Impact of hydrophobic modification on biocompatibility of Alaska pollock gelatin microparticles

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Abstract

This study investigates the impact of hydrophobic modification on the immunogenicity, cytotoxicity, and inflammatory response of Alaska pollock gelatin (ApGltn) microparticles (MPs). Gelatin, known for its inherent biocompatibility, was modified with decyl group (C10) to explore potential alterations in its interaction with the immune system. Immunogenicity was evaluated through the measurement of material-specific IgM and IgG responses, indicating no significant increase post-modification. Cytotoxicity against Caco-2 cell lines and NF-kB-mediated LPS-induced inflammation were also assessed, revealing no exacerbation by the modified MPs. Furthermore, C10-modification with different types of linkage such as secondary amine and amide structure did not influence immune reactivity. These findings suggest that C10-modification maintains the non-immunogenicity and biocompatibility of gelatin MPs, supporting their potential use in biomedical applications.

Keywords: Alaska pollock gelatin microparticle, decyl-group modification, immunogenicity, biocompatibility

1. Introduction

Gelatin, a versatile biopolymer derived from collagen, has been widely used in various industries, including food, pharmaceutical and cosmetics, owing to its remarkable properties that enhance viscosity, texture, and stability of products [1]. Traditionally, gelatin has been sourced primarily from pig skins, bovine hides, and bones. However, recent attention has shifted towards alternative sources, particularly fish and fish by-products, such as fish bones and skins, as promising biomaterials for gelatin production [2]. The distinctive characteristics of fish gelatin, such as its lower gelling and melting points compared to gelatin derived from porcine or bovine sources [3], enable its use in applications requiring higher fluidity at room temperature.

Alaska pollock gelatin (ApGtln) has been explored as a basic material for biodegradable surgical sealants due to its low transition temperature, low imino acids content and low viscosity [4]. Introduction of several hydrophobic groups such as cholesterol, alkyl chains with various length have been demonstrated to enhance interfacial strength under wet conditions [5–7]. Among these modifications, the incorporation of the decyl group (C10) onto ApGltn has been highlighted for its notable enhancement of interfacial strength with blood vessels [6]. Interestingly, microparticles formed by the self-assembly of C10-modified ApGltn swelled into a stable hydrogel layer in physiological saline, which was demonstrated to protect the wound surface and facilitate the tissue regeneration process in wound healing models [7]. Notably, in addition to internal gastrointestinal tissue, efficacy in preventing postoperative adhesion on duodenum serosal tissue was also demonstrated [8].

Given that most C10 modifications were targeted to the primary amine groups of ApGltn, the exposure of primary amine groups may have implications for immunogenicity and inflammatory response. For example, primary amines on polymers have been reported to form hydrogen bonds and electrostatic interactions with phosphate groups in lipids, disrupting the organization of lipid bilayers and potentially increasing toxicity [9]. Furthermore, *in vivo* toxicity associated with cationic nanoparticles such as chitosan and bPEI nanoparticles, which induce complement activation and Toll-like receptor 4 (TLR-4) activation respectively, underscores the importance of understanding the impact of primary amine exposure. Interestingly, modifying primary amines to secondary and tertiary amines has shown promise in mitigating these adverse effects [10], suggesting a potential strategy for enhancing biocompatibility and minimizing risk of potential inflammation in biomedical applications.

Thus, in this study, C10-modification was performed on amine groups in ApGltn with different types of linkage formation: amide (C10-am-ApGltn) and secondary amine (C10-sa-ApGltn), prior to microparticle fabrication, allowing a comprehensive evaluation of the immunogenicity, cytotoxicity and inflammatory response associated with these microparticles. By investigating the interactions between these modified microparticles and biological systems, insights can be gained into their biocompatibility post-modification and potential suitability for biomedical applications.

