 g/cm^3, 1.19 g/cm^3, and 1.12 g/cm^3, respectively. The VC, CVC, and VUC had 3.40 m^2/g, 3.68 m^2/g, and 4.77 m^2/g surface areas, respectively. Particle xray diffraction (XRD) revealed the β-TCP of the composites expressed typical calcium and phosphate picks at 2 Theta under CuK-alpha radiation (Fig. 4).

VC and CVC released 2.67 ± 0.31 mg (100%) and 1.71 ± 0.13 mg (63.1%) of vancomycin on Day 1, respectively. At the end of 6 weeks, CVC released 2.49 ± 0.64 mg (91.9%) of its vancomycin (Fig. 5). For the antimicrobial susceptibility test, zones that were 9 mm or less were considered resistant. Zones between 10 and

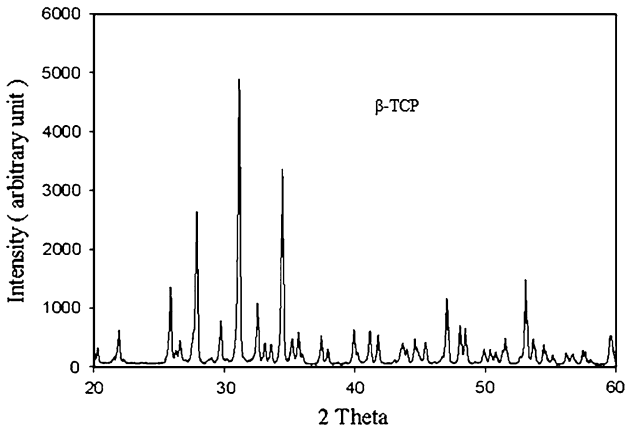

Fig. 4 A powder xray diffraction (XRD) analysis of the β-TCP expressed typical calcium and phosphate picks at 2 Theta under CuK-alpha radiation.

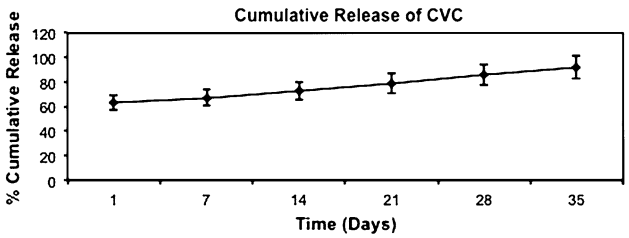

Fig. 5 The dip-coated vancomycin-containing PLLA/β-TCP (CVC) composite vancomycin release curve is shown.

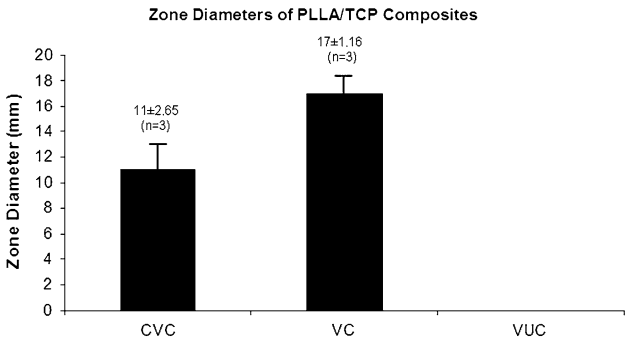

Fig. 6 Inhibition zones of MRSA of the CVC and VC groups are presented. VC = vancomycin-containing PLLA/ß-TCP composite; CVC = dip-coated vancomycin-containing PLLA/ ß-TCP composite; VUC = vancomycin-free PLLA/ß-TCP composite.

11 mm were considered intermediate and those that were 12 mm or greater were considered sensitive to MRSA [46]. The CVC, VC, and control groups developed 11 mm, 17 mm, and 18 mm inhibition zone diameters, respectively (Fig. 6). The VUC did not develop any inhibition zones.

MSC and Saos type 2 cells attached to the composites on Day 3. Adhesion of Saos type 2 cells on Day 7, however,

Table 2. Cell adhesion results

Cell type	Vancomycin-containing PLLA/β-TCP composite (VC)		Dip-coated vancomycin containing PLLA/β-TCP composite (CVC)		Cells only (no composite)	
	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
MSC	30,000	40,000	40,000	45,000 ± 7071	35,000 ± 7071	45,000 ± 7071
Saos type 2 cells	55,000 ± 21,213	70,000 ± 14,142	50,000	70,000	50,000	115,000 ± 21,213

MSC = mesenchymal stem cells.

Table 3. Cell proliferation (MTT) test absorbance results*

Cell type	VC		CVC		Cells Only	
	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
MSC	0.328	0.572	0.452	0.658	0.154	0.193
Saos type 2 cells	0.925	0.607	0.788**	1.703‡	0.141†	0.182#

* Median (mean − maximum); VC = vancomycin-containing PLLA/β-TCP composite; CVC = dip-coated vancomycin containing PLLA/β-TCP composite; MSC = mesenchymal stem cells; **p = 0.016; †p = 0.016; ‡p = 0.004; #p = 0.004.

was lower in the VC and the CVC groups compared with the control group (p = 0.04) (Table 2). All composites stimulated MSC proliferation on Days 3 and 7 (Table 3). All composites except the CVC group, showed no difference when compared with the cell-only group which stimulated Saos type-2 cell proliferation on Days 3 (p = 0.02) and 7 (p = 0.004). We observed mineralization in all composites but no differences between the groups at either time (Table 4).

