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Studies of Polyacrylate Based Nanoparticle Emulsions

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Studies of Polyacrylate Based Nanoparticle Emulsions

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DEDICATION

I dedicate my dissertation to the Mahzamani family. To my parents, Vali and Nahid Mahzamani whose words of wisdom and years of guidance helped me achieve this milestone. To my brother, Farrokh Mahzamani for all of his moral and financial support during difficult times, and to his children Amber, Aiden, and Ashton for the joy they bring into our lives. A special and deep feeling of gratitude to my loving wife and my other half, Danielle Mahzamani; your love and support were immeasurable and provided the path for me to continue. To the newest member of the Mahzamani family, my daughter Ava Mahzamani you have brought a light of endless joy into our lives.

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LIST OF ABBREVIATIONS

Nm = nanometers  
MRSA = methicillin-resistant *Staphylococcus aureus*  
HCl = hydrochloric acid  
LDH = layered double hydroxide  
UV = ultraviolet light  
MIP = molecular imprinted polymers  
CMS = Chiral mesoporous silica  
ACMS = Achiral mesoporous silica  
B-CMS = Biomimetic - Chiral mesoporous silica  
N-PLA = N-palmityl-L-alanine  
APTES = 3-aminopropyltriethoxysilane  
TEOS = tetraethoxysