Polysaccharide from *Atractylodes macrocephala* Koidz binding with zinc oxide nanoparticles: Characterization, immunological effect and mechanism

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*Atractylodes macrocephala* Koidz (*A. macrocephala*) has been used both as a traditional medicine and functional food for hundreds of years in Asia. And it has a variety of biological activities, such as enhancing the ability of immunity and modulating effect on gastrointestinal motility. In this study, a water-soluble polysaccharide with molecular weight of $2.743 \times 10^3$ Da was isolated from the root of *A. macrocephala*. Polysaccharide from *A. macrocephala* (AMP) consisted of arabinose, galactose, glucose, xylose, mannose, ribose, galactose uronic acid, glucose uronic acid, with a percentage ratio of 21.86, 12.28, 34.19, 0.43, 0.92, 0.85, 28.79, and 0.67%, respectively. Zinc plays an important role in immune system. Therefore, we supposed that AMP binding with zinc oxide (ZnO) nanoparticles (AMP-ZnONPs) might be an effective immunostimulator. AMP-ZnONPs was prepared by Borch reduction, and its structural features were characterized by Scanning Electron Microscope (SEM), Transmission electron microscope (TEM), TEM-energy dispersive spectroscopy mapping (TEM-EDS mapping), Fourier transform infrared spectroscopy (FT-IR), X-ray photoelectron spectrometer (XPS), X-ray diffraction (XRD), particle size and zeta-potential distribution analysis. Then, its immunostimulatory activity and the underlying mechanism were evaluated using RAW264.7 cells. The results showed that AMP-ZnONPs remarkably promoted cell proliferation, enhanced phagocytosis, the release of nitric oxide (NO), cytokines (IL-6 and IL-1β) and the expression of co-stimulatory molecules (CD80, CD86 and MHCII). Moreover, AMP-ZnONPs could promote...
the expression of Toll-like receptor 4 (TLR4), Myeloid differentiation factor 88 (MyD88), TNF receptor associated factor 6 (TRAF6), phospho-IκBα (P-IκBα) and phospho-p65 (P-p65), and TLR4 inhibitor (TAK242) inhibited the expression of these proteins induced by AMP-ZnONPs. Therefore, AMP-ZnONPs activated macrophages by TLR4/MyD88/NF-κB signaling pathway, indicating that AMP-ZnONPs could act as a potential immunostimulator in medicine and functional food.

KEYWORDS
polysaccharide from Atractylodes macrocephala Koidz, zinc oxide nanoparticles, immunostimulatory activity, TLR4 signaling pathways, potential immunostimulator

Introduction

In recent years, immunoregulatory polysaccharides are considered important macromolecules for stimulation of immune response, then gradually become a major research hot spot (1, 2). Atractylodes macrocephala Koidz (A. macrocephala) has been used both as traditional medicine and functional food for hundreds of years in Asia, and it was approved as a functional food by the National Health Commission of the People's Republic Health of China (3–5). Polysaccharide from A. macrocephala (AMP) has a variety of biological activities, such as enhancing the ability of immunity, modulating effect on gastrointestinal motility and decreasing the blood glucose level (6–8). However, a lot of natural polysaccharides exhibit only weak bioactivities due to the limitation of structural and conformational properties (9). Thus, further research about enhancing bioavailability of AMP is necessary.

Zinc (Zn) deficiencies in the body is a serious problem, which severely harms the health of the organism and causes the etiology of myocardial apoptosis, deregulated homeostasis (10–12). In addition, zinc is important for cellular homeostasis and also serves as a regulatory signaling molecule for immune cells (13, 14). Zinc oxide (ZnO) is listed as “commonly considered as safe” by the US Food and Drug Administration (FDA) (15). Some studies have shown that Zn has a significant role in the development and activation of effector cells of the innate and adaptive immune systems (16–18). ZnO nanoparticles (ZnONPs) have been exploited in biomedical and preclinical research for their advantages such as non-toxicity and low cost (19, 20). However, ZnONPs are limited their application in drug delivery due to their poor water solubility, strong agglomeration and less dispersion. Hence, it is imperative to develop an effective, safe and high-content Zn-supplement. To improve the dispersion of particles in water, a silane coupling agent (KH550) was applied to modify its surface. Then, AMP-ZnONPs was successfully prepared by the binding of KH550-ZnONPs and AMP via the Borch reduction between -NH2 group and -CHO group (Figure 1). Its structural properties were characterized and then its immunostimulatory activities including cell viability, phagocytosis, surface molecules, cytokines release were evaluated using RAW264.7 cells. To further reveal the mechanism of immune stimulation, the effects of AMP-ZnONPs on the TLR4/MyD88/NF-κB signaling pathways were analyzed. This study is expected to provide new ideas for the development and utilization of polysaccharides and microelements in the food and pharmaceutical industry.