Discussion

IRO is difficult to treat and in addition to suffering and cost decreases the quality of life for patients. Various biodegradable polymers [33, 48] or bioceramics [35] have been developed and used for controlled antibiotic release to treat MRSA experimentally. PLLA and TCP have been combined [4] as a bone substitute; however, this composite has not been used for release of vancomycin to treat osteomyelitis arising from MRSA. One disadvantage of biodegradable polymers used for controlled release is the initial burst of antibiotics into the environment and an advantage of β-TCP is its osteoconductivity. Given the longer degradation times of PLLA-TCP composites, we reasoned these composites could be combined with vancomycin to provide longer antibiotic delivery. We therefore developed a vancomycin-containing PLLA/β-TCP composite, characterized it according to its pore structure, pore size, internal volume of pores, apparent density and surface area, assessed vancomycin release, and determined adhesion, proliferation, and mineralization of MSC and Saos type 2 cells.

There are some limitations of this approach and of our study. First, this is an in vitro study with limited conditions and times of assessment; further in vivo and clinical studies would be required to confirm the validity of the approach. Second, beta-TCP was not determined since the particles formed clusters. When the clusters were mixed with vancomycin and PLLA they formed a functional composite that met the study aim of delayed antibiotic release. Third, vancomycin release in VC was limited to Day 1 that strengthened the rationale of the dip-coating method. Vancomycin release was extended up to 6 weeks by dip coating. Fourth, the absorbance value of the degrading PLLA at 280 nm wavelengths in spectrophotometry could have overlapped with vancomycin release. High performance liquid chromatography [36, 47] would be an alternative method to measure vancomycin release in degrading composites. Nonetheless, the amount and activity of released vancomycin was still effective to control MRSA. Fifth, we assumed a larger surface area would increase cell attachment to the composites [34]. This was not the case in our study. The VUC had the largest surface area; however, it was not tested in vitro owing to failure of sterilization.

We found pore structure, size, internal volume, and the apparent density of the composites were similar to those previously reported [31]. The VC was coated with PLLA by the dip-coating method to control vancomycin release. Morphologic features of the surface of the CVC changed, whereas the apparent density and surface area remained unchanged after 10 seconds of dip coating, and this approach extended vancomycin release up to 6 weeks. Coating formed an homogenous porous morphologic feature on the surface of the CVC; these findings are in line with those of a previous study [7]. The gelling method has been used to coat bioceramics to control drug release [41]. Gelling and regelling methods resulted in an homogeneous surface and regulated drug release [37]. The regelling method is technically demanding [37]. Although Li et al. [28] recently presented the nanocoating method for cefazolin release from PLL and PLGA or PLL/$PLGA_{20,}$ the

Table 4. Cell mineralization results (mg/dL Ca^{+2})

Cell type	Vancomycin-containing PLLA/β-TCP composite (VC)		Dip-coated vancomycin containing PLLA/β-TCP composite (CVC)		Cells only (no composite)	
	Day 10	Day 21	Day 10	Day 21	Day 10	Day 21
MSC	24.07	26.72	24.23	26.40	9.17	26.8
Saos type 2 cells	24.46	29.23	24.27	25.52	24.77	19.81

MSC = mesenchymal stem cells.

dip-coating method we used was easy and effective for VC coating.

One hundred percent and 63.1% of the vancomycin released from the VC and CVC groups on Day 1, respectively. Microbiologically active vancomycin continued releasing for 6 weeks in the CVC group. Adams et al. [1] and Liu et al. [29] found vancomycin released from PMMA and from poly(D,L)-lactide-co-glycolide for 28 and 55 days, respectively. Cevher et al. [8] reported release of vancomycin decreased as the polymer to drug ratio increased. Vancomycin release profiles in our study were comparable to published profiles [29] when the VC was coated and the CVC was established. VC and CVC may provide early control of MRSA and maintain vancomycin release up to 6 weeks when used combined.

VC and CVC promoted cell adhesion, proliferation, and mineralization. Findings indicated that these implants were biocompatible with MSC and Saos type 2 cells. Chen et al. [9] stated that composite surface promoted more osteoblast-like cell attachment and β-TCP played the lead role for cell proliferation [4]. Also, MSC adhesion increased the osteogenic property of the biomaterial [20].

We developed a porous vancomycin-containing PLLA/β-TCP composite that is bioactive and appears biocompatible. Dip coating of the composite extended vancomycin release up to 6 weeks. Vancomycin-containing and dip-coated vancomycin-containing composites allowed attachment and proliferation of MSC and Saos type 2 cells. Although these findings must be confirmed, our initial observations suggest porous vancomycin-containing PLLA/β-TCP composites have the potential to prevent and/or control IRO. However, composites must be studied in expanded in vitro and in vivo investigations before clinical use.

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