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Characterization and biocompatibility evaluation of a Ti@HA-GO@IL-4 bioactive composite coating for guided bone regeneration in dental implant applications

Congcong Li¹, Li Zhao¹, Xiaotao Chen¹, Aikepaer Aierken¹, Min Yu¹, Chuifan Kong¹ and Wufanbieke Baheti^{1,2*}

Abstract

Background Pure titanium and titanium alloy implants have been widely utilized in dental implantology due to their excellent biocompatibility and favorable elastic modulus. However, the inherent bioinertness of titanium-based implant surfaces often results in suboptimal osseointegration with surrounding bone tissue. Consequently, surface modification is essential to enhance the osseointegrative capacity of titanium implants.

Methods We fabricated hydroxyapatite-graphene oxide (HA-GO) coatings via electrochemical deposition and systematically compared four distinct protein loading methods. Ultimately, we developed a hydroxyapatite-graphene oxide/interleukin-4 (Ti@HA-GO@IL-4) coating on pure titanium substrates to investigate its physicochemical properties and biocompatibility.

Results The composite coating was successfully fabricated on pure titanium substrates, inducing significant surface modifications including the incorporation of bioactive functional groups, enhanced hydrophilicity, and altered surface morphology. Comparative characterization revealed that the composite coating exhibited superior hydrophilic properties, improved mechanical compatibility with bone tissue, stable Interleukin-4 (IL-4) adsorption and sustained-release behavior, as well as excellent biosafety confirmed by proliferation and adhesion assays using mouse embryo osteoblast precursor (MC3T3-E1) cells, when compared to unmodified titanium controls.

Conclusions Ti@HA-GO@IL-4 represents a composite coating material with exceptional physicochemical properties and biological performance. This material demonstrates efficient IL-4 loading capacity and sustained release characteristics, thereby effectively promoting osteogenesis. These findings provide valuable insights for developing bioactive functional materials to enhance implant osseointegration.

Keywords Graphene oxide (GO), Hydroxyapatite (HA), IL-4, Implant composite coating, Engineered materials

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Introduction

Dental defects and edentulism are significant issues in oral medicine [1], and dental implants provide an effective solution for their restoration [2]. However, titanium-based implants currently face challenges such as insufficient osseointegration [3], which requires surface modifications to enhance their bone integration capabilities [4]. Among bioactive coatings, hydroxyapatite (HA) is commonly used due to its excellent chemical composition and crystal structure, which facilitate osteoblast differentiation. HA is frequently applied to modify implant surfaces [5] and is one of the most widely used materials for bone regeneration [6]. Studies have shown that altering the composition, morphology, and surface characteristics of HA can improve its mechanical strength, control drug release, and enhance biodegradability, making it suitable for use in drug delivery systems, biosensors, tissue engineering scaffolds, and regenerative medicine applications [7]. Graphene-based materials, due to their unique physicochemical properties and excellent biocompatibility, are widely utilized in various biological fields [8]. Graphene oxide (GO) has been shown to promote the proliferation of mesenchymal stem cells (MSCs) and osteoblasts [9], as well as the osteogenic differentiation of MSCs [10]. GO's large surface area and high porosity make it an attractive and promising nanofiller and drug carrier. As a result, there has been increasing research into the preparation of hydroxyapatite-graphene oxide (HA-GO) composite materials [11–13]. In recent years, biomaterials scientists have explored methods for preparing HA-GO composites, with electrochemical techniques receiving particular attention [14, 15]. For example, Akram et al. [16] successfully fabricated biocompatible, non-toxic, and antibacterial medical-grade hydroxyapatite/zinc oxide (HA/ZnO) nano-composite coatings via electrochemical methods, while Wu et al. [17] developed lincomycin/sr-loaded HA-GO nano-composites with excellent mechanical properties, antibacterial abilities, and bone-inductive characteristics. Previous studies have also successfully coated HA-GO composites onto titanium implants using electrochemical methods, and found that at a 2% GO content, osteogenic differentiation of mesenchymal stem cells was significantly enhanced. Despite these promising osteogenic outcomes, challenges remain in implant applications [18], such as poor osseointegration related to the foreign body characteristics of implants, leading to sterile inflammatory reactions and even bone degradation, which pose significant threats to implant survival. Peri-implantitis is a major cause of dental implant failure [19], highlighting the importance of immune response after implantation in prognosis. Research on implant materials and their immune modulation effects is crucial for advancing surface design and improving clinical outcomes.

Interleukin-4 (IL-4) is an anti-inflammatory cytokine that induces M2 polarization of macrophages [20]. Recent studies have shown that loading IL-4 onto biomaterial surfaces significantly promotes osteogenic differentiation of stem cells and tissue healing [21], and that IL-4 has therapeutic effects in various inflammatory disease models [22, 23]. Therefore, IL-4-loaded implants can induce macrophage polarization and immune responses, modulating the local bone immune microenvironment and offering significant advantages and potential in promoting bone formation. In this study, IL-4 was loaded onto HA-GO composite coatings using an immersion method, and its physicochemical properties and biocompatibility with mouse embryo osteoblast precursor (MC3T3-E1) osteoblast precursor cells were systematically investigated. These findings provide valuable insights for selecting bioactive functional materials for implant applications, as shown in Scheme 1.

Materials and methods

The specimens were divided into four experimental groups: ①pure titanium discs (Ti), ②interleukin-4-loaded titanium discs (Ti@IL-4), ③hydroxyapatite-graphene oxide coated titanium discs (Ti@HA-GO), and ④hydroxyapatite-graphene oxide/interleukin-4 coated titanium discs (Ti@HA-GO@IL-4).

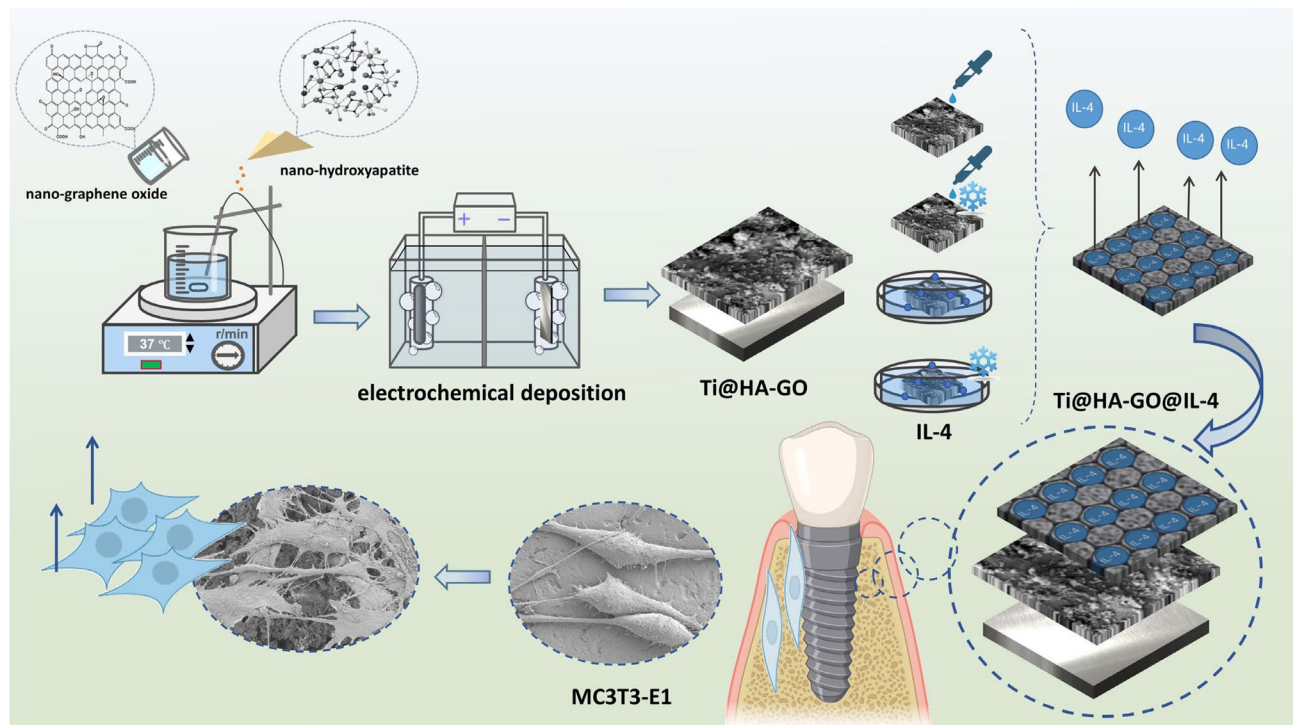
Material preparation

Titanium disc polishing and cleaning

Commercially pure titanium discs (TA1 grade, $\Phi 1\text{cm} \times 0.1\text{ cm}$; Haiyuan Scientific Metals Co., Ltd., Guangdong, China) were sequentially polished using #600–#1200 grit silicon carbide sandpaper with edge refinement. The polished discs underwent ultrasonic cleaning (SK5200LH, Kedao Ultrasonic Instrument Co., Ltd., Shanghai, China; 40 kHz) in three stages: acetone (30 min), absolute ethanol (30 min), and deionized water (15 min), followed by storage in anhydrous ethanol.

