

Zeolitic Imidazolate Framework Nanoencapsulation of CpG for Stabilization and Enhancement of Immunoadjuvancy

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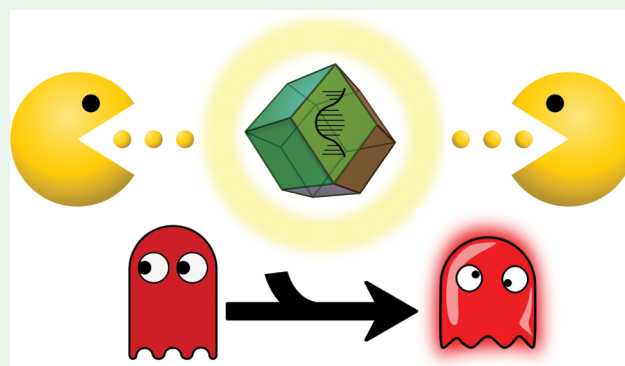
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ABSTRACT: Metal–organic frameworks (MOFs) have been used to improve vaccine formulations by stabilizing proteins and protecting them against thermal degradation. This has led to increased immunogenicity of these proteinaceous therapeutics. In this work, we show that MOFs can also be used to protect the single-stranded DNA oligomer CpG to increase its immunoadjuvancy. By encapsulation of the phosphodiester CpG in the zinc-based MOF, zeolitic imidazolate framework-8, the DNA oligomer is protected from nuclease degradation and exhibits improved cellular uptake. As a result, we have been able to achieve drastically enhanced B-cell activation in splenocyte cultures comparable to the current state-of-the-art phosphorothioate CpG. Furthermore, we have made a direct comparison of micro- and nanosized MOFs for optimization of the particulate delivery of immunoadjuvants to maximize immune activation.

KEYWORDS: metal–organic framework, zeolitic imidazolate framework 8, CpG, immunoadjuvant, phosphodiester DNA, B cell activation, nuclease resistance



INTRODUCTION

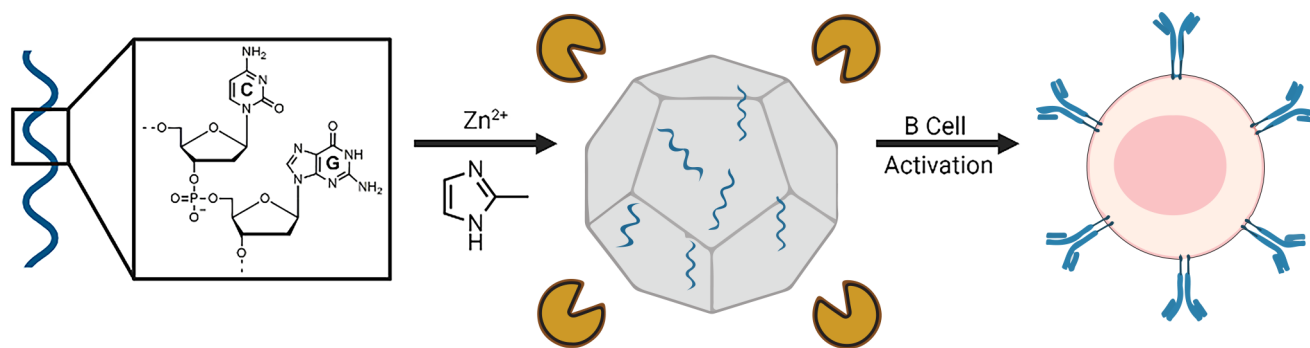
Metal–organic frameworks (MOFs) have been used to stabilize a wide variety of biomacromolecules including proteins,^{1,2} viruses,³ and liposomes⁴ against thermal degradation as a means to overcome the “cold chain”. This technology has been revolutionary in overcoming the notorious instability of biological therapeutics and offers the possibility of significantly reducing their cost and increasing their accessibility, specifically in developing areas that lack the infrastructure to maintain the required refrigeration for storage and transport.^{5,6} These powerful polymeric frameworks are formed through coordination bonds between a metal node and organic linkers.^{7–9} Biomacromolecules act as nucleating agents that catalyze MOF formation, resulting in encapsulation through a process called biomimetic mineralization.^{10–13} Once encapsulated, the biomacromolecule is protected from enzymatic degradation¹⁴ and thermal denaturation.^{15,16} The resulting structure is thermodynamically stable^{17–19} but is kinetically labile and easily degrades in the presence of strong metal chelators,^{20,21} low pH,^{22,23} and inorganic phosphates,²⁴ which allows for the recovery of the preserved biomacromolecule. In this work, we apply this technology for encapsulation of the single-stranded DNA (ssDNA) immunoadjuvant CpG.