Materials and methods

Reagents and materials

A. macrocephala was purchased from the Tongrentang Company in Beijing. The plant material was identified by Prof. Jingui Li. The purified AMP was prepared in our laboratory and the polysaccharide content was 96% (UV). RAW264.7 cells were obtained from American Type Culture Collection (ATCC,
FIGURE 1
Schematic illustration of AMP-ZnONPs synthesis. The surface of ZnONPs was modified with KH550 through Borch reduction and then AMP was bond to the KH550-ZnONPs.

Extraction and purification of polysaccharide from *Atractylodes macrocephala*

AMP was extracted by water extraction and alcohol precipitation methods. Briefly, *A. macrocephala* was first extracted with alcohol for 2 times to remove the impurity. Second, *A. macrocephala* was decocted in water. The aqueous extract was concentrated under a vacuum. After that, a threefold volume of alcohol was added, the precipitated was washed three times with anhydrous ethanol, acetone and diethyl ether.
Preparation of AMP-ZnONPs

In order to fully hydrolyze KH550, 4 mL KH550 was added to 400 mL of equal volumes of alcohol and water, the mixture was reacted for 10 min under ultrasonication, and then agitated for 20 min on a magnetic stirrer. The pH of the solution was adjusted to between 6.5 and 7.0 with 0.2 M HCl to generate silicon-oxygen bonds for grafting the ZnONPs. Then, 4.5 g of ZnONPs was added to this solution, sonicated for 30 min, agitated for 30 min at 200 rpm on a magnetic stirrer (80° C), and then the mixture was collected and lyophilized. The surface of ZnONPs was modified with KH550 by these processes. KH550-ZnONPs (10 mg) was added to water (20 mL). After sonication for 1 h, AMP was added to the KH550-ZnONPs (m_{AMP}: m_{KH550:ZnONPs} = 4:1) and stirred for 24 h. The -CHO of AMP and the -NH2 of ZnONPs were linked to assemble AMP-ZnONPs by Borch reduction.

Characterization of AMP-ZnONPs

Scanning Electron Microscope (SEM, Zeiss Supra55, Germany) was used to detect the samples of ZnONPs, KH550-ZnONPs, AMP and AMP-ZnONPs, the image magnification was 5,000 x. The morphology of samples was also observed via a Transmission electron microscope (TEM, HT7800, Hitachi, Japan). The element distribution was observed by Transmission electron microscope-energy dispersive spectroscopy mapping (TEM-EDS mapping, Tecnai G2 F30 S-TWIN, FEI, US) to verify the connection of the ZnONPs and AMP. The Fourier transform infrared spectroscopy (FT-IR, Thermo Electron Corporation, United States) spectra were recorded in the mid-infrared region. The samples were determined at room temperature on an X-ray diffraction (XRD, D8 Advance, Germany), and operated at 40 kV and 40 mA. The samples were determined with X-ray photoelectron spectrometer (XPS, ESCALAB 250Xi, United States). Data were analyzed using the Avantage software. The laser particle size analyzer (NanoPlus 3, Micromeritics Instrument Corp., United States) was applied to measure average particle size, polydispersity index (PDI) and zeta-potential.

Cell culture

RAW264.7 cells were cultured in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cells were maintained under a humified atmosphere at 37°C with 5% CO2.