Preparation of HA-GO electrolyte

A homogeneous suspension was prepared by dispersing 200 mg nano-hydroxyapatite powder (TX21249, Yingxin Laboratory Equipment Co., Ltd., Shanghai, China) in 50 mL absolute ethanol under constant magnetic stirring (Kexing Instrument Co., Ltd., Shanghai, China; 180 rpm, 37°C) for 3 h. Subsequently, 0.8 mL graphene oxide solution (XF224-1, XFNANO Materials Tech Co., Ltd., Jiangsu, China; 5 mg/mL) [13] was added dropwise with continuous stirring for an additional 3 h. The electrolyte pH was precisely adjusted to 3.0–4.0 using nitric acid (HNO_3).



Scheme 1 Schematic diagram of the preparation of hydroxyapatite-graphene oxide/interleukin-4 (Ti@HA-GO@IL-4) composite coating material

Coating fabrication

The hydroxyapatite-graphene oxide (HA-GO) coating was synthesized through electrochemical deposition in a temperature-controlled electrolytic cell (37°C). The deposition system comprised a graphite anode and titanium cathode (inter-electrode distance: 10 mm) under constant magnetic agitation (180 rpm). Electrodeposition was performed using a regulated power supply (MAT-SUSADA Precision Inc., Japan) at 30 V with a current density of 0.8 mA/cm² for 2 min. The coated specimens (Ti@HA-GO) were subsequently air-dried at ambient temperature for 24 h.

IL-4 immobilization

Solution Preparation

Recombinant mouse interleukin-4 (IL-4, abs04697, Absin Bioscience Inc., Shanghai) was dissolved in deionized water to prepare a 2 µg/mL IL-4 solution [24].

Loading methods

Immersion-Drying Method

Ti and Ti@HA-GO samples were immersed in the IL-4 solution for 24 h under light-protected conditions. After incubation, unbound IL-4 was removed by washing with phosphate-buffered saline (PBS), followed by drying at room temperature (RT) for 2 h, yielding Ti@HA-GO@IL-4 (immersion-drying method) and Ti@IL-4 (immersion-drying method).

Immersion-freeze-drying method

Similarly, Ti and Ti@HA-GO samples were soaked in the IL-4 solution for 24 h (light-protected), then frozen overnight at -20°C and lyophilized for 24 h. Unbound IL-4 was washed away with PBS, resulting in Ti@HA-GO@IL-4 (immersion-freeze-drying method) and Ti@IL-4 (immersion-freeze-drying method).

Pipette-drying method

A 2 µg/mL IL-4 solution was uniformly pipetted onto the surfaces of Ti and Ti@HA-GO substrates to ensure complete coverage. The samples were incubated at room temperature (RT) for 2 h, and this adsorption process was repeated three times to enhance loading efficiency. After a final 2 h drying at RT, unbound IL-4 was removed by PBS washing, yielding Ti@HA-GO@IL-4 and Ti@IL-4 composites prepared via the pipette-drying method.

Pipette-freeze-drying method

The same pipetting procedure was performed, but after incubation, the samples were frozen at -20 °C overnight and lyophilized for 24 h. Unbound IL-4 was eliminated via PBS washing, yielding Ti@HA-GO@IL-4 (pipette-freeze-drying method) and Ti@IL-4 (pipette-freeze-drying method).

Material characterization

Macroscopic morphological features were documented using a digital single-lens reflex (DSLR) camera. Surface

microstructures were examined and imaged using scanning electron microscopy (SEM; Zeiss Supra55 VP, Germany). The elemental composition of coatings was quantitatively and qualitatively analyzed by energy-dispersive X-ray spectroscopy (EDS; Bruker X-Flash SDD 5010, Germany). Surface roughness and topographical characteristics were measured via atomic force microscopy (AFM; Bruker Dimension Icon, Germany). The crystalline structure and chemical composition of coatings were characterized by X-ray diffraction (XRD; D8 ADVANCE Plus, Germany). Surface chemical composition and electronic states were analyzed using X-ray photoelectron spectroscopy (XPS; PHI 5000 VersaProbe, USA). Functional groups present in the coatings were identified by comparing characteristic frequencies with spectral libraries using Fourier-transform infrared spectroscopy (FTIR; Spotlight 400/400 N, PerkinElmer, USA). Molecular structure and composition were investigated through Raman spectroscopy (LabRAM HR Evolution, Horiba, Japan). Surface wettability was evaluated by measuring water contact angles using a goniometer (SZ-CAMC32, Shanghai Xunzhun Instrument Co., China), with representative images captured for analysis.

Stability testing of Ti@HA-GO coatings

Ti@HA-GO-coated samples were immersed in 24-well plates containing 1 mL PBS per well. After 14 days, the supernatant was collected, and absorbance was measured at wavelengths ranging from 300 to 700 nm. The absorbance of fresh PBS was also determined within the same wavelength range using a Nano-100 spectrophotometer (Allsheng, Hangzhou, China). For Raman analysis, Ti@HA-GO-coated samples were similarly immersed in PBS for 14 days and subsequently examined using a Raman microscope system.

Protein adsorption assay

Samples were incubated in 24-well plates containing 1 mg/mL bovine serum albumin (BSA; Baisha Biology, Anhui, China) for 24 h. Non-adherent proteins were removed by phosphate-buffered saline (PBS) washing, while adherent proteins were eluted using 2% sodium dodecyl sulfate (SDS; BL517A, Sigma-Aldrich). Protein concentrations were quantified using a BCA protein assay kit (PC0020, Solarbio, Beijing, China) to evaluate the protein adsorption capacity of different scaffold materials.

IL-4 release study

IL-4-loaded samples were incubated in 2 mL microcentrifuge tubes containing 1 mL PBS at 37 °C. At pre-determined time points, supernatant was collected and replaced with equal volumes of fresh PBS to maintain system volume. IL-4 concentrations were quantified

using an ELISA kit (46410, Thermo Fisher Scientific) according to the manufacturer's protocol, with cumulative release calculated based on standard curves.

For visualization, IL-4 was conjugated with fluorescein isothiocyanate (FITC; 46425, Solarbio, Beijing) following the manufacturer's instructions to create FITC-labeled IL-4. The labeled IL-4 was then loaded onto material surfaces following the same procedure. Residual FITC-IL-4 on material surfaces was observed and imaged at specific time points using fluorescence microscopy (Eclipse TS100-F, Nikon, Japan).

Biocompatibility evaluation of Hydroxyapatite-Graphene oxide nanocoatings

MC3T3-E1 cell culture

Mouse MC3T3-E1 cell line (CL0813, Fenghui Biotechnology, Hunan, China) was cultured in complete medium consisting of high-glucose DMEM (90%; C11965500BT, GIBCO), heat-inactivated fetal bovine serum (10%; 13011-8611, Sijiqing Biotech, Hangzhou), and penicillin-streptomycin solution (1%, containing 100 U/mL penicillin and 0.1 mg/mL streptomycin; P7630, Solarbio). Cells were maintained at 37 °C in a 5% CO₂ humidified incubator (Smart Cell HF-90, Likang Instruments, Shanghai).

Cell proliferation assay

Cell proliferation on different coatings was assessed using CCK-8 assay. Cells (5×10^4 cells/mL) were seeded onto coating surfaces, and proliferation was measured after 4 and 7 days using a CCK-8 kit (FC101-03, TransGen Biotech) following manufacturer's instructions.

Cell adhesion evaluation

DAPI Staining Cell adhesion was examined by DAPI staining after 4 days of culture. Cells were fixed with 4% paraformaldehyde (MUXMT-90021, Aladdin) for 20 min at room temperature, stained with DAPI (4',6-diamidino-2-phenylindole; A003-1-2, Nanjing Jiancheng) for 5 min, and imaged under fluorescence microscopy for nuclear counting.

SEM Observation After 4 days of culture, adherent cells were fixed with glutaraldehyde (323381, Aladdin), dehydrated through ethanol gradients (Sinopharm), and sputter-coated for SEM examination of cell morphology and adhesion patterns.

Statistical analysis

Data were analyzed using SPSS 27.0 and visualized with GraphPad Prism 10.1.2 and Origin 2021. Normally distributed data were expressed as mean $\pm$ SD and analyzed by one-way ANOVA (with Bonferroni or Tamhane's T2 post-hoc tests). Non-normal data were analyzed using Kruskal-Wallis H test with Bonferroni correction.

Two-tailed tests were performed with statistical significance set at $p < 0.05$. All experiments were performed in triplicate. Statistical methods were reviewed by biostatisticians at Xinjiang Medical University.

Results and discussion

Characterization and evaluation of the physicochemical properties of the samples in each group

Through a comparative analysis of four distinct protein loading methodologies—Immersion- Drying Method, Immersion-Freeze-Drying Method, Pipette-Drying Method, and Pipette- Freeze-Drying Method—the Immersion-Drying Method was ultimately selected for loading IL-4 onto Ti@IL-4 and Ti@HA-GO@IL-4 substrates. Loading status was assessed via scanning electron microscopy (SEM, visualizing protein aggregates) and fluorescence tracking labeling, while quantitative ELISA analysis characterized the 8-day sustained release profile (detailed data in sections covering IL-4 adsorption/release, SEM imaging, and water contact angle measurements). Successful IL-4 loading was confirmed by three independent analyses: (i) SEM visualization of protein aggregates, (ii) EDS detection of nitrogen signatures, and (iii) FTIR identification of characteristic amide bonds ($1,650\text{--}1,660\text{ cm}^{-1}$). Loading efficiency was evaluated based on sustained release kinetics (ELISA) and comparative fluorescence intensity. Subsequently, these functionalized samples will undergo further physicochemical characterization.