2. Materials and Methods

2.1 Materials

Alaska pollock gelatin (ApGtln, molecular weight (MW) = 34,352 Da, amine group content: 364 μmol/g and MW = 34,323 Da, amine group content: 355 μmol/g) was purchased from Nitta Gelatin Inc. (Osaka, Japan). Decanal and decanoic anhydrate were obtained from Tokyo Chemical Industry Co., Ltd. (Osaka, Japan). Ethanol and 2-picoline borane were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Dulbecco's Modified Eagle Medium (DMEM), Antibiotic-Antimycotic Mixed Stock Solution, MEM Non-Essential Amino Acids Solution, Dulbecco's Phosphate-buffered Saline (DPBS) and dimethyl sulfoxide (DMSO) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Nichirei Bioscience Inc. (Tokyo, Japan). Trypsin-EDTA was purchased from Gibco (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Sodium carbonate, 4-Nitrophenyl phosphate disodium salt hexahydrate, albumin from mouse serum (MSA), albumin from chicken egg white (OVA), lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 and TWEEN® 20 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Sodium hydrogen carbonate, magnesium chloride hexahydrate, G418 sulfate and sulfuric acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Anti-mouse horseradish peroxidase (HRP)-conjugated IgM and IgG were purchased from Bethyl Laboratories, Inc (Montgomery, TX, USA). 3, 3', 5, 5' tetramethyl benzidine (TMB) was purchased from BioLegend, Inc. (San Diego, CA, USA).

2.2 Synthesis of C10-sa-ApGltn

C10-sa-ApGltn was synthesized following a previously established method [11, 12]. In brief, ApGltn (50 g) was dissolved into 175 mL of ultrapure water at 50 °C under continuous stirring at 400 rpm. Subsequently, 35 mL of ethanol and 35.5 mmol of decanal (two equivalent molar ratio of amine groups in ApGltn) dissolved into ethanol were added into the ApGltn solution, followed by 1 hour of stirring at 50 °C. Then, 53.3 mmol of 2-picoline borane (1.5 equivalent molar ratio of decanal), used as a reductant, was added to the ethanol solution under continuous stirring at 400 rpm. The resulting solution had a 20 w/v% ApGtln concentration (water: ethanol = 175:75 (mL)). After stirring the reaction for 17 hours, the solution was dropped into 2,500 mL of chilled ethanol (maintained at −7 to 4 °C) to induce precipitation. The precipitate was washed three times with 1,250 mL of ethanol to remove residual decanal and 2-picoline borane, then vacuum dried overnight (< 3 mbar) to obtain C10-sa-ApGltn powder. The degree of substitution (DS) was measured by TNBS method where residual amine groups were labeled by 2,4,6-trinitrobenzene sulfonic acid (TNBS) to measure the absorbance [13].

2.3 Synthesis of C10-am-ApGltn

C10-am-ApGltn was synthesized through a reaction with primary amine and decanoic anhydrate. Briefly, ApGltn (10 g) was dissolved in 35 mL of 0.1 M phosphoric acid buffer solution (pH 8.0) at 50 °C with continuous stirring at 400 rpm. Subsequently, 7.06 mmol of decanoic anhydrate (2-equivalent molar ratio of amine groups in ApGltn) was dissolved in 15 mL of ethanol and added into the ApGltn solution. The resulting solution had a 20 w/v% ApGtln concentration (water: ethanol = 35:15 (mL)). After stirring for 1 hour, the obtained solution was dropped into 500 mL of iced ethanol (maintained at −7 to 4 °C) to reprecipitate. The precipitate was washed three times

with 250 mL of ethanol to remove residual decanoic acid. The washed precipitate was dissolved in a water/ethanol mixture (35/15 mL) and then dialyzed against 2 L of a water/ethanol mixture at a ratio of 7/3 (v/v) for 2 days at room temperature. The dialyzed solution was freeze dried to obtain C10-am-ApGltn powder. The DS was measured by TNBS method.

2.4 Preparation and characterization of C10-sa-MPs and C10-am-MPs

C10-sa- and C10-am-MPs were prepared using the coacervation method, following a previous method [7, 8, 12, 14]. In this process, a poor solvent for C10-sa- and C10-am-ApGltn was used to induce coacervation. C10-sa- or C10-am-ApGltn was dissolved in ultrapure water at a concentration of 5 w/v%. An equal volume of ethanol, serving as a poor solvent, was added dropwise to the ApGltn solution, inducing the formation of a coacervate. The resulting coacervate solutions were freeze dried to remove water and ethanol. Subsequently, thermal treatment at 150 °C for 3 hours under vacuum conditions (< 3 mbar) was applied to facilitate the formation of chemical bonds between C10-sa- or C10-am-ApGltn molecules. Org-MPs derived from Org-ApGltn, non-modified Alaska pollock gelatin, were prepared using the same procedure. The obtained MPs were examined using scanning electron microscopy (SEM; JCM- 7000, JEOL, Japan) and the particle diameter was determined using Image J software.

2.5 Mice

Female Balb/c mice (6 weeks old) were purchased from Kyudo Co. Ltd. (Saga, Japan) and acclimatized for one week before the commencement of experiments. All animals were housed under standard laboratory conditions on a 12-hour light-dark cycle. During the acclimatization and experimental periods, mice were provided with CE-2 (CLEA Japan, Inc.) and had *ad libitum* access to water. The diet and water were replaced and replenished as needed to ensure the well-being of the animals. Experiment was conducted with approval from the Institutional Animal Care and Use Committee of Kyushu University.

2.6 Immunogenicity of MPs

Female Balb/c mice were randomly divided into groups of five. Each group received four consecutive subcutaneous injections per week with the following substances: Org-MPs (0.4 mg/mL), C10-am-MPs (0.4 mg/mL), C10-sa-MPs (0.4 mg/mL), MSA (0.4 mg/mL) and OVA (0.4 mg/mL), respectively. Following the final injection, mice sera were collected one week later for analysis.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was conducted following the protocol published by Li et al. [15] with slight modifications. In brief, ELISA plates were coated with 100 µL of sample solution (10 µg/mL), containing the substances to be tested (Org-MPs, C10-am-MPs, C10-sa-MPs, MSA and OVA, respectively), prepared in 0.1 M sodium carbonate buffer (pH 10.5) and incubated at 4°C overnight. Following the coating procedure, plates underwent five washes with PBS-T and were subsequently blocked with 1% BSA for 1 hour at room temperature. After blocking, plates were washed again five times with PBS-T before being incubated with diluted mice sera (in 1% BSA solution) for 1 hour at

room temperature. Following this incubation, plates were washed five times with PBS-T and then incubated with diluted HRP-conjugated IgM and IgG (1:50,000) for 1 hour at room temperature. Subsequently, plates were washed five times with PBS-T and incubated with TMB substrate for 15 minutes at room temperature. The reaction was stopped by adding 0.2 M H_2SO_4 solution. Absorbance was measured at 450 nm with a reference wavelength of 570 nm using Infinite® 200 PRO M Plex (Tecan, Switzerland).

2.8 Cell cultures

Human colorectal adenocarcinoma (Caco-2) cells were cultured and maintained in complete DMEM, supplemented with 10% heat-inactivated FBS, 1% antibiotic-antimycotic mixed stock solution and 1% MEM non-essential amino acid solution. RAW264.7 macrophages transfected with secreted alkaline phosphatase (SEAP) gene under the transcriptional control of NF-κB responsive promoter, were cultured and maintained in complete DMEM, supplemented with 10% heat-inactivated FBS, 1% antibiotic-antimycotic mixed stock solution and 500 µg/mL G418. All cell lines were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.9 Cytotoxicity of MPs

Cytotoxicity against Caco-2 cells was assessed using Cell Counting Kit-8 assay. Briefly, Caco-2 cells were seeded at a density of 2 x 10^4 per well in a 96-well plate and allowed to pre-incubate for 24 hours at 37°C in a 5% CO_2 atmosphere. Subsequently, the cells were treated with MPs at concentrations ranging from 6.25 to 200 µg/mL or with 10% DMSO (v/v) for either 24 hours or 48 hours, at 37°C in a 5% CO_2 atmosphere. MPs solutions were prepared by first dispersing it in DPBS, followed by 2-fold serial dilutions using DPBS. After the treatment period, CCK-8 was added to each well according to the manufacturer's instruction and incubated for 2 hours at 37°C in a 5% CO_2 atmosphere. Absorbance was then measured at 450 nm using Infinite® 200 PRO M Plex (Tecan, Switzerland). Cell viability was calculated using the following formula: -

$$\% \text{ viability} = \frac{A_1 - A_0}{A_c - A_0} \times 100$$

where A_0 is the absorbance value of wells lacking the presence of CCK-8, A_1 is the absorbance value of wells containing both samples and CCK-8, and A_c is the absorbance value of wells containing CCK-8 alone, without any samples.