Cell activity assay

The cell activity of AMP and AMP-ZnONPs on RAW264.7 cells was determined according to the CCK-8 method. Cell viability of RAW264.7 cells was evaluated after treatment with AMP and AMP-ZnONPs (0.06–250 μg·mL−1) for 24 h. Following this, the supernatants were discarded, then added fresh DMEM medium (100 μL-well−1) containing CCK8 (10
µL-well⁻¹) and cultivated for 1.5–4 h at 37°C. Finally, the absorbance at 450 nm was measured by microplate reader. The cell survival rate was calculated as follows:

\[
\text{Cell activity (\%)} = \frac{A_2}{A_1} \times 100\%
\]

(Where \(A_1\) and \(A_2\) are the absorbances of the control and test samples, respectively).

**Measurement of nitric oxide**

In brief, RAW264.7 cells (1 × 10⁵ cells·mL⁻¹) were separately exposed to ZnONPs, AMP-ZnONPs and AMP (1.95 µg·mL⁻¹) for 24 h at 37°C in a constant temperature incubator ventilating with 5% CO₂. At the end of incubation, the NO amount in the supernatant was measured by a Griess reagent system kit K.

**Quantitative real-time polymerase chain reaction**

Real-time polymerase chain reaction (PCR) was employed for the determination of cytokines (IL-6 and IL-1β) and TLR4, MyD88, TRAF6 mRNA expression. The total RNA of RAW264.7 cells was obtained with Trizol reagent, and then synthesized the corresponding cDNA. The Hieff qPCR SYBR Green Master Mix was employed to perform Quantitative real-time PCR assay. The primer sequences of genes were displayed in Table 1. The following PCR protocol was referenced by our previous report (32). The expression of each gene was analyzed by the 2⁻ΔΔct comparative method.

**Determination of phagocytic function using CytExpert flow cytometer**

RAW264.7 cells (1 × 10⁶·mL⁻¹) were cultured in 6 well plates at 37°C for 12 h, and then exposed to ZnONPs, AMP, AMP-ZnONPs (1.95 µg·mL⁻¹) or LPS (0.5 µg·mL⁻¹) for 24 h, respectively. The cells were incubated with 1 mg·mL⁻¹ FITC-dextran for 1 h, then the reaction was stopped by cold PBS. The FITC-dextran intensity of cell samples was analyzed by CytExpert flow cytometer (Beckman Coulter, CA, United States).

**High-resolution laser confocal microscopy**

Cells were seeded at 1 × 10⁵ cells·mL⁻¹ on coverslips in a 24 well plate, then stimulated with ZnONPs, AMP, AMP-ZnONPs (1.95 µg·mL⁻¹), or LPS (0.5 µg·mL⁻¹), and incubated with 1 mg·mL⁻¹ FITC-dextran (1 mg·mL⁻¹) at 37°C for 1 h. After incubation, RAW264.7 cells were fixed with 4% paraformaldehyde for 10 min. Cell samples were stained with phalloidin for 1 h under dim light, followed by DAPI staining. The green fluorescence was measured by laser confocal microscopy (LSCM) (TCS SP8 STED, Germany).

**Expression of cell surface molecule CD80, CD86, and MHCII**

The cells (1 × 10⁶ cells·mL⁻¹) were treated with AMP-ZnONPs for 24 h in a 6-well plate. Then, the cells were suspended and incubated with anti-CD80, anti-CD86 and anti-MHCII at 4°C for 30 min, and analyzed by the CytExpert flow cytometer.

**Cell morphological observation**

The cells (1 × 10⁵ cells·mL⁻¹) were plated on coverslips in 24-well plates, and then treated with ZnONPs, AMP, AMP-ZnONPs (1.95 µg·mL⁻¹), LPS (0.5 µg·mL⁻¹) or DMEM. The RAW264.7 cells with glutaraldehyde-treated were prepared for 24 h. Next, the cells were evaporated using a 30, 50, 70, 80, 90, 95, and 100% ethanol gradient (10–15 min each time); then displaced in Na₂SO₄, dried at a tipping point, and finally scanned by SEM (HT7700, Hitachi, Japan) at 1,000 and 8,000×.