Vaccines often have a limited ability to activate the B- and T-cell-mediated components of the immune system and therefore must be combined with an immunoadjuvant to promote and direct the immune response.^{25–27} Synthetic adjuvants designed to mimic pathogen-associated molecular patterns (PAMPs) can bind to pattern recognition receptors to begin the process of switching immune cells from a passive naive state to an active state ready to fight infection.^{28,29} CpG is a synthetic immunoadjuvant composed of unmethylated, bacterial ssDNA.^{30,31} In mammals, 70–80% of CpG cytosines are methylated; therefore, unmethylated CpG is recognized by the immune system as a PAMP.^{32,33} The immunoadjuvant CpG can activate both plasmacytoid dendritic cells (pDCs) and B cells to trigger a proinflammatory response.³⁴ This is important because pDCs are the body’s vanguards against infection by foreign pathogens and proliferation of cancer, while B cells are responsible for producing different types of proteins that bind

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Scheme 1. Illustration of the Encapsulation of CpG in ZIF-8 for Enhanced Immunoadjuvancy^a

^aThe ssDNA immunoadjuvant CpG is encapsulated in the zinc-based MOF ZIF-8 by biomimetic mineralization using Zn^{2+} and HMIM. Encapsulation of CpG within ZIF-8 affords nucleic acid protection from nucleases and promotes cellular uptake for enhanced B cell activation.

to and neutralize infection, in particular, different types of immunoglobulins or antibodies.

There are two main types of CpG, each specializing in activating a specific part of the innate immune system. Class A CpG (also referred to as D-type) more strongly activates pDCs and promotes a T-cell-mediated cellular immune response,³⁵ whereas class B CpG (K-type) more strongly activates B cells and promotes a humoral immune response.³⁶ Because of their ability to boost antibody production, class B CpG immunoadjuvants have been the focus of human clinical trials for vaccines, infectious diseases, and cancer.^{37–39} The major limitation undermining the success of CpG in clinical trials comes from the nuclease susceptibility of the DNA backbone.⁴⁰ The current state-of-the-art technology has reengineered the labile phosphodiester (PO) backbone by thiolation of the sugar moiety to form a nuclease-resistant phosphorothioate (PS) bond, resulting in an increased *in vivo* half-life of 30–60 min (5–10 min for PO).⁴¹ However, this modification has been found to lower the immunoadjuvancy of CpG and induce acute toxicity.^{42,43}

As an alternative to PS backbone modification, we propose using MOFs to improve the *in vivo* stability of PO CpG. In this way, we can protect CpG from nucleases while also retaining the innate immunoadjuvancy of the oligonucleotide. Recent work with zeolitic imidazolate framework 8 (ZIF-8), a zinc-based MOF, has shown it to be possible to grow a MOF shell around biomacromolecules in a simple one-pot synthesis under ambient conditions.^{44–47} The resulting formulation does not require refrigeration, unlike other currently used nanocarriers such as lipid nanoparticles,⁴⁸ virus-like particles,⁴⁹ and liposomes.⁵⁰ ZIF-8 forms a protective barrier that inhibits the enzymatic degradation of biomacromolecules.⁵¹ ZIF-8 has previously served⁵² as a nanoparticle carrier of PS CpG by electrostatically binding the negatively charged CpG onto the cationic surface of the crystals to promote cellular uptake and enhance immunoadjuvancy in macrophages. In this work, we encapsulate PO CpG within ZIF-8 to protect the DNA from nucleases and enhance immunoadjuvancy by improving B cell activation (Scheme 1). Furthermore, we have optimized our formulation by tuning the ZIF-8 metal-to-ligand ratios to synthesize both micro- and nanosized CpG@ZIF.

EXPERIMENTAL SECTION

Materials. PO CpG (ODN 1826-Class B) and fluorescein isothiocyanate (FITC)-labeled PO CpG were purchased as custom DNA oligomers from Invitrogen [Waltham, MA; sequence 5'-

tccatgacgttctctgacgtt-3' (20 mer); 5' FAM modification]. PS CpG (ODN 1826), FITC-labeled PS CpG (ODN 1826 FITC), and PS GpC (ODN 1826 control: ODN 2138) were purchased from InvivoGen (San Diego, CA). Nuclease-free water, zinc acetate dihydrate, 2-methylimidazole, β -mercaptoethanol, RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), FB Essence, penicillin-streptomycin, phosphate-buffered saline, and desoxyribonuclease I (DNase I) were purchased from Sigma-Aldrich (St. Louis, MO), Thermo Fisher Scientific (Waltham, MA), or VWR (Radnor, PA). Lactate dehydrogenase (LDH) cytotoxicity assay kit, cell staining buffer, RBC lysis buffer, Alexa Fluor 700 antimouse CD19 antibody, Alexa Fluor 647 antimouse CD80 antibody, and PE antimouse CD86 antibody were purchased from BioLegend (San Diego, CA).

CpG@ZIF Synthesis and Characterization. *Synthesis of CpG@ZIF.* Stock solutions of 1 M $Zn(OAc)_2$ and 3 M 2-methylimidazole (HMIM) were made in nuclease-free water. For the synthesis of CpG@ μ ZIF, 213 μ L of 3 M HMIM (final concentration 640 mM) was combined with 708 μ L of nuclease-free water. A total of 39 μ L of 100 μ M PO CpG (final concentration 25 μ g/mL) was added, and the solution was vortexed for 10 s. Last, 40 μ L of 1 M $Zn(OAc)_2$ (final concentration 40 mM) was added, and the solution was vortexed for another 30 s. The solution immediately turned turbid and was allowed to react at room temperature (RT) for 1 h. The same was done for the synthesis of CpG@nZIF using 853 μ L of 3 M HMIM (final concentration 2560 mM), 28 μ L of nuclease-free water, 39 μ L of 100 μ M PO CpG (final concentration 25 μ g/mL), and 80 μ L of 1 M $Zn(OAc)_2$ (final concentration 80 mM). For the synthesis of pristine ZIF, the same conditions were used except 39 μ L of additional nuclease-free water was added in place of 39 μ L of PO CpG. The resulting solutions were centrifuged at 17000g for 10 min to obtain a pellet of the crystals. The crystals were then washed with 1000 μ L of methanol (MeOH), a mixture of 500 μ L of MeOH and 500 μ L of nuclease-free water, and 1000 μ L nuclease-free water using the same centrifugation method. The final pellet was resuspended in nuclease-free water. The same procedure was used for the encapsulation of FITC-labeled CpG. For adsorption of CpG onto the surface of ZIF (CpG+ZIFs), 39 μ L of 100 μ M PO CpG was combined with pristine ZIF in a total reaction volume of 100 μ L and incubated on a rotisserie for 1 h at RT. The samples were purified by centrifugation (17000g for 10 min); however, no subsequent washings were employed to prevent dislodging of the surface-adsorbed CpG.

Characterization of CpG@ZIF. Scanning Electron Microscopy (SEM). The surface morphology of the CpG@ZIFs was examined using a Zeiss Supra 40 scanning electron microscope at 2.5 kV and 6–10 mm working distance. A total of 5 μ L of the prepared crystals in nuclease free water was loaded onto a silicon wafer and allowed to dry for 10 s, and the excess was wicked off using Whatman Filter Paper No. 1. The samples were then sputtered with a \sim 40 Å layer of gold before being imaged.

Dynamic Light Scattering (DLS). The size and polydispersity of the CpG@ZIFs were quantified using a Malvern Analytical Zetasizer Nano ZS. A total of 1 mL of the crystals diluted in nuclease-free water

was loaded into a 1 mL disposable cuvette and read at 25 °C, a 175° scattering angle, a medium refractive index of 1.33, a 633 nm laser, and a material refractive index of 1.51.

ζ Potential. The charge of the CpG@ZIFs was quantified using a Malvern Analytical Zetasizer Nano ZS. A total of 1 mL of the crystals diluted in nuclease-free water was loaded into a 1 mL disposable folded capillary cell and read at 25 °C.

Powder X-ray Diffraction (PXRD). The crystallinity of the CpG@ZIFs was determined with a Rigaku SmartLab X-ray diffractometer with Cu Kα (1.54060 Å) at 30 mA and 40 kV. The samples were washed with MeOH and put under vacuum overnight before being analyzed.

Brunauer–Emmett–Teller (BET) Nitrogen Isotherms. The surface areas of the CpG@ZIFs were quantified using a Micrometrics ASAP 2020 surface area analyzer. Nitrogen adsorption measurements were taken at 77 K. The samples were activated in MeOH for 4 h, dried under vacuum for 24 h, soaked with dichloromethane for 4 h, and finally dried under vacuum for another 24 h. Before analysis, the samples were put under vacuum and degassed at 120 °C for 12 h. The data were processed by the BET method for calculation of the surface area, and the pore sizes were quantified by nonlocalized density functional theory with a carbon slit pore model.

Confocal Microscopy. The fluorescence of the FITC-labeled PO CpG encapsulated in ZIF-8 was qualitatively observed using an Olympus FV3000 RS confocal microscope. A total of 10 μL of the sample was loaded onto a glass slide, covered with a glass coverslip, and left to dry overnight in the dark. The slide was then sealed and imaged using 100× magnification. Images were processed using *ImageJ* software.

Fluorimetry. The encapsulation efficiency of FITC-labeled PO CpG in ZIF-8 before and after denaturing washes was quantified by measuring the FITC fluorescence intensity of the supernatant during synthesis. For washing, CpG@ZIFs were treated with either 10% Sodium dodecyl sulfate (SDS) for 30 min at RT or 2 units of DNase I for 10 min at 37 °C. After, the solution was centrifuged at 17000g for 10 min, and 100 μL of the supernatant was added to a black 96-well plate in triplicate. Fluorescence readings at $\lambda_{\text{ex}} = 495$ nm and $\lambda_{\text{em}} = 520$ nm were performed on a BioTek Synergy H4 hybrid microplate reader. The encapsulation efficiency was calculated as a percent of the FITC fluorescence intensity of the starting material.

CpG@ZIF Stability against DNase I. The digestion was performed according to the manufacturer's protocol. In brief, 10 μg each of encapsulated and unencapsulated PO CpG (as well as PS CpG) were incubated with 1 μL (2 units) of DNase I and 10 μL of 10× DNase reaction buffer (final concentration 1×) in a final reaction volume of 100 μL using nuclease-free water. The mixture was incubated for 10 min at 37 °C. The reaction was quenched with 1 μL of 0.5 M ethylenediaminetetraacetic acid (EDTA). The digested and undigested CpG@ZIFs samples were centrifuged at 17000g for 10 min and the supernatants decanted. A total of 100 μL of 0.5 M EDTA was used to dissolve the ZIF-8 crystals and recover CpG. The recovered CpG samples were run on a 5% agarose gel containing 0.5 mg/mL ethidium bromide at 100 V for 10 min with a 1× TBE running buffer alongside an ultralow-range DNA ladder. The same was done for the adsorbed samples (CpG+ZIFs) except EDTA exfoliation was not required to recover CpG.

CpG@ZIF Performance in Vitro. Cytotoxicity. Cell viability assay was performed according to the manufacturer's protocol. In brief, RAW 264.7 murine macrophages were grown in DMEM supplemented with 10% FB Essence and 1% penicillin–streptomycin. The cells were then seeded at a concentration of 1×10^6 cells/mL in a 96-well plate (100 μL/well) and allowed to adhere overnight. All incubations took place in a 37 °C CO₂ incubator. The following day the cells were treated with the CpGs, CpG@ZIFs, or ZIFs at a CpG concentration of 3.3 μg/mL (100 μL/well) for 4 h. Next, 10 μL of lysis buffer was added to a set of untreated cells for 30 min to create the negative control. After that, 100 μL of the working solution was added to all of the wells for 30 min in a light-protected area. Last, 50 μL of the stop solution was added to all wells before the absorbance

was read at 490 nm on a BioTek Synergy H4 hybrid microplate reader.

Uptake. Spleens from naïve BALB/c mice were collected from euthanized mice in accordance with Protocol 19-06 approved by the Institutional Animal Care and Use Committee (IACUC), The University of Texas at Dallas. Spleens were homogenized into single-cell suspensions using cell pestles and 100 μm cell strainers, and red blood cells were lysed with 1× RBC lysis buffer. Splenocytes were seeded at 1×10^6 cells/mL in a 24-well plate (2 mL/well). The splenocytes were treated with FITC-labeled CpGs, CpG@ZIFs, or ZIFs (3.3 μg/mL CpG concentration) in RPMI supplemented with 10% FB Essence, 1% penicillin–streptomycin, and 50 μM β-mercaptoethanol for 4 h at 37 °C in a CO₂ incubator. After that, the cells were washed three times with 0.5 M sodium acetate buffer at pH 5 to remove surface-bound material, washed three times with 1× phosphate-buffered saline (PBS), stained with Alexa Fluor 700 antimouse CD19 antibody to identify the B cells, washed three times with a cell-staining buffer, and finally resuspended in 1 mL of a cell-staining buffer. Quantitative analyses were completed using a BD LSR Fortessa flow cytometer, with approximately 100000 events collected per sample. Data processing was performed on *FlowJo* software, version 10.6.1.

B Cell Activation. Spleens from naïve BALB/c mice were collected from euthanized mice in accordance with Protocol 19-06 approved by the IACUC, The University of Texas at Dallas. Spleens were homogenized into single-cell suspensions using cell pestles and 100 μm cell strainers, and red blood cells were lysed with 1× RBC lysis buffer. Splenocytes were seeded at 1×10^6 cells/mL in a 24-well plate (2 mL/well). The splenocytes were treated with FITC-labeled CpGs, CpG@ZIFs, or ZIFs (3.3 μg/mL CpG concentration) in RPMI supplemented with 10% FB Essence, 1% penicillin–streptomycin, and 50 μM β-mercaptoethanol for 4 h at 37 °C in a CO₂ incubator. The cells were washed three times with 0.5 M sodium acetate buffer at pH 5 to remove surface-bound material, washed three times with 1× PBS, stained with Alexa Fluor 700 antimouse CD19 antibody, Alexa Fluor 647 antimouse CD80 antibody, and PE antimouse CD86 antibody to identify activated B cells, washed three times with a cell-staining buffer, and finally resuspended in 1 mL of a cell-staining buffer. Quantitative analyses were completed using a BD LSR Fortessa flow cytometer, with approximately 100000 events collected per sample. Data processing was performed on *FlowJo* software, version 10.6.1.

RESULTS AND DISCUSSION

Biomimetic mineralization of ZIF-8 on the surface of CpG was done by iteratively adjusting aqueous solutions of zinc acetate and HMIM as the metal node and organic ligand, respectively. Tuning the metal-to-ligand ratio allows us to not only capture the oligonucleotide but also control the size of ZIF-8. From our initial screen, we found that we could produce CpG encapsulated in microsized crystals (CpG@μZIF) when we used 40 mM Zn(OAc)₂, 640 mM HMIM, and 25 μg/mL CpG. Further, we found that when we used 80 mM Zn(OAc)₂, 2560 mM HMIM, and 25 μg/mL CpG, we could synthesize nanosized crystals (CpG@nZIF). Prior work using⁴ time-resolved X-ray spectroscopy showed that ZIF-8 formation begins within seconds; consequently, the time that the DNA resides at these high concentrations of metals and ligand is extremely short before it becomes encapsulated. SEM micrographs of the resulting microcrystals (Figure 1A) and nanocrystals (Figure 1B) show the characteristic rhombic dodecahedral shape of ZIF-8 that is consistent with pristine ZIFs (Figure 1C,D). Furthermore, the crystallinity of the CpG@ZIFs was measured by PXRD, with the patterns matching that of pristine and simulated ZIF-8 (Figure 1E). Following activation, we found that the resulting composites were still porous. As expected, the nitrogen isotherms of CpG@μZIF and CpG@nZIF show diminished surface areas,

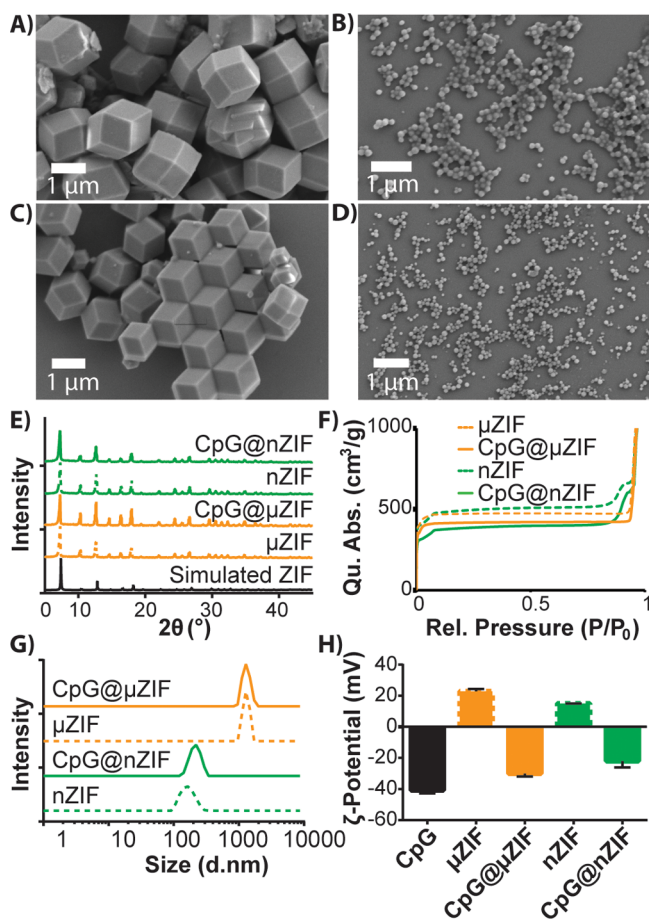


Figure 1. Encapsulation of CpG in micro- and nanosized ZIF-8. SEM micrographs of (A) CpG@ μ ZIF, (B) CpG@nZIF, (C) pristine μ ZIF, and (D) pristine nZIF. (E) PXRD patterns of CpG@ μ ZIF and CpG@nZIF compared to those of pristine and simulated ZIF-8. (F) BET nitrogen isotherms assessing the reduction in the surface area as a result of CpG encapsulation in μ ZIF and nZIF. (G) DLS characterization of the size distribution of CpG@ μ ZIF and CpG@nZIF. (H) ζ -potential measurements illustrating the reduction in the negative charge of CpG after encapsulation in the positively charged ZIFs.

which is attributed to the presence of CpG in ZIF-8 (Figure 1F). The hydrodynamic radius of the resulting composites was measured via DLS. From these data, we found CpG@ μ ZIF to have a size of around $\sim 1.3 \mu\text{m}$ (PDI: 0.374), whereas CpG@nZIF was $\sim 215 \text{ nm}$ (PDI: 0.470) (Figure 1G). The ability to control the size of the composites is important in the formulation of vaccines and adjuvants. Polymeric vaccine formulations often advocate for a larger particle size ranging from 500 nm to several microns, with the advantage of providing a sustained release system.⁵³ With these constructs, we have seen a heightened humoral immune response and prolonged immunity; however, literature reports that particles should be less than 500 nm for optimal uptake by immune cells.^{54,55} Having two sizes of CpG@ZIF both above and below this cutoff allows us to make a direct comparison of micro- and nanosized MOFs for optimal delivery of CpG and subsequent activation of B cells. Further characterization of both micro- and nanoformulations found that the encapsulation of CpG in ZIF-8 resulted in a slight shielding of the strong negative charge of the DNA by the positively charged MOF (Figure 1H). It was hypothesized that this factor may also play

an important role in mediating cell uptake because mammalian cell membranes are negatively charged owing to the presence of phosphatidyl serine; thus, cargo with strong negative charges are thought to be electrostatically repelled from cell surfaces.^{56,57}