2.10 NF-κB-mediated inflammatory response of MPs

The NF-κB-mediated inflammatory response of MPs was assessed by quantification of secreted alkaline phosphatase (SEAP) activity based on the hydrolysis of para-nitrophenylphosphate (pNPP) into para-nitrophenol (pNP), a yellow compound detectable at 405 nm. Briefly, RAW264.7 macrophages transfected with the SEAP gene (SEAP-RAW264.7) were seeded at a density of 2 x 10^4 per well in a 96-well plate and pre-incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Subsequently, the cells were treated with MPs at concentrations ranging from 2 to 200 µg/mL for 6 hours at 37°C in a 5% CO_2 atmosphere. MP solutions were prepared by first dispersing it in DPBS, followed by 10-fold serial dilutions using DPBS. After the 6-hour treatment period, LPS was added to each well at a final concentration 20 ng/mL, and the cells were further incubated for 18 hours at 37°C in a 5% CO_2 atmosphere. Following incubation, culture supernatants were collected and heated at 65°C for 5 minutes to

inactivate other alkaline phosphatases in the cells and serum [16]. An equal volume of culture supernatant and 4-Nitrophenyl phosphate (1 mg/mL) in substrate buffer (composed of 13.2 mM Na_2CO_3, 35 mM $NaHCO_3$, 1mM $MgCl_2$ at pH 9.6 [17]) was mixed and incubated at 37°C for 3 hours in the dark. Absorbance was then measured at 405 nm using Infinite® 200 PRO M Plex (Tecan, Switzerland).

2.11 Statistical analysis

Statistical analyses were conducted using GraphPad Prism 9 (La Jolla, CA, USA). One-way ANOVA was employed to assess the significance of means across all groups, followed by Dunnett's multiple comparisons test to compare each mean to the control group. Significance levels were denoted as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3. Results and Discussion

3.1 Characterization of MPs

The DS of C10-sa-ApGln and C10-am-ApGltn were 47 mol% and 44 mol% against total amount of primary amine. As reported previously, C10-sa-ApGltn and C10-am-ApGltn were successfully synthesized based FT-IR spectra and ^{1}H NMR spectra [13]. In the paper, the increased C-H bond stretching peak of -CH2- structure at 2,927 cm^{-1} in both C10-sa-ApGltn and C10-am-ApGltn FT-IR spectra compared to Org-ApGltn spectra and increased C-H peak at 1.24 ppm in both C10-sa-ApGltn and C10-am-ApGltn ^{1}H NMR spectra were confirmed. From these result and literature, the modification of decyl group was considered successful.

Afterward, three types of microparticles (MPs) were successfully prepared by coacervation method as described previously [7, 8, 12, 14]. Scanning electron microscopy (SEM) observations of all MPs revealed the consistent formation of monodispersed, spherical, and smooth MP structures (Figure 1b-d). The average diameter of MPs prepared by Org-ApGltn was approximately 2.26 μm (Figure 1e). However, MPs prepared by C10-sa-ApGltn and C10-am-ApGltn exhibited a slight increase in average diameter, with a difference of approximately 0.8 μm (Figure 1f, g). Through C10 modification, the increase in hydrophobicity of ApGltn introduces additional sites for intermolecular interaction within the gelatin matrix. This promotes stronger interactions between ApGltn chains and particles, leading to the formation of larger clusters of ApGltn molecules and, consequently, resulting in an increase in microparticle size. However, there was no difference in particle size between C10-sa-MPs and C10-am-MPs, indicating that the type of linkage between C10 group and ApGltn does not significantly influence the self-assembly process of microparticle formation. This suggest that the overall hydrophobicity of the ApGltn, rather than the type of C10 group modification method, is the primary factor that drive the observed change in particle size.