**Western blotting analysis**

The BCA protein assay kit was employed to detect the protein concentrations. Equal amounts (30 µg) of total protein were separated and transferred to the NC membrane (33). The membrane was incubated with 5% skim milk for 2 h and then incubated with gentle shaking with primary antibodies at 4°C overnight. Later incubated NC at 4°C with antibodies of TLR4 (rabbit, 1: 1,000), TRA6 (rabbit, 1: 1,000), MyD88 (rabbit, 1: 1,000), P-p65 (rabbit, 1: 1,000), P-IκBα (rabbit, 1: 1,000), TRAF6 (rabbit, 1: 1,000), MyD88 (rabbit, 1: 1,000). After incubation with the primary antibody, the membrane was exposed to goat anti-rabbit secondary antibody (1: 5,000) at room temperature for 1 h. The membrane was washed with TBST for 3 times, membrane-bound antibodies were visualized using the ECL Enhanced Chemiluminescence system, the protein band intensity was analyzed with Image J Analysis Software.

**Statistical analysis**

Graphpad Prism 5.0 Software was utilized for statistical analysis. Data were analyzed by one-way analysis of variance.
Results and discussion

Molecular weight and monosaccharide composition of polysaccharide from Atractylodes macrocephala

As shown in Figure 2A, the results of HPGPC implied a good homogeneity of AMP. The retention time was 24.98 min. The chromatographic result of HPGPC showed that AMP had one peak, which indicated that AMP was homogeneous polysaccharides. Based on the regression equation of the dextran standard curve, $y = 11.864079 - 0.338831 x + 0.0005080 x^2 + 0.000195 x^3$, the molecule weight of AMP was calculated as $2.743 \times 10^3$ Da (Figure 2B). The result of monosaccharide composition obtained from AMP was described by HPAEC (Figures 2C,D). AMP was composed of arabinose, galactose, glucose, xylose, mannose, ribose, galactose uronic acid, glucose uronic acid, with a percentage ratio of 21.86, 12.28, 34.19, 0.43, 0.92, 0.85, 28.79, and 0.67%, respectively.

Characteristics of AMP-ZnONPs

Morphological characteristics of AMP-ZnONPs

Ultrastructure of ZnONPs, KH550-ZnONPs, AMP and AMP-ZnONPs was obtained with the SEM (Figure 3A). The AMP exhibited an irregular surface with many folds. ZnONPs showed rod morphology and a nano-lamellar structure. The KH550-ZnONPs after the surface modification displayed particles with uniform size, good monodispersity, and no obvious agglomeration. The surface of AMP appeared to be covered by rod-shaped ZnONPs, which was attributed to the strong interaction between amino in ZnONPs and hemiacetal in AMP because of hydroamination. Ultrastructure of ZnONPs, KH550-ZnONPs, AMP and AMP-ZnONPs as shown in Figure 3B. The ZnONPs showed strong clustering and exhibited aggregation. The KH550-ZnONPs had smaller clusters and showed a homogenous dispersion, all aggregation was disrupted. As shown in the AMP-ZnONPs, KH550-ZnONPs were connected to the surface of AMP. The TEM-EDS mapping and EDS spectra showed that C, O and Zn elements were present in AMP-ZnONPs (Figure 3C). Therefore, the results strongly supported the formation of AMP-ZnONPs.