To quantitatively and qualitatively confirm DNA encapsulation within ZIF, fluorescently labeled CpG, FITC-CpG, was used. The encapsulation efficiency was first quantified by measuring the amount of unencapsulated material in the supernatant during synthesis. Fluorometric analyses show that CpG was encapsulated quantitatively ($\sim 95\%$; 23–24 μg of CpG per mL of ZIF) in both sizes of ZIF (Figure 2A,B), an important observation given the high cost of CpG. Qualitatively, confocal images of the micro- and nanoformulations found that the crystals were obviously fluorescent in the FITC channel (Figure 2C). Furthermore, SDS (Figure 2D) and DNase (Figure 2E) washes were employed to remove any surface-bound material. From these experiments, we note that nZIF seems to adsorb CpG onto the surface more than the larger μ ZIF, a phenomenon previously detailed by Li et al.⁵⁸ Given the high concentrations of metal and ligand, we were concerned that hydrolysis of the DNA backbone might occur. Curiously, ZIF growth has never been shown to degrade any biomacromolecules, even with such high concentrations of Lewis acids and alkaline ligands. To the contrary, ZIF shells grow and protect even very delicate systems like protein-embedded liposomes,⁴ enzymes,^{59,60} whole yeast,^{61,62} and bacteria.^{46,63} To confirm that the CpG was not damaged during the encapsulation, the ZIF shells of CpG@ μ ZIF and CpG@nZIF were removed by treatment with 0.5 M EDTA to pull Zn^{2+} from the coating and recover the DNA. Using a 5% agarose gel stained with ethidium bromide, we found that the CpG was unaltered (Figure 2F).

After confirming that CpG was properly encapsulated in ZIF-8, we sought to test the ability of ZIF to protect CpG from nuclease degradation. We subjected both CpG@ μ ZIF and CpG@nZIF, as well as CpG controls, to DNase I digestion. After incubation with DNase I for 10 min at 37 $^{\circ}\text{C}$, the CpG@ZIFs samples were exfoliated using 0.5 M EDTA to remove the ZIF shell. The recovered CpG was run on a 5% agarose gel and visualized with ethidium bromide (Figure 2F). It was found that DNase I degraded the unencapsulated CpG, whereas the encapsulated CpG and PS CpG remained intact. This confirms that ZIF successfully protects CpG for nucleases. Furthermore, we tested CpG+ZIF to confirm that the nuclease protection was truly due to the encapsulation of CpG within ZIF (Figure 2F). Mere surface adsorption did not provide the same protection as encapsulation, with complete degradation of CpG observed following the treatment of CpG+ZIF with DNase I. In addition, it was noted that CpG was adsorbed to the surface of nZIF more strongly than μ ZIF, as was made evident by the reduced electrophoretic mobility of CpG, which corroborates our earlier findings.

With our nuclease-resistant formulations of CpG, we then moved *in vitro*. First, we confirmed the biocompatibility of both formulations using an LDH cytotoxicity assay and RAW 264.7 murine macrophages (Figure 3A), where both formulations were found to be nontoxic after a 4 h incubation at the CpG concentrations that we needed to use to induce B cell maturation (3.3 $\mu\text{g}/\text{mL}$). We then moved forward with cellular assays using splenocytes prepared as a single-cell suspension from spleens of naive BALB/c mice. Splenocytes are a mixture of T cells, B cells, monocytes, granulocytes,