Surface morphology

Gross observation revealed that after sequential polishing with water sandpaper, the surface of the Ti group was smooth and reflected a characteristic metallic luster. The Ti@IL-4 group retained a mirror-like smooth surface, resembling that of the Ti group. The Ti@HA-GO group exhibited a highly saturated milky white surface, with areas around the edges in contact with the electrode clamp showing no deposited coating, exposing the underlying metal substrate. In contrast, the Ti@HA-GO@IL-4

group displayed a less saturated milky white surface, with a uniformly coated layer, as shown in Fig. 1.

Energy dispersive x-ray spectroscopy (EDS)

The EDS analysis results showed that the Ti group only exhibited the distribution of Ti elements. The Ti@IL-4 group, in addition to Ti, contained trace amounts of C elements. Although a distinct N peak was observed in the spectral data of Ti@IL-4, the quantitative results showed a nitrogen content of 0.00 at%. This apparent contradiction arose because the extremely poor hydrophilicity of the titanium substrate resulted in low adsorption efficiency of the IL-4 aqueous solution. The subsequent PBS rinse further reduced the amount of surface-bound IL-4, causing the nitrogen content to drop below the detection threshold (typically 0.1–1 at%). Therefore, the nitrogen content was reported as 0.00 at%, indicating that the actual value was below the detectable limit. The Ti@HA-GO group showed the presence of O, C, P, Ca, and N elements, with a relatively uniform distribution. Compared to the Ti@HA-GO group, the Ti@HA-GO@IL-4 group exhibited an increase in the N element content, and both C and N element contents were higher than those in the Ti@HA-GO group, as shown in Fig. 2; Table 1.

Graphene oxide primarily consists of a graphene backbone with carbon-carbon double-bonded aromatic rings, which are bonded to oxygen-containing functional groups [25], while hydroxyapatite has a crystal structure composed of Ca^{2+} and PO_4^{3-} ions [26]. Therefore, both the Ti@HA-GO and Ti@HA-GO@IL-4 groups contained O, C, P, and Ca elements. The absence of detectable Ti element content indicates that the coatings in both Ti@HA-GO and Ti@HA-GO@IL-4 groups exhibited high coating density. The slight increase in C elements in the Ti@IL-4 group, and the higher levels of C and N elements in the Ti@HA-GO@IL-4 group, suggest successful loading of IL-4, as IL-4 contains C, N, and O elements.

Atomic force microscopy (AFM)

The two-dimensional and three-dimensional surface microstructures of the samples in each group are shown in Fig. 3. Roughness parameters, such as the average roughness (Ra) and the root-mean-square roughness (Rq) of the Z data, are provided in Table 2. The Ti@HA-GO@IL-4 group exhibited a Ra of $30.93 \pm 7.30\text{ nm}$ and an Rq of $40.20 \pm 7.94\text{ nm}$, which were rougher than the surfaces of the other three groups: the Ti group with Ra of $2.82 \pm 0.78\text{ nm}$ and Rq of $3.58 \pm 1.02\text{ nm}$, the Ti@IL-4 group with Ra of $4.84 \pm 2.90\text{ nm}$ and Rq of $7.61 \pm 5.01\text{ nm}$, and the Ti@HA-GO group with Ra of $14.70 \pm 3.63\text{ nm}$ and Rq of $19.80 \pm 5.00\text{ nm}$.

The surface topography of dental implants comprises both macro- and micro-scale morphological

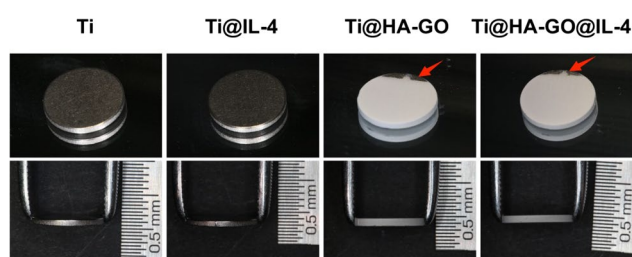


Fig. 1 Gross observation of the surface of samples in each group. Ti: pure titanium discs, Ti@IL-4: interleukin-4-loaded titanium discs, Ti@HA-GO: hydroxyapatite-graphene oxide coated titanium discs, Ti@HA-GO@IL-4: hydroxyapatite-graphene oxide/interleukin-4 coated titanium discs. Red arrows highlight uncoated margins at electrode clamp contact zones

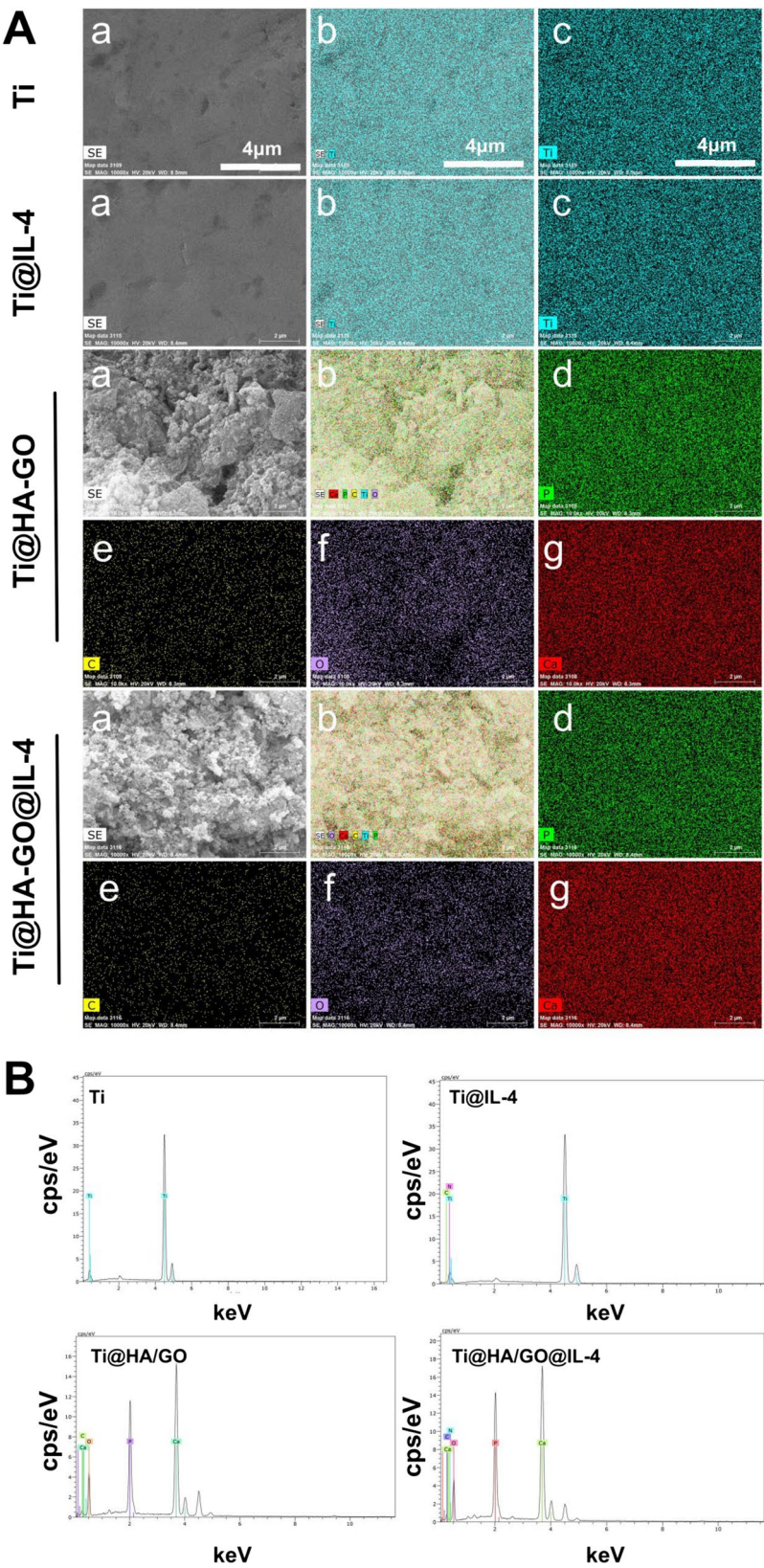


Fig. 2 X-ray energy dispersive spectroscopy (EDS) results of the samples in each group: **(A)** shows the X-ray energy dispersive maps for each group, where (a) represents the scanning electron microscope (SEM) image and (b) is the merge image. Panels (c) to (g) display the elemental distribution maps for Ti, P, C, O, and Ca, respectively. **(B)** presents the X-ray energy dispersive spectra for each group