Fourier transform infrared spectroscopy analysis

As shown in Figure 4A, FT-IR spectra of AMP revealed that the characteristic peaks of polysaccharide at 3366.9 cm$^{-1}$, 2931.4 cm$^{-1}$, and 1427 cm$^{-1}$ were attributed to O-H stretch vibration of hydroxyl group, C-H stretch vibration and O-H bending vibration, respectively (34). Moreover, the weak bands around 935.6 cm$^{-1}$ and 818.9 cm$^{-1}$ indicated that there were α-configuration and β-configuration (35, 36). FT-IR spectra of ZnONPs showed an intense peak at 571.1 cm$^{-1}$ and a broad peak at 3442.1 cm$^{-1}$. The weak absorption peak at 3442.1 cm$^{-1}$ could be attributed to stretching vibration of associating hydroxyls formed by weak hydrogen bonding as well as van der Waals interaction (37). The stretching vibration band at 571.1 cm$^{-1}$ corresponding to the Zn-O bond (38). Compared with ZnONPs, KH550-ZnONPs showed a new typical characteristic absorption peak of -NH$_2$ at 1582.6 cm$^{-1}$, which was attributed to the characteristic absorption peak of KH550. The AMP-ZnONPs spectra showed the characteristic absorption peaks of ZnONPs, KH550 and AMP. In addition, the signal in 1058.9 cm$^{-1}$ was mainly assigned to the stretching vibration of the C-O-C group, the absorption peak at 1,629 cm$^{-1}$ corresponded to N-H stretching vibrations (39, 40). FT-IR spectra of AMP showed that the band at 1636.2 cm$^{-1}$ corresponding to the C = O bond (41). There was no N-H bond in AMP, after connecting to ZnONPs, the presence of an N-H bond in AMP-ZnONPs indicated a possible connection. The result provided evidence for the successful grafting of the KH550-ZnONPs by the NH$_2$ groupings in KH550 agents onto AMP.

X-ray diffraction analysis

XRD, as a valuable instrument (42), could be used to further confirm the composition of AMP-ZnONPs (Figure 4B). The

| Gene | Sense (5′-3′) | Antisense (5′-3′) |
|------|--------------|------------------|
| IL-6 | TTCCATCCAGTTGCGCTTCTTG | ATTTAGGCTCGACTTTGGA |
| IL-1β | ATCTGGCAGCGACGACATCA | CGACGAGTTATCATCATATCC |
| TLR4 | TGGTGCGGTGGTGCTTATCC | GCCCTTTCCTCGTCGTATTCT |
| MyD88 | TCGATGCGTATTCGTGACTTG | GGTCGAGACACACACACTTA |
| TRAF6 | GCTGAGCGCAACATCTCCTAA | TCTTACGGGATGGACATTACAC |
| GADPH | ATGGTGAAGGTGGTGGTGAA | CTTTGACTGTGGCGTTGAA |
characteristic peaks located at $2\theta = 31.7, 34.4, 36.2, 47.5, 56.6, 62.8, 66.3, 67.9, 72.5, 76.9, 78.9, 80.1, 82.8, 85.9, 87.1, 90.2, 92.3, 94.4, 96.5, 98.6,$ corresponding to the (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), (202) planes, respectively, match well with characteristic reflections of ZnONPs (P63mc, JCPDS no. 89-0511). A similar curve of ZnONPs modified by KH550 proved that modification of KH550 did no effect on the phase formation of ZnONPs. The XRD results of AMP recorded between $10^\circ$ and $30^\circ$ suggested the presence of crystalline components, with major reflections at $12.0^\circ, 17.7^\circ$ and $21.8^\circ$. This profile was also observed in other different polysaccharides (43, 44). The XRD profile of AMP-ZnONPs showed that the main characteristic peaks of ZnONPs, confirming that the hexagonal structure of the ZnONPs was not affected after binding with AMP. Moreover, as sreov-shapedoder affected aft $\theta = 10–20$, the modified AMP molecule maybe undergo a chemical structure change and convert to amorphous materials under this circumstance.

X-ray photoelectron spectrometer analysis
XPS was depicted in Figure 4C to investigate the surface compositions of the ZnONPs (45, 46). Compared with ZnONPs, the XPS spectra of the KH550-ZnONPs exhibited the characteristic peak components of Zn2p3, O1s, N1s, C1s and Si2p, suggesting that silane had successfully modified on the surface of ZnONPs. The AMP showed a Zn-free surface, the two peaks at 285, and 532 eV correspond to C1s and O1s, respectively. The C1s core-level spectrum of the KH550-ZnONPs was divided into three peak components: C-C, C-N and C-Si (Figure 4D). The C1s core-level spectrum of the AMP was divided into two peak components: C-C and C-O (Figure 4E). The peaks of C-O (286.6 eV), C-C (284.8 eV), C-N (286.3 eV) and C-Si (283.5 eV) were also observed in the C1s spectrum of the AMP-ZnONPs (Figure 4F), the C-N single bond peak was introduced by KH550, and the C-O peak was introduced by AMP. The presence of a C-N bond confirmed that there was a cross-linking reaction between the AMP and the KH550-ZnONPs. Although the Zn2p3 peak was reduced due to the AMP shielding, some active sites of ZnONPs remain even. These results were in good agreement with previous results of XRD and FT-IR, indicating that the successful grafting of AMP and KH550-ZnONPs.

Particle size, polydispersity index and zeta potential
The particle size (Table 2 and Figure 5A) of the ZnONPs was larger than that of the KH550-ZnONPs, this result revealed that after KH550 being modified on the surface of ZnONPs promoted the particle size to be smaller. The particle size of AMP-ZnONPs was slightly larger than KH550-ZnONPs, which may be caused by the AMP binding on KH550-ZnONPs. The PDI values of AMP-ZnONPs was lower than 0.3, which was considered optimal for the dispersion and homogeneity (47). The zeta potential of AMP-ZnONPs was more negatively charged than KH550-ZnONPs (Figure 5B), with AMP-ZnONPs gaining additional negative charge of $-4.43$ mV. Negatively
charged nanoparticles are more likely to be internalized by cells than positively charged nanoparticles, this property underlies the fact that AMP-ZnONPs stimulates phagocytosis of RAW264.7 cells more significantly than either ZnONPs or AMP alone.

**Cell viability**

The cell viability of ZnONPs, AMP and AMP-ZnONPs on RAW264.7 cells were shown in Figure 6A. Compared with the control group, ZnONPs, AMP and AMP-ZnONPs exerted no damaging effect and promoted cell proliferation to a certain extent at a concentration of 0.24–1.95 µg·mL⁻¹. When the concentration of AMP-ZnONPs was 0.49–3.91 µg·mL⁻¹, the proliferation effect was proportional to the concentration. To compare the immune effects of ZnONPs, AMP and AMP-ZnONPs at the same concentration level, the concentration of ZnONPs, AMP and AMP-ZnONPs at 1.95 µg·mL⁻¹ were chosen in the following experiments.

**AMP-ZnONPs induced cells nitric oxide production**

NO is an important active substance associated with the immunomodulatory effect (48), which participates in apoptosis regulation and host defense function (49). The NO production was calculated from the standard curve formula. The results of NO release in AMP-ZnONPs were shown in Figure 6B. As a positive control, the NO content of LPS group (0.5 µgPS⁻¹) showed significantly higher than the control group (P < 0.001). And the release of NO in AMP-ZnONPs group was higher than that of the control, ZnONPs group (P < 0.001) and AMP group (P < 0.05). Therefore, AMP-ZnONPs could stimulate NO
FIGURE 4
Spectroscopic characterization and curve-fitting spectra of AMP-ZnONPs. The FT-IR pattern (A), XRD pattern (B), XPS spectra (C) of AMP-ZnONPs (a), AMP (b), KH550-ZnONPs (c) and ZnONPs (d). The curve-fitting of C1s (D) spectra of KH550-ZnONPs. The curve-fitting of C1s (E) spectra of AMP. The curve-fitting of C1s (F) spectra of AMP-ZnONPs.
TABLE 2  The particle size, PDI, and zeta potential of ZnONPs, KH550-ZnONPs and AMP-ZnONPs (n = 3).

| Samples         | ZnONPs       | KH550-ZnONPs | AMP-ZnONPs |
|-----------------|--------------|--------------|------------|
| Size (nm)       | 211.89 ± 8.98| 136.73 ± 3.12| 391.37 ± 3.27|
| PDI             | 0.095 ± 0.053| 0.391 ± 0.137| 0.206 ± 0.086|
| Zeta potential (mV) | –33.20 ± 1.11| –10.97 ± 0.32| –15.40 ± 0.35|

release more than AMP and ZnONPs in RAW264.7 cells, this suggested that ZnONPs displayed synergy with AMP.