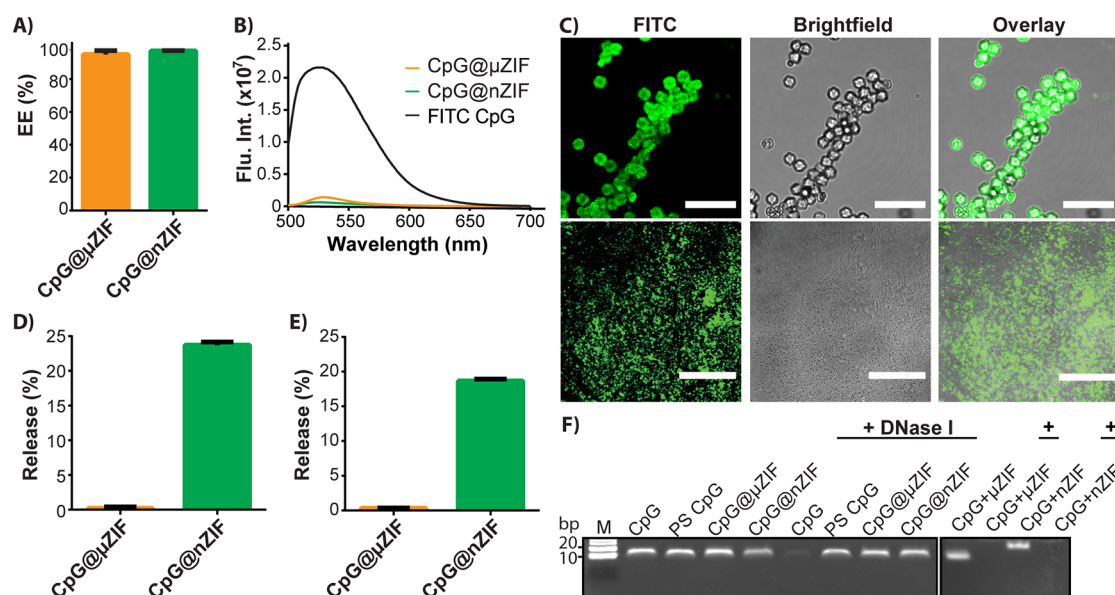


Figure 2. Characterization of CpG encapsulated in ZIF-8. (A) Encapsulation efficiency of CpG in ZIF-8 as measured by fluorescence of the supernatant ($n = 3$; $\lambda_{\text{ex}} = 495 \text{ nm}$; $\lambda_{\text{em}} = 520 \text{ nm}$). (B) Representative fluorescence spectra of the supernatant used to measure the encapsulation efficiency ($\lambda_{\text{ex}} = 495 \text{ nm}$). (C) Confocal images of FITC-labeled CpG@ μ ZIF (top, scale bar = $10 \mu\text{m}$) and CpG@nZIF (bottom, scale bar = $20 \mu\text{m}$). (D) Quantification of surface-adsorbed CpG via a SDS wash and fluorescence measurement of the resulting supernatant ($n = 3$; $\lambda_{\text{ex}} = 495 \text{ nm}$; $\lambda_{\text{em}} = 520 \text{ nm}$). (E) Quantification of surface-adsorbed CpG via a DNase wash and fluorescence measurement of the resulting supernatant ($n = 3$; $\lambda_{\text{ex}} = 495 \text{ nm}$; $\lambda_{\text{em}} = 520 \text{ nm}$). (F) 5% agarose gel characterizing the intactness of CpG before and after DNase I digestion to demonstrate the nuclease protection afforded by ZIF encapsulation.