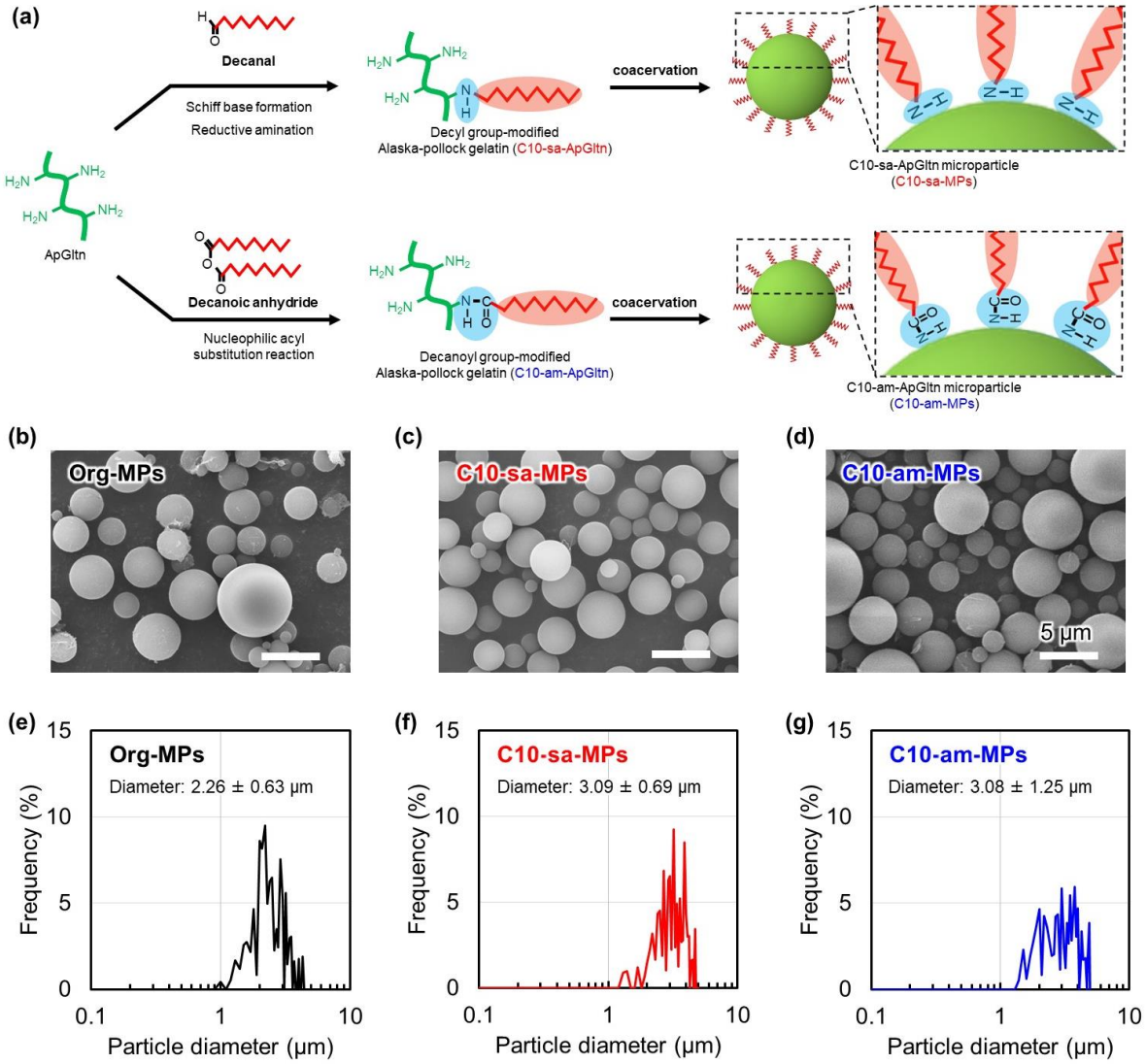

Figure 1: (a) Synthesis of C10-sa-ApGltn and C10-am-ApGltn to prepare C10-sa-MPs and C10-am-MPs. Characterization of Org-, C10-sa-, C10-am-MPs. (b-d) Scanning electron microscopy (SEM) images of Org-, C10-sa-, C10-am-MPs. The scale bars represent 5 μm. (e-g) Particle diameter distribution of Org-, C10-sa-, C10-am-MPs. Particle diameter analysis was performed by ImageJ software. 100 particles were analyzed for each analysis.

3.2 Immunogenicity of MPs

While previous studies have highlighted the superior characteristics of microparticles (MPs) as a surgical sealant, it is crucial to assess their potential risk in triggering unnecessary immune responses. This consideration is especially significant due to the inherent risk associated with polymers, which often induce the production of material-specific antibodies that may compromise their effectiveness in biomedical applications. To evaluate the immunogenicity of MPs, subcutaneous injection in mice was employed. The subcutaneous route was selected based on its relevance, as anti-drug antibodies are frequently associated with drugs administered via this route [18].