AMP-ZnONPs induced cells cytokines secretion

Cytokines are the central logical targets for immune modulation as they influence the formation of a phenotype. They act as immunoregulators by either inducing or suppressing the production and maturation of immune cells (50). IL-1β is a major mediator of inflammation secreted by various activated innate immune cells, such as macrophages, monocytes and dendritic cells (51). IL-6 is also one of the important mediators that can stimulate antibody production and participate in immune response. Both IL-1β and IL-6 are of great importance for immune homeostasis and barrier immunity. To further investigate the immunological activity of AMP-ZnONPs on RAW264.7 cells, the cytokine (IL-1β and IL-6) contents in cells were evaluated by RT-qPCR in this study (Figures 6C,D). LPS stimulated the production of IL-1β and IL-6 by more than 2,000 times compared to the control group (P < 0.001), which indicated that LPS could promote inflammation and lead to excessive cytokine release (52). Notably, AMP-ZnONPs treatment exerted a significant action on IL-1β and IL-6 secretion than both AMP and ZnONPs (P < 0.001), which showed ZnONPs exerted a synergistic effect with
AMP. The results indicated that the AMP-ZnONPs had immunostimulatory effect, but it did not cause cell inflammation like LPS.

**AMP-ZnONPs enhanced cells phagocytosis**

Macrophages exist in virtually all tissues, phagocytosis, which is a classic index to evaluate macrophage activation, plays a critical role in the uptake and degradation (53–55). In addition, it is a signal-inducing process in which phagosomes bind to the antibody on the cell surface, consequently, cell morphology and signaling pathways are affected (56). The enhanced phagocytosis is one of the remarkable characteristics of activated macrophages, meanwhile indicating the activation of innate immunity. The results showed that compared with the control and ZnONPs, AMP-ZnONPs and AMP could significantly promote cell phagocytosis of macrophages (Figures 7A,B). Meanwhile, it should be highlighted that the stimulating effect of AMP-ZnONPs on phagocytosis was remarkably higher than AMP (P < 0.05). Furthermore, FITC-dextran accumulation in RAW264.7 was measured by LSCM. As shown in Figure 7C, AMP-ZnONPs treatment markedly enhanced the fluorescence intensity of tested cells relative to AMP and ZnONPs, and the FITC-dextran were mainly distributed in the cytoplasm. These results demonstrated that the AMP binding with ZnONPs significantly improved the immune activity of RAW264.7, which indicated ZnONPs and AMP acted in synergy of immune system.

**AMP-ZnONPs promoted cells costimulatory molecules expression**

The activation and further differentiation of T cells and cellular immune function are closely related to the function of antigen-presenting cells (APC), especially macrophages, considered as professional antigen-presenting cells (57). CD80
FIGURE 7
The phagocytosis was evaluated by flow cytometry (A). The histogram showed the positive rate of cells for FITC-dextran (B). ***P < 0.001 vs. AMP-ZnONPs. Enhanced FITC-dextran uptake into RAW264.7 cells following incubation with AMP-ZnONPs. FITC-dextran (green) was mixed with ZnONPs, AMP, AMP-ZnONPs, LPS and DMEM medium (control) overnight avoiding light. Cytoskeleton and cell nuclei were stained with phalloidin (red) and DAPI (blue) respectively. (C).
and CD86 may differentially control the T-cell activation because of the distinct properties of each molecule. Once presented to T cells by MHCII, peptide antigens generally stimulate a typical T cell-dependent immune response and the induction of immune memory (58). In this study, the expression of phenotypic markers of CD80, CD86 and MHCII up-regulated after the RAW264.7 cells were exposed to LPS for 24 h (Figure 8A). Figures 8B–D documented a significant increase in the percent of RAW264.7 cells positive for the expression of CD80, CD86, and MHCII following incubation with AMP-ZnONPs as compared to the control, ZnONPs and AMP. The results proved that AMP-ZnONPs significantly increased the expression of CD80, CD86 and MHCII compared with ZnONPs or AMP alone, which indicated ZnONPs and AMP showed synergetic effect with each other.