Table 1 Mass fraction (%) of each element in the samples of each group

Atom	Ti		Ti@IL-4		Ti@HA-GO		Ti@HA-GO@IL-4	
	Weight%	Atomic %	Weight%	Atomic %	Weight%	Atomic %	Weight%	Atomic %
Ti	100	100	99.18	96.81	0	0	0	0
O	0	0	0	0	44.94	61.77	42.1	57.05
Ca	0	0	0	0	36.68	20.13	36.47	19.73
P	0	0	0	0	13.88	9.85	13.31	9.32
C	0	0	0.82	3.19	4.51	8.26	5.22	9.42
N	0	0	0	0	0	0	2.9	4.49

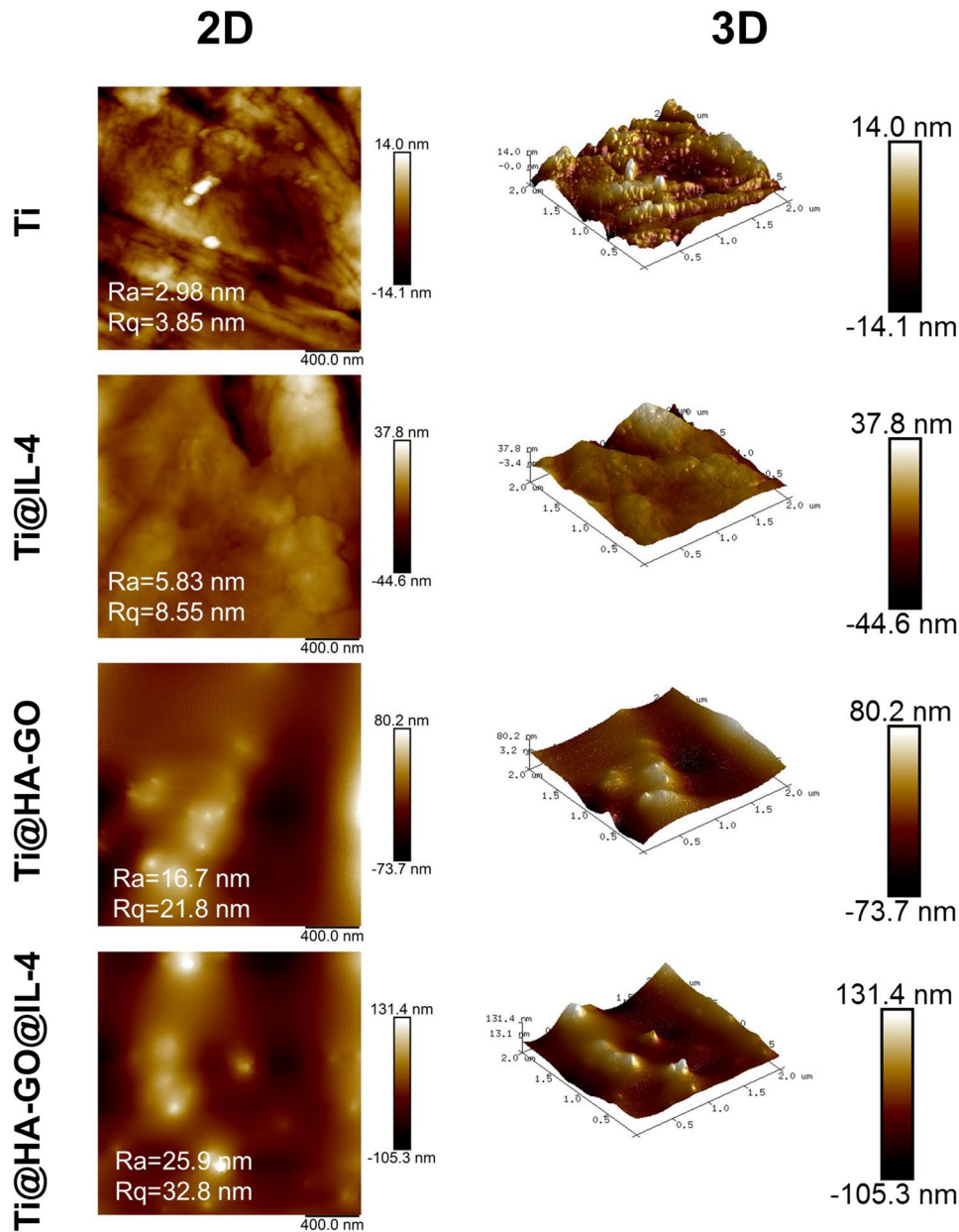


Fig. 3 Atomic force microscopy (AFM) was used to examine the two-dimensional (2D) and three-dimensional (3D) surface microstructure of the samples in each group

Table 2 AFM analysis of the roughness of samples in each group

sample	Ti	Ti@IL-4	Ti@HA-GO	Ti@HA-GO@IL-4
Ra value (nm)	2.82 ± 0.78	4.84 ± 2.90	14.70 ± 3.63	30.93 ± 7.30 ^a
Rq value (nm)	3.58 ± 1.02	7.61 ± 5.01	19.80 ± 5.00 ^a	40.20 ± 7.94 ^a

^a*p* < 0.05 compared to the Ti sample

characteristics. Titanium implants with optimized surface roughness demonstrate enhanced primary stability, improved bone-to-implant contact, and significantly increased removal torque values. The osteogenic effects of surface roughness are mediated through two primary mechanisms: (1) substantial increase in effective surface area, and (2) modulation of cellular morphology and behavior. These topological features have been well-documented to promote osteoblastic differentiation, accelerate bone formation, and optimize bone remodeling processes [27]. Notably, the surface roughness of natural bone tissue approximates 32 nm [28]. In our study, the Ti@HA-GO@IL-4 group exhibited surface roughness values closest to this biological benchmark ($R_a = 30.93 \pm 7.30$ nm), suggesting that this biomimetic surface characteristic may provide a more favorable microenvironment for bone tissue regeneration.

X-ray diffraction (XRD)

Both Ti@HA-GO@IL-4 and Ti@HA-GO groups exhibited characteristic diffraction peaks of HA and GO, along with peaks from the titanium substrate [12]. Specifically, the HA phase was identified by its distinctive reflections at $2\theta = 25.9^\circ$ (002), 31.8° (211), and 32.2° (112) (JCPDS 09-0432) [29]. In contrast, only the titanium diffraction peak was detected in the Ti@IL-4 and Ti groups, as shown in Fig. 4A. This analysis further confirms the successful deposition of the HA-GO coating on the titanium substrate surface using the electrochemical method.

X-ray photoelectron spectroscopy (XPS)

The XPS spectra of the surfaces of the samples in each group are shown in Fig. 4B. Compared to the Ti and Ti@IL-4 groups, the Ti@HA-GO and Ti@HA-GO@IL-4 groups exhibited peaks corresponding to elements such as Ca_{2p} and P_{2p} , which are components of hydroxyapatite [30]. However, no Ti_{2p} peaks were detected in these two groups, indicating that the coating materials were fully applied to the titanium substrate surface. This result is consistent with the X-ray energy dispersive spectroscopy (EDS) analysis.

Fourier transform infrared (FTIR)

The FTIR spectroscopy results are presented in Fig. 4C. Characteristic absorption peaks corresponding to Ti–O bonds were observed at 747.89 cm^{-1} , 821.83 cm^{-1} , and

962.32 cm^{-1} in both the Ti and Ti@IL-4 groups [31]. The peak at 1159.57 cm^{-1} was attributed to the P–O vibration from residual phosphate species. In the Ti@IL-4 group, the C–H stretching vibrations (1456.53 cm^{-1} , 2869.92 cm^{-1} , 2927.15 cm^{-1} , and 2954.94 cm^{-1}) [32] and the C=O stretching (1170.32 cm^{-1}) likely originate from IL-4, as these functional groups are characteristic of proteins and organic molecules. For the Ti@HA-GO and Ti@HA-GO@IL-4 groups, the phosphate (PO_4^{3-}) peaks at 1034 cm^{-1} [33] and 1090.46 cm^{-1} are attributed to HA [34], while the carbonate (CO_3^{2-}) signal at 1416 cm^{-1} suggests partial substitution in the HA lattice [35]. Additionally, GO typically exhibits C=O (carboxyl) and C–O (epoxy/alkoxy) stretches around 1720 cm^{-1} and $1050\text{--}1200\text{ cm}^{-1}$ [12], though these may overlap with HA and IL-4 signals. The presence of IL-4 is further confirmed by the amide I (C=O, $\sim 1650\text{ cm}^{-1}$) and amide II (N–H, $\sim 1540\text{ cm}^{-1}$) bands.

Raman

Raman spectroscopy confirmed the presence of characteristic D (1350 cm^{-1}) and G (1580 cm^{-1}) peaks in Ti@HA-GO and Ti@HA-GO@IL-4 groups (Fig. 4D), verifying successful GO incorporation into the coating. While these peaks are intrinsic to GO's sp^2 carbon structure and disorder characteristics [36], they cannot confirm chemical bonding between GO and HA. Supporting this, FTIR analysis (Fig. 4C) showed no emergent absorption peaks (e.g., P–O–C at $900\text{--}1100\text{ cm}^{-1}$), indicating that GO–HA integration occurs predominantly via non-covalent interactions (hydrogen bonding, electrostatic forces) rather than covalent bonding, consistent with prior reports on similar composites [37]. Furthermore, the absence of significant Raman spectral shifts after IL-4 loading demonstrates that IL-4 attaches to the coating surface primarily through physical adsorption (hydrophobic interactions, van der Waals forces), not chemical conjugation. This conclusion is corroborated by two key observations: (1) no new chemical bonds detected in FTIR spectra (Fig. 4C), and (2) complete IL-4 release kinetics observed in Fig. 6C. The low IL-4 loading concentration ($2\text{ }\mu\text{g/mL}$) also explains the negligible perturbation of the coating's Raman signatures.

Comparative evaluation of the efficiency of IL-4 loading methodologies

Based on a comprehensive comparison of the efficiency of various IL-4 loading methods, the optimal coating modification strategy was identified, demonstrating superior performance in terms of loading capacity, binding mode, release kinetics, and stability.

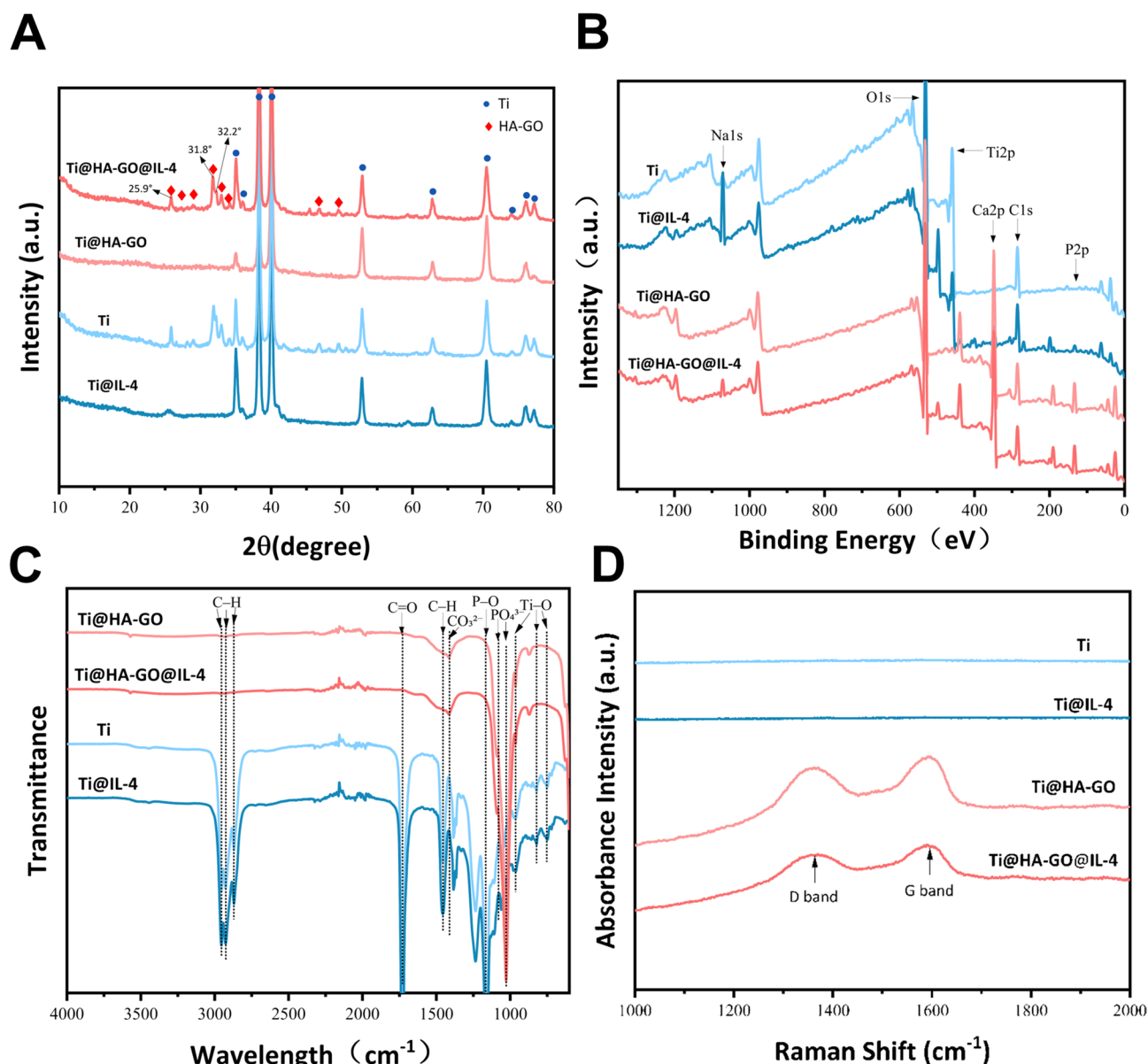


Fig. 4 **A** X-ray diffraction spectra of the four groups of samples, **B** X-ray photoelectron spectroscopy (XPS) spectra of the four groups of samples, **C** Fourier transform infrared (FTIR) spectra of the four groups of samples, **D** Raman spectra of the four groups of samples

Scanning electron microscopy (SEM)

The microstructural morphology of four titanium sheet groups (Ti, Ti@HA-GO, Ti@IL-4, and Ti@HA-GO@IL-4) was examined by scanning electron microscopy (SEM) in Fig. 5A and B, with all IL-4-containing groups prepared via immersion. At low magnification, Ti and Ti@IL-4 exhibited characteristic striated polishing marks, while Ti@HA-GO and Ti@HA-GO@IL-4 showed complete substrate coverage without polishing artifacts. The Ti@HA-GO surface displayed nanoscale GO wrinkles that enhance cell adhesion [38, 39], whereas Ti@HA-GO@IL-4 presented a smoother morphology with microcracks attributed to interfacial stress from

differential contraction during IL-4 immersion-drying. High-magnification analysis revealed pronounced polishing grooves on Ti/Ti@IL-4, a “coral-like” microstructure in Ti@HA-GO (block-like GO sheets supporting “salt-crystal” HA crystallites) [40], and clustered spherical protein-like structures on Ti@HA-GO@IL-4 (Fig. 5C) confirming IL-4 adsorption.

Comparative analysis of IL-4 loading methods (Fig. 5A) confirmed striated abrasion marks on Ti and all Ti@IL-4 groups, with Ti displaying greater roughness. For coated groups (Fig. 5B), Ti@HA-GO exhibited uniform corrugated structures with needle-like protrusions and no cracking. Freeze-drying methods induced microcracks

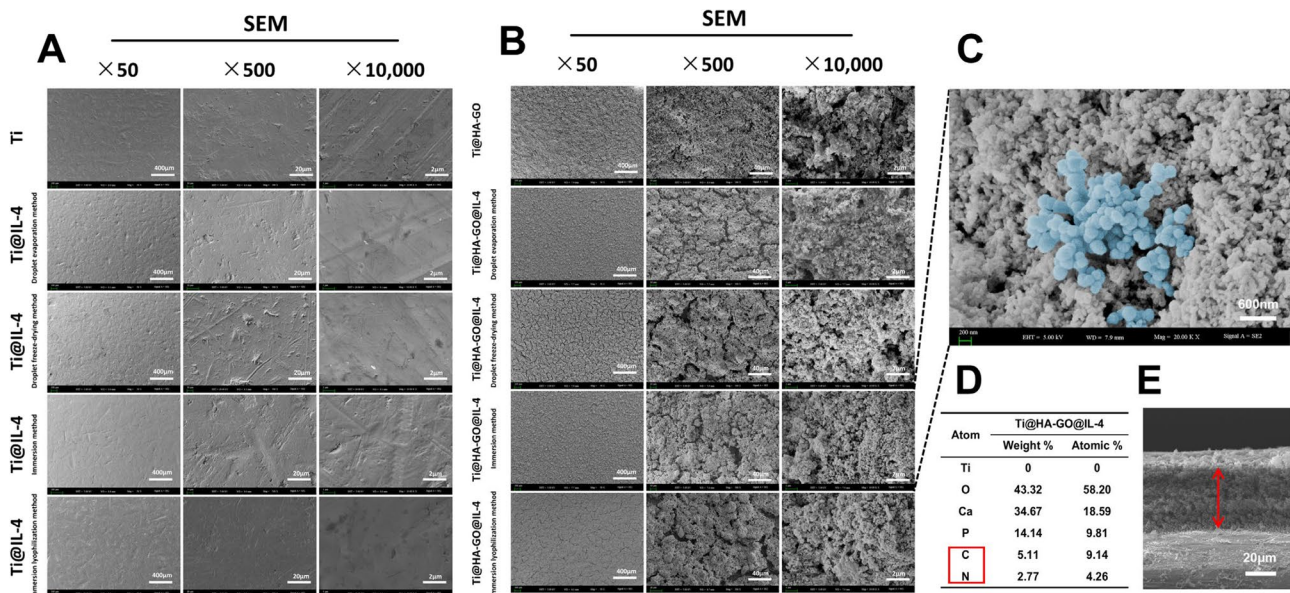


Fig. 5 **A** Comparative SEM analysis of Ti control and Ti@IL-4 samples with varied IL-4 loading methods on pure titanium substrates, **B** SEM characterization of Ti@HA-GO and Ti@HA-GO@IL-4 composites fabricated through different IL-4 loading approaches, **C** High-resolution SEM image (x10,000) of Ti@HA-GO@IL-4 specimen prepared via immersion-mediated IL-4 loading, **D** The mass fractions (%) of elements in the clustered circular protein-like structures on the surface of Ti@HA-GO@IL-4 in Fig. 5C, **E** Cross-sectional SEM image (x500) of the Ti@HA-GO@IL-4 sample prepared by the impregnation method IL-4, as a protein, is primarily composed of C, N, and O

in Ti@HA-GO@IL-4 due to rapid water sublimation fracturing GO layers [12], while conventional drying maintained crack-free coatings. All Ti@HA-GO@IL-4 specimens replaced corrugated features with globular protrusions, creating smoother surfaces. Immersion techniques generated clustered spherical protein-like structures, with immersion-dried specimens showing distinct blue-marked IL-4 aggregates (irregular spheroids with smooth surfaces, Fig. 5C) confirmed by EDS proteinaceous elements (Fig. 5D). Furthermore, cross-sectional SEM analysis revealed that the coating thickness was approximately 44.794 μm (Fig. 5E).

Characterization of coating durability and interleukin-4 release dynamics

The coating stability of Ti@HA-GO is shown in Fig. 6A. Comparative analysis of absorbance values between PBS containing Ti@HA-GO and control PBS over 14 days demonstrated nearly identical optical densities, indicating excellent stability of the coating in PBS without detectable HA-GO particle detachment or dissolution. Raman spectroscopy (Fig. 6B) revealed no significant changes in characteristic peaks after 14-day PBS immersion, further confirming the structural stability of the coating. Protein adsorption capacity (Fig. 6D) was significantly higher on Ti@HA-GO than on Ti ($P < 0.001$). This characteristic is more favorable for subsequent IL-4 loading.

Enzyme-linked immunosorbent assay (ELISA) results (Fig. 6C) showed sustained IL-4 release from Ti@

HA-GO@IL-4 for up to 7 days, whereas Ti@IL-4 exhibited a typical burst release ($>75\%$ within 24 h). No statistically significant differences ($P > 0.05$) were observed in IL-4 release profiles among the four loading methods (immersion-drying method, immersion-freeze-drying method, pipette-drying method, and pipette-freeze-drying method) between Ti@IL-4 and Ti@HA-GO@IL-4 groups. Time-dependent fluorescence monitoring (Fig. 7) further validated these findings. FITC-labeled IL-4 tracking demonstrated prolonged cytokine retention on Ti@HA-GO@IL-4 surfaces. At day 0, protein adsorption was significantly higher in Ti@HA-GO@IL-4 across all four loading methods compared to Ti@IL-4 groups. By day 3, near-complete IL-4 release occurred from Ti@IL-4, while Ti@HA-GO@IL-4

maintained sustained release for approximately 7 days. Experimental data revealed that both the immersion-drying method and immersion-freeze-drying method achieved significantly higher IL-4 loading than the pipette-drying method and pipette-freeze-drying method in both Ti@IL-4 and Ti@HA-GO@IL-4 groups. These results demonstrate that although the immersion-based methods enable higher IL-4 loading, the sustained-release performance of Ti@HA-GO coatings is independent of loading method, with the coating significantly prolonging IL-4 release duration.

Water contact angle test

Comparative analysis of SEM observations and IL-4 release experiments demonstrated superior performance

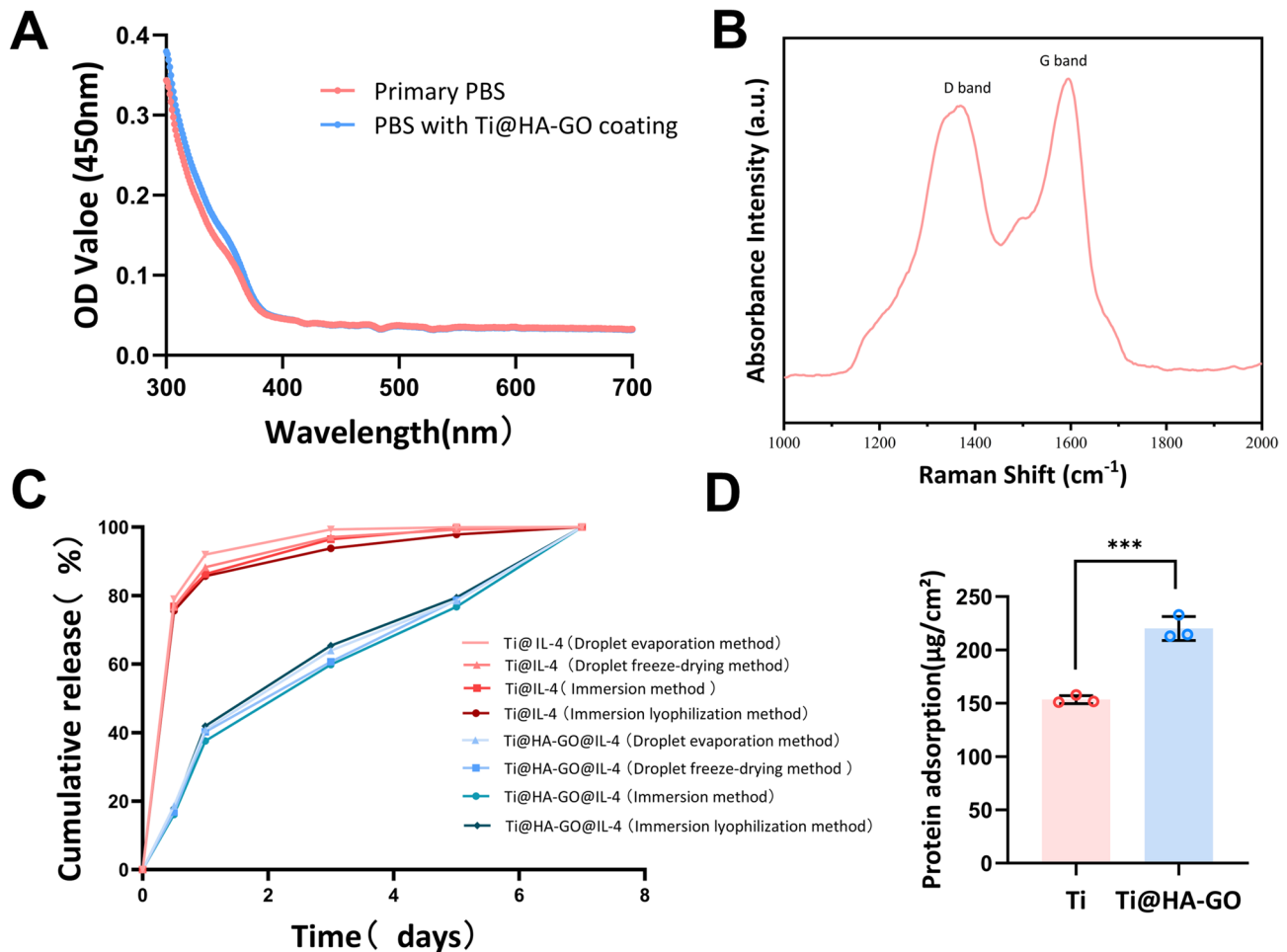


Fig. 6 **A** Absorbance of Ti@HA-GO coatings after 14 days of immersion in PBS compared with blank PBS control. **B** Raman spectra of Ti@HA-GO coatings following 14-day immersion in PBS. **C** ELISA results demonstrating the release kinetics of IL-4 from Ti@IL-4 and Ti@HA-GO@IL-4 groups. **D** Comparison of protein adsorption capacity between Ti and Ti@HA-GO coatings. *** denotes $p < 0.001$ versus the Ti group

of both the immersion-drying method and immersion-freeze-drying method among the four protein loading approaches (immersion-drying method, immersion-freeze-drying method, pipette-drying method, and pipette-freeze-drying method) in terms of protein loading capacity, distribution uniformity, and sustained-release characteristics. Based on these findings, we further conducted comparative analysis of surface wettability between the immersion-drying method and immersion-freeze-drying method. The surface wettability of each material group was evaluated by water contact angle measurements (Fig. 8A). Quantitative analysis (Fig. 8B) demonstrated distinct patterns at both 1 s and 30 s time points: For intra-group comparisons, the Ti@IL-4 (immersion-drying method) group exhibited significantly lower contact angles than both the Ti control group and Ti@IL-4 (immersion-freeze-drying method) group ($P < 0.001$). Similarly, in the Ti@HA-GO@IL-4 series, the immersion-drying method group showed superior wettability compared to both the Ti@HA-GO control and

immersion-freeze-drying method group ($P < 0.001$). Inter-group analysis revealed that the Ti@HA-GO group displayed markedly reduced contact angles relative to the Ti group ($P < 0.001$). Notably, within both IL-4-loaded groups, samples prepared via the immersion-drying method consistently demonstrated lower contact angles than their immersion-freeze-drying counterparts ($P < 0.001$), with the Ti@HA-GO@IL-4 (immersion-drying) group showing significantly enhanced wettability compared to Ti@IL-4 (immersion-drying) ($P < 0.01$).