To evaluate the production of MPs-specific antibodies, mice received a total of four subcutaneous injections of the respective MPs, administered once per week. Serum samples were collected, and a series of diluted serum samples were used to quantify antibody titers using a sandwich ELISA with plates coated with the respective MPs [15]. In this experiment, ovalbumin (OVA) is used as a positive control due to its well-known immunogenic properties [19], providing a standard reference for evaluating the immunogenicity of all test MPs. Conversely, mouse serum albumin (MSA) was used as negative control as it is a common protein native to mice itself, and its immunogenicity is expected to be minimal [20]. This was confirmed in Figure 2, where OVA induced a high titer of OVA-specific IgG while MSA activity was negligible.

By comparing to OVA treatment group, Org-MPs, which was prepared from unmodified ApGltn, did not induce specific IgM or IgG responses, demonstrating the non-immunogenic nature of ApGltn. Additionally, specific IgG response to C10-modified ApGltn, either at its linkage structures: amide (C10-am-MPs) or secondary amine (C10-sa-MPs), were not observed. While the length of alkyl side chain has been previously demonstrated to confer immunogenicity to peptide vaccine [21], it itself did not induce any immunogenic responses in this study.

On the other hand, in the IgM production evaluation, specific IgM seemed to be detected in mice injected with C10-modified ApGltn. However, similar levels of IgM were also detected in the C10-modified ApGltn-untreated group (Figure S1), suggesting that the observed IgM may be due to nonspecific adsorption and not directly related to the substance itself.

In other words, these results suggest that all tested MPs did not induce targeted antibody responses and are unlikely to induce an immune reaction or sensitization upon exposure.

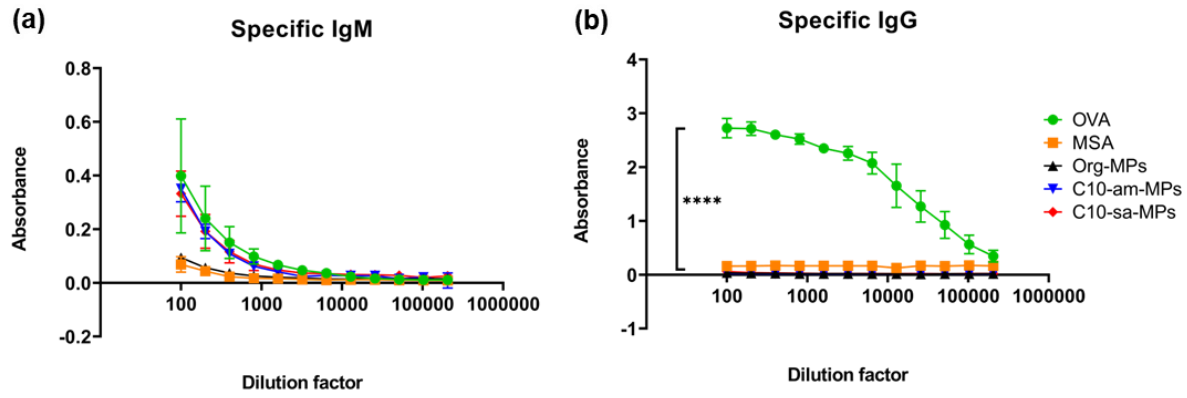

Figure 2: Immunogenicity of Org-, C10-sa-, C10-am-MPs. (a) MP-specific IgM levels (b) MP-specific IgG levels measured by ELISA. 5 mice were used for each treatment group. Ovalbumin (OVA) and mouse serum albumin (MSA) were used as positive and negative control, respectively. The error bars represent the standard deviation of the mean for a sample size of n = 5.

3.3 ***In vitro*** **cytotoxicity of MPs**

The potential cytotoxic effects of MPs were evaluated on Caco-2 cell line to provide valuable insights into its cytocompatibility. The viability assay utilized 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8), which is reduced by cellular dehydrogenases to form a water-soluble, orange-colored product (formazan). The amount of formazan dye produced correlates with the number of living cells, providing a measure of cell viability [22].

According to Figure 3, the cytotoxicity assay against Caco-2 cell lines revealed that all MPs, when compared to the untreated group, did not exhibit cytotoxicity up to a concentration of 200 µg/mL. This observation suggests that C10-modification with different linkage type: amide and secondary amine does not compromise the non-cytotoxic characteristic of hydrophilic ApGltn. Furthermore, cell viability was not significantly affected at both the 24-hour and 48-hour time points, indicating the long-term safety of MPs. Interestingly, tendency of increase in cell viability was observed upon treatment with Org-MPs and C10-sa-MPs at the 24-hour timepoint; however, this effect did not extend to the 48-hour mark. This indicates that these MPs treatment show some beneficial effect on cell viability in the early stages, potentially promoting cell proliferation or enhancing cellular functions [23], while C10-am-MPs do not possess this effect.

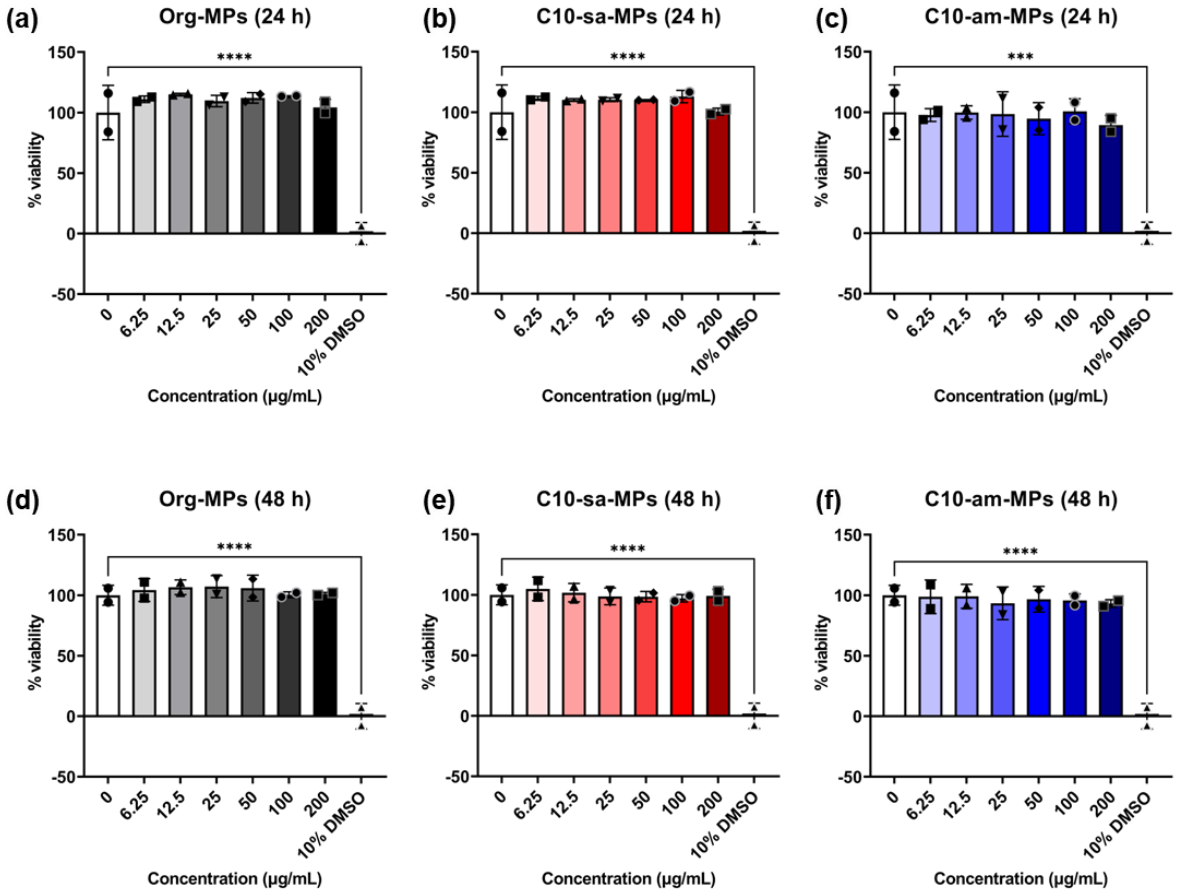

Figure 3: Cytotoxicity against Caco-2 cell lines after 24 hours (a-c) and 48 hours (d-f) of exposure to (a, d) Org-MPs, (b, e) C10-sa-MPs, and (c, f) C10-am-MPs. 10% DMSO were used as positive control. The error bars represent the standard deviation of two independent experiments, each conducted with a sample size of n = 3.