**FIGURE 8**
The production of CD80, CD86, MHCII in RAW264.7 cells were analyzed by flow cytometry (A). The histogram showed the positive rate of CD80 (B), CD86 (C), MHCII (D) in cells. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. AMP-ZnONPs). SEM analysis for morphological changes of RAW264.7 cells in different groups (E).
FIGURE 9
The relative expressions of proteins (A,B) and mRNAs (C) of the critical nodes in the TLR4/MyD88/NF-κB signaling pathways. Effects of TAK242 on TLR4, MyD88, TRAF6, P-κBα and P-p65 expression stimulated by AMP-ZnONPs or not (D,E). The results were expressed as the mean ± SD (n = 3) (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. AMP-ZnONPs).

FIGURE 10
Schematic illustration depicts that a new approach was carried out to bind AMP with ZnONPs as an immunostimulator. AMP-ZnONPs could significantly activate RAW264.7 cells by TLR4/MyD88/NF-κB signaling pathway to improve its immune function. Therefore, AMP-ZnONPs remarkably enhanced phagocytosis, the release of NO, cytokines and the costimulatory molecules of RAW264.7 cells.

AMP-ZnONPs induced cells morphological changes
Macrophages engulf nutrients and pathogens by stretching their arms (59). The morphology was observed by SEM as shown in Figure 8E. Cells in the control group had a round shape and microvilli-like structures on the cell surface. RAW264.7 cells showed elongated and polygonal with many filopodia in the LPS group than in the other groups. In the AMP-ZnONPs group, even in the case of round-shaped RAW264.7 cells, their arms were stretching in various directions. The size, microvilli-like structures and surface folds of the AMP-ZnONPs treated group were more than ZnONPs and AMP. These results indicated that AMP-ZnONPs could induce RAW264.7 cells activation visibly, which was inconsistent with the results of phagocytosis and cytokine secretion.

AMP-ZnONPs regulated the expression of TLR4/MyD88/NF-κB associated proteins
As an important member of the TLR family, TLR4 has been widely reported to recognize and bind to different pathogen-related molecular patterns, initiate intracellular signal transduction pathways, cause the release of cytokines or...
chemokines, and play an effective innate immune response (60). Whether AMP-ZnONPs could mediate the immunomodulatory effect on RAW264.7 cells by the TLR4 signaling pathway was explored. The RT-qPCR and Western blot were used to detect the mRNA and proteins of key nodes in the TLR4/MyD88/NF-κB signaling pathways. As shown in Figures 9A,B, compared with AMP group, the protein expression levels of TLR4, MyD88, TRAF6, P-IκBα and P-p65 were upregulated in AMP-ZnONPs group. As shown in Figure 9C, AMP-ZnONPs significantly upregulated the mRNA expression of TLR4, MyD88, TRAF6 in the RAW264.7 cells, compared with those in control, ZnONPs and AMP group. Therefore, the results indicated that AMP-ZnONPs was more effective than ZnONPs and AMP in activating the TLR4/MyD88/NF-κB signaling pathway.

Conclusion

In summary, by Borch reaction between AMP and ZnONPs modified by KH550, the AMP-ZnONPs was successfully prepared and its characterization was evaluated. AMP-ZnONPs showed excellent immunostimulatory activity on macrophages and the activities were much better than those of ZnONPs or AMP applied alone. Furthermore, this study clarified that AMP-ZnONPs could significantly activate RAW264.7 cells by TLR4/MyD88/NF-κB signaling pathway to improve its immune function. These data demonstrated that AMP binding with ZnONPs could potentially be used as an easily available source for immunomodulatory nutraceutical or immune adjuvant, which can be widely used in the food or medicine industry in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

RB: conceptualization, methodology, software, data curation, writing—original draft, review and editing, visualization, supervision, project administration, and funding acquisition. XL: conceptualization, methodology, investigation, software, data curation, writing—original draft, and visualization. JW: methodology, investigation, and software. SW: investigation and writing—review and editing. XW: investigation and software. YT and SX: visualization and software. ML: supervision. JL: conceptualization, methodology, project administration, and funding acquisition. HP: conceptualization and methodology. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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