These findings collectively indicate that the incorporation of hydroxyapatite-graphene oxide composites substantially enhances surface hydrophilicity [33]. This enhancement arises from synergistic mechanisms: (1) GO's oxygen-containing groups (carboxyl/hydroxyl/epoxy) increase surface polarity [25]; (2) GO-HA nanoscale topography ($R_a \approx 30.93$ nm) mimics bone roughness and traps water via capillary action [27]; (3) HA's phosphate/carbonate moieties form hydrogen bonds; (4) IL-4's hydrophilic residues ($-OH/-NH_2$)

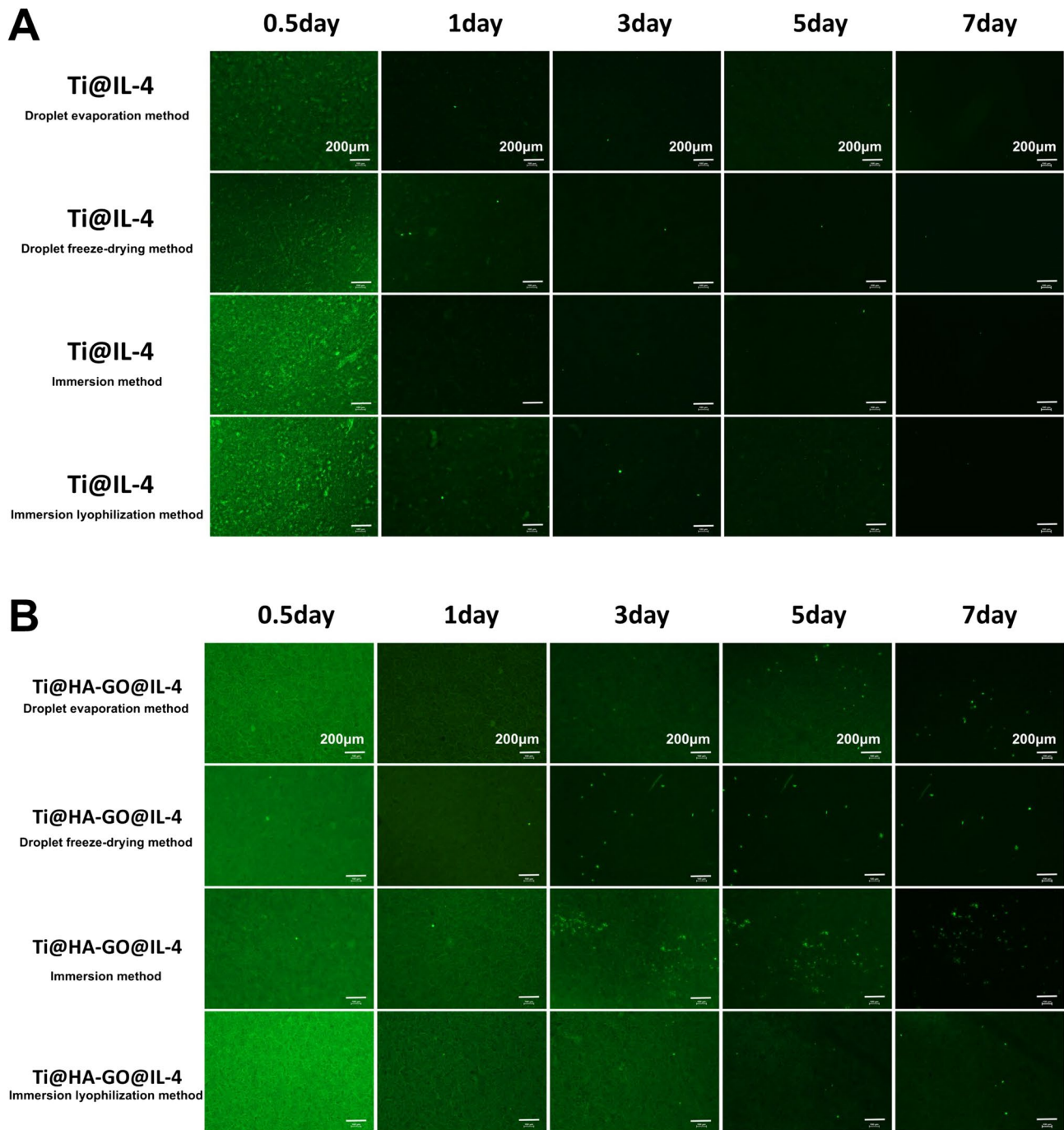


Fig. 7 Fluorescence imaging of FITC-labeled IL-4 on Ti@IL-4(A) and Ti@HA-GO@IL-4 (B) surfaces at 0, 0.5, 1, 3, 5, and 7 days using inverted fluorescence microscopy to visualize IL-4 release and retention. Scale bar = 200 μ m

reduce contact angles [24]; and (5) crack-free coating integrity preserves hydrophilic sites (Fig. 5). Cumulatively, these mechanisms reduce water contact angles, facilitating protein adsorption, osteoblast adhesion, and anti-inflammatory responses.

The loading method of IL-4 significantly influences wettability. The immersion-freeze-drying method compromises hydrophilicity, potentially due to protein film

formation during lyophilization altering surface contact area. Conversely, the immersion-drying method effectively improves surface wettability. Enhanced hydrophilicity mitigates inflammatory responses while actively promoting osteogenesis [41]. Such surfaces facilitate osteoblast and bone marrow mesenchymal stem cell (BMSC) adhesion and differentiation in vitro and in vivo. This facilitatory effect aligns with co-culture studies

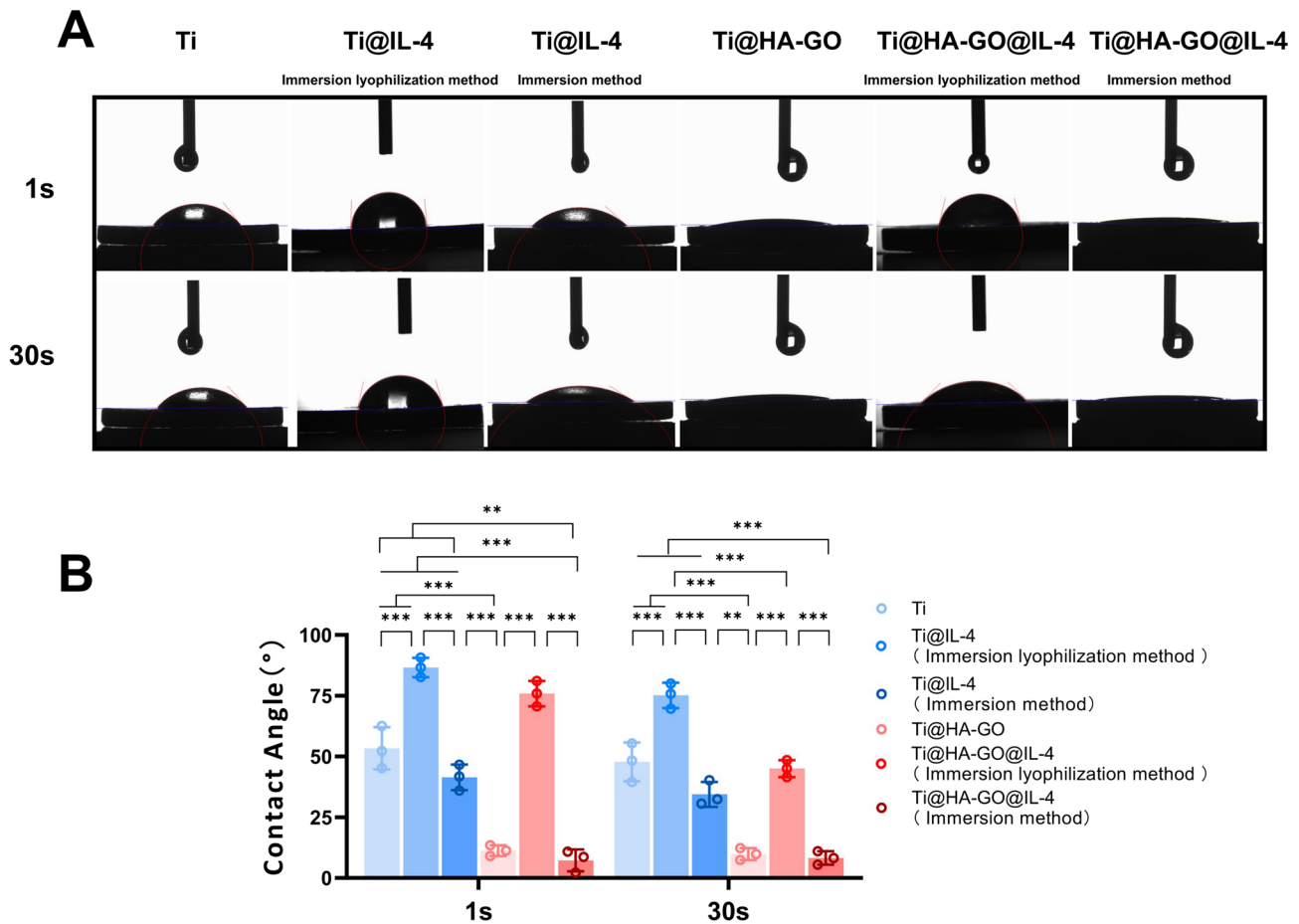


Fig. 8 **A** Static water contact angle images of samples from each group, **B** Contact angle analysis results of samples from each group. ** $p < 0.01$, *** $p < 0.001$

where hydroxyapatite-graphene oxide composite coatings promote proliferation and adhesion of both BMSCs [42] and RAW 264.7 murine macrophages [43].