3.4 Anti-inflammatory properties of MPs

Immune activation begins when receptors on phagocytes are engaged, initiating signaling cascade. For instance, lipopolysaccharides (LPS), the major components of outer membrane of Gram-negative bacteria, bind to Toll-like receptor-4 (TLR-4) on macrophages, initiating an inflammatory response. This binding triggers the activation of nuclear factor kappa B (NF-κB) pathway, a crucial mechanism in inflammation [24]. RAW264.7 macrophages transfected with the SEAP (alkaline phosphatase) reporter gene (SEAP-RAW264.7) were utilized to evaluate NF-κB activity. The inflammatory response induced by MPs was evaluated by quantifying secreted alkaline phosphatase (SEAP) activity, measured based on the hydrolysis of para-nitrophenylphosphate (pNPP) to para-nitrophenol (pNP), a yellow-colored compound detectable by absorbance.

The effect of SEAP secretion upon exposure to varying concentration of each MPs was depicted in Figure 4. In the absence of LPS, SEAP activity was negligible compared to the control group (PBS). As expected, the addition of LPS increased SEAP activity, indicating the initiation of an inflammatory response. Treatment of RAW264.7 macrophages with C10-sa-MPs and C10-am-MPs tended to increase SEAP activity compared with Org-MP treatment. Considering that hydrophobic C10 chains are predominantly localized on the surface [7], these hydrophobic counterparts may interact more readily with the cell membrane. Consequently, this interaction leads to an observable trend of increased SEAP activity.

In addition, treatment with Org-MPs showed dose-dependent changes in inflammatory responses. At the highest concentration (200 μg/mL) of Org-MPs, SEAP activity mirrored that of the control (PBS), but at the lowest concentration (2 μg/mL), SEAP activity was significantly decreased. Although a decreasing trend was also observed in C10-sa-MPs and C10-am-MPs, it was not statistically significant. The decreasing trend suggests that Org-MPs have the potential to suppress inflammatory responses. However, at higher concentrations, Org-MPs may induce cellular stress, which could counteract its anti-inflammatory activity. Overall, all three MPs did not exhibit an additional effect on SEAP activity beyond that induced by LPS alone, suggesting their neutral impact on LPS-induced inflammation.

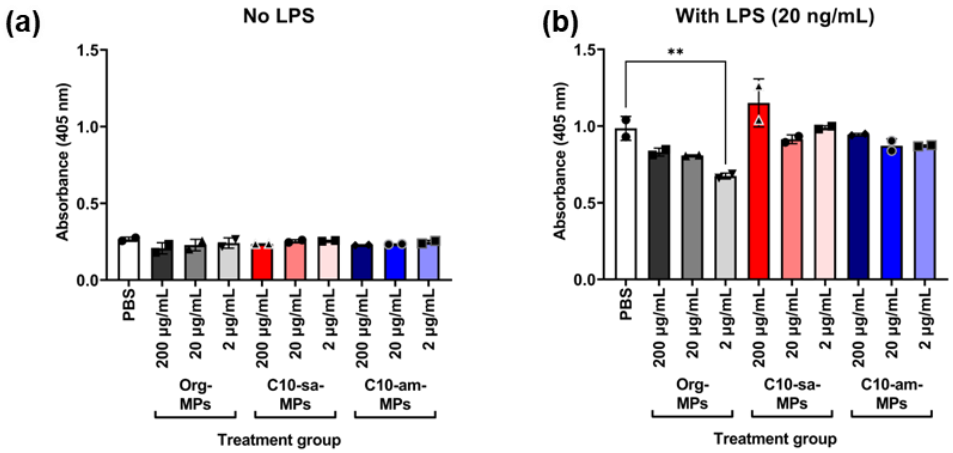

Figure 4: Anti-inflammatory properties of MPs (a) alone and (b) LPS-induced NF-κB activation in SEAP-RAW264.7 macrophages. PBS was used as negative control. The error bars represent the standard deviation of two independent experiments, each conducted with a sample size of n = 3.

4. Conclusion

In conclusion the non-immunogenic, non-cytotoxic, and anti-inflammatory characteristics of C10-modified MPs were demonstrated. While C10 modification improves the interfacial strength of MPs under wet conditions in previous studies, these findings demonstrate that C10-modification with amide or secondary amine linkage showed similar properties and do not compromise the inherent biocompatibility of ApGltn, underscoring their potential safe use in biomedical applications. Further investigation is required to elucidate the underlying mechanisms and to solidify their implications for biomedical applications.

Statements and Declarations

The authors declare that they have no conflicts of interest relevant to this manuscript.

Data availability

The data presented in this study are available upon request from the corresponding authors.

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Graphical Abstract