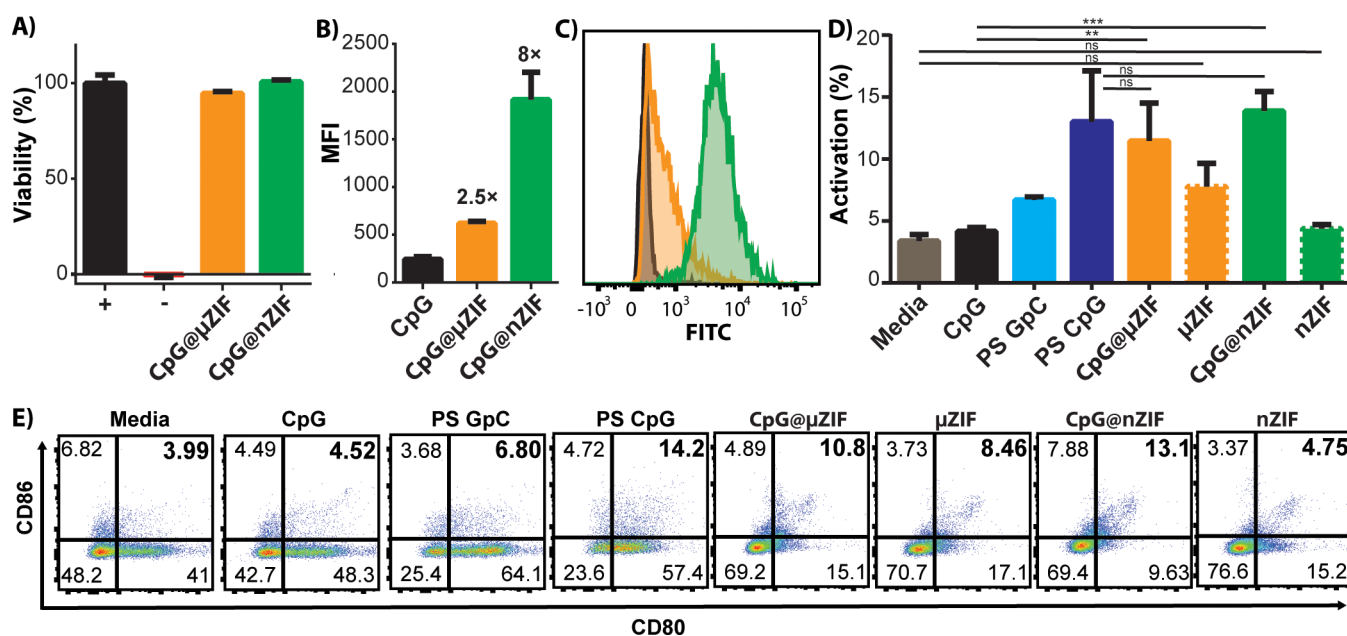


Figure 3. Evaluation of micro- and nanosized CpG@ZIF *in vitro*. (A) LDH cytotoxicity assay quantifying the biocompatibility of CpG@ μ ZIF and CpG@nZIF with RAW 264.7 murine macrophages after 4 h ($n = 3$). (B) Uptake of FITC-labeled CpG@ μ ZIF and CpG@nZIF by CD19⁺ B cells after 4 h incubation as measured by flow cytometry ($n = 3$). (C) Representative histogram of the uptake of FITC-labeled CpG, CpG@ μ ZIF, and CpG@nZIF in CD19⁺ B cells. (D) Percent of activated B cells (CD19⁺, CD80⁺, and CD86⁺) after 48 h of stimulation with CpG@ μ ZIF and CpG@nZIF, as measured by flow cytometry ($n = 3$). (E) Representative flow plots of B cell activation by CpGs and CpG@ZIFs. The statistical significance was calculated by ordinary one-way ANOVA with Tukey's multiple comparison test [* , $p < 0.05$; ** , $p < 0.01$; *** , $p < 0.0005$; **** , $p < 0.0001$; ns = not significant ($p > 0.05$)].

dendritic cells, natural killer cells, and macrophages and are commonly used for *in vitro* immune stimulation experiments. Using FITC-labeled CpG, we were able to quantify the uptake of CpG@ μ ZIF and CpG@nZIF by B cells using flow cytometry. After incubation of the CpG@ZIFs with

splenocytes for 4 h, the cells were washed three times with a low pH buffer to dissolve any surface ZIF.^{46,64,65} This ensured that the uptake observed indicated the degree of internalization of the particles. The cells were then stained with Alexa Fluor 700 antimouse CD19 antibody to identify B cells before being

analyzed by flow. From these results, we found that both CpG@ZIF formulations were able to improve the uptake of CpG (Figure 3B,C). We attribute this to the improved *in vitro* stability as well as the shielding of the strong negative charge of the DNA by the positively charged carrier. Furthermore, we found that the nanosized formulation, CpG@nZIF, was taken up more efficiently compared with the larger CpG@ μ ZIF, following literature examples that nanoparticles are more optimized for cellular uptake. Following uptake by endocytosis, the ZIF is degraded by the acidic pH of the lysosome and releases the CpG cargo to activate B cells.^{16,44,45,52,66–68} To test this, we incubated CpG@ μ ZIF and CpG@nZIF with splenocytes for 48 h, washed the cells with a low pH buffer, and subsequently stained the cells with three fluorescently labeled antibodies that allow us to differentiate between naïve and activated B cells: Alexa Fluor 700 antimouse CD19 antibody, Alexa Fluor 647 antimouse CD80 antibody, and PE antimouse CD86 antibody. In this study, we employed PS CpG as a positive control and PS GpC, an antisense complement to PS CpG, as a negative control. Using flow cytometry, we were able to quantify B cell activation, where we found that both CpG@ μ ZIF and CpG@nZIF were able to improve B cell activation, even matching the performance of the “gold standard” PS CpG (Figure 3D,E). Curiously, despite the significantly enhanced uptake of CpG@nZIF compared to CpG@ μ ZIF, there was no significant difference in B cell activation in these two formulations.

CONCLUSIONS

In this work, we demonstrate how the zinc-based MOF ZIF-8 can be used to encapsulate the ssDNA immunoadjuvant CpG. By tuning the metal-to-ligand ratios, we were able to synthesize both micro- and nanosizes of the encapsulated formulation. Encapsulation of CpG within ZIF-8 was shown to stabilize the PO nucleic acid by protecting it from nuclease degradation. Furthermore, encapsulation of the negatively charged biomacromolecule in the positively charged nanocarrier, ZIF-8, improved the cellular uptake of CpG in B cells, with nanosizes significantly outperforming microsized. Together, these improvements have demonstrated that ZIF encapsulation has the potential to enhance the function of CpG in adjuvanting an immune response through the activation of B cells to a comparable degree to the state-of-the-art PS CpG. From this work, we hope to shed light on the prospect of using MOFs for the stabilization of PO DNAs.

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Author Contributions

O.R.B. optimized the synthesis of CpG@ZIFs and executed SEM, gel electrophoresis, and all *in vitro* studies. F.C.H. performed DLS and ζ potential characterizations. Y.H.W. performed PXRD characterizations. S.D.D. performed BET nitrogen isotherm experiments under the supervision of R.A.S. R.N.E. performed confocal fluorescence microscopy and fluorimetry experiments and aided in all *in vitro* studies. A.S. also performed fluorimetry experiments. A.R. aided in gel electrophoresis characterizations. O.R.B. and J.J.G. composed the manuscript. J.J.G. conceived the project. All authors have read and given their approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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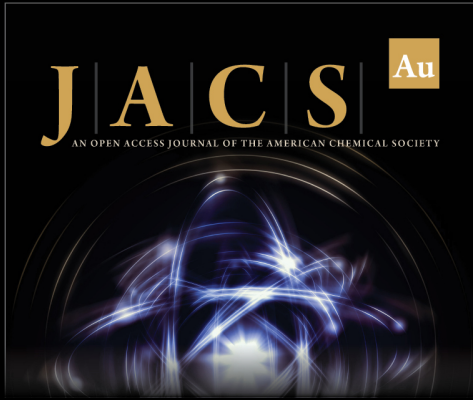
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