The physicochemical characteristics and wettability of implant surfaces critically determine protein adsorption and release kinetics [44]. Here, the Ti@HA-GO coating demonstrated superior hydrophilicity versus pure titanium, facilitating greater protein loading capacity. This enhancement is attributed to Ti@HA-GO's nanoscale architecture providing increased specific surface area for protein interaction. Furthermore, the coating sustains protein release through hydrophobic interactions, electrostatic forces, and hydrogen bonding with protein molecules [45]. These multifactorial interactions underlie improvements in both initial protein adsorption and sustained release profiles.

Biological evaluation of coatings in different groups

The complex biological process of osseointegration encompasses coordinated interactions among various cellular components, particularly osteogenic lineage cells and immunomodulatory cells. As the key effector cells in bone formation, osteoblasts mediate extracellular matrix

synthesis, secretion and subsequent mineralization processes [46]. To investigate the material-cell interactions, we conducted comprehensive in vitro evaluations using passage 3 (P3) Mouse calvaria-derived 3t3 established 1 (MC3T3-E1) subclone 4 murine pre-osteoblasts cultured on four different composite coatings, with particular focus on their proliferative capacity, morphological development and adhesive properties [47].

SEM observations (Fig. 9A) revealed that: (1) Ti@IL-4 group exhibited more pronounced pseudopod extension and significantly increased cell spreading area compared to Ti group; (2) Ti@HA-GO group displayed characteristic adhesive morphology with cells embedded into the material surface, showing tight interfacial bonding and significantly larger spreading area than both Ti and Ti@IL-4 groups; (3) Ti@HA-GO@IL-4 group formed dense pseudopod networks at both cell-cell and cell-coating interfaces, with significantly higher pseudopod density than other groups. After 7 days of culture: (1) Ti group showed increased spreading area and flattened morphology compared to day 4; (2) Both Ti@IL-4 and Ti@HA-GO groups demonstrated increased pseudopod quantity and enhanced surface roughness; (3) Ti@

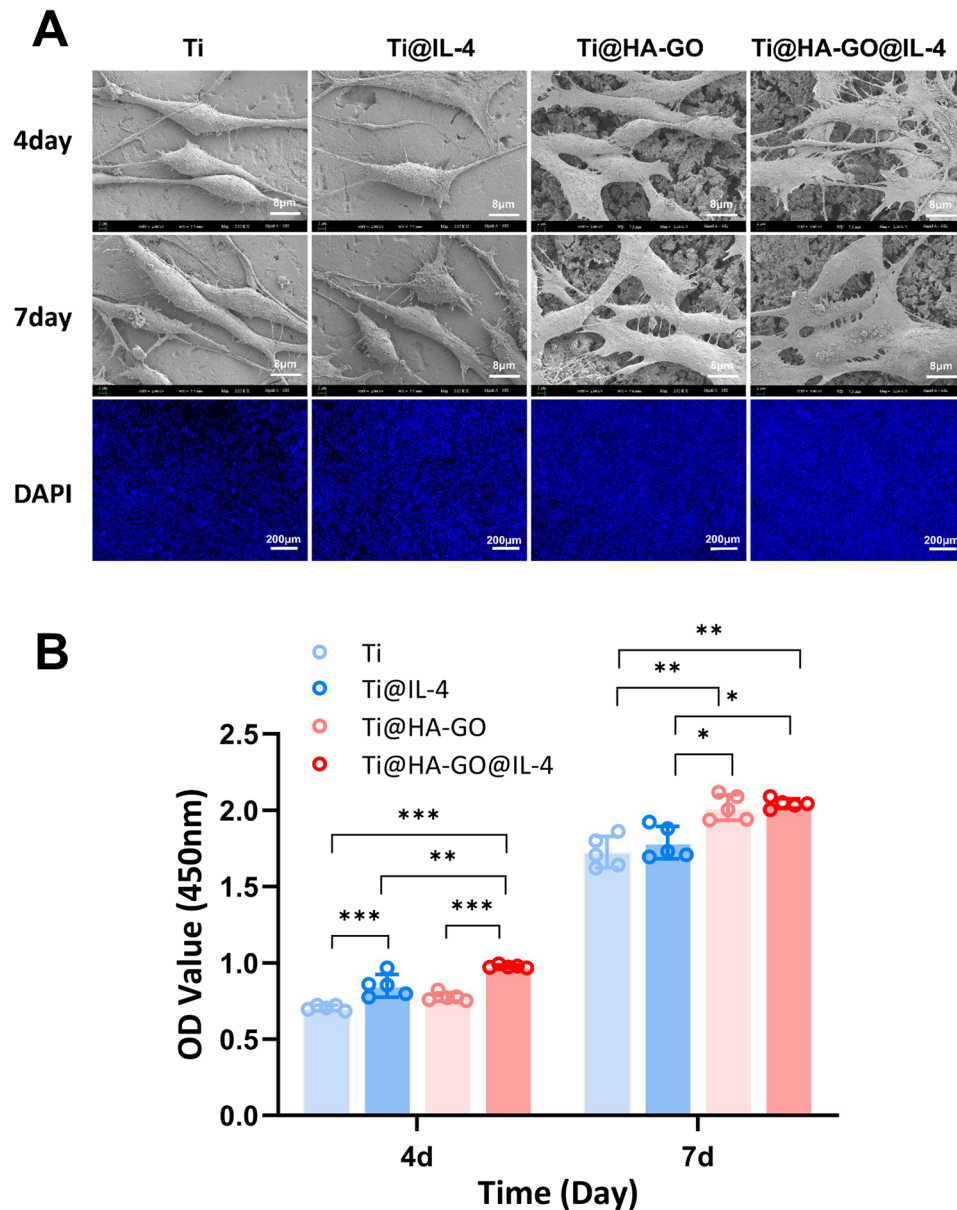


Fig. 9 **A** Adhesion of MC3T3-E1 cells on coating surfaces (SEM) and DAPI staining images, **B** Comparison of MC3T3-E1 cell proliferation on different coating surfaces. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

HA-GO@IL-4 group exhibited tighter intercellular connections, with some pseudopods penetrating into micro/nano-scale structural pores of the coating surface and forming firm anchoring.

4',6-diamidino-2-phenylindole (DAPI) staining results (Fig. 9A) indicated clustered growth patterns of MC3T3-E1 cells in all groups after 7-day culture, with Ti@HA-GO@IL-4 group showing the largest nuclear fluorescence coverage area, consistent with cell counting kit-8 (CCK-8) assay data. CCK-8 quantitative analysis (Fig. 9B) demonstrated: (1) Progressive cell proliferation in all groups over time; (2) At day 4, Ti@HA-GO@IL-4 group absorbance was significantly higher than other three

groups ($P < 0.01$), while Ti@IL-4 group was significantly higher than Ti group ($P < 0.001$); (3) At day 7, Ti@HA-GO group absorbance was significantly higher than Ti group ($P < 0.01$), and Ti@HA-GO@IL-4 group was significantly higher than Ti@IL-4 group ($P < 0.05$). Consistent with previous findings demonstrating IL-4's capacity to enhance proliferation and migration of mouse bone marrow mesenchymal stem cells [24], our results corroborate these pro-regenerative effects. Emerging evidence indicates that IL-4 mediates cellular responses via interleukin-4 receptor alpha subunit (IL-4R α) signaling, thereby promoting cellular growth/adhesion while exerting anti-inflammatory and wound-healing properties [48]. Future

investigations will elucidate the underlying molecular mechanisms of IL-4's action through systematic biological analyses.

Conclusions

This study successfully developed a novel Ti@HA-GO@IL-4 composite coating through innovative electrochemical deposition and optimized immersion-drying techniques, achieving three key breakthroughs: (1) First demonstration of synergistic Ti@HA-GO@IL-4 integration for dental implants, combining enhanced hydrophilicity, biomimetic surface roughness ($R_a = 30.93 \pm 7.30$ nm), and sustained IL-4 release (7-day duration); (2) The composite coating significantly promotes the proliferation and adhesion of osteoblasts (MC3T3-E1 cells); and (3) Clinical-ready platform for customizable cytokine delivery. These findings establish a new paradigm in implant surface engineering, transitioning from passive biocompatibility to active osteogenic microenvironment modulation, demonstrating significant potential for improving dental implant outcomes.

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Authors' contributions

C.L. contributed to experiment implementation, data curation, writing the original Draft, writing review & editing, and project administration. M.Y. and C.K. was committed to software use, visualization, writing-review & editing. X.C. contributed to data curation and supervision. A.A. helped with the experiment implementation. W.B. helped with writing-review & editing, supervision. W.B. and L.Z. contributed to conceptualization, methodology, writing review & editing, supervision, and Funding acquisition.

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Data availability

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not required.

Competing interests

The authors declare no competing interests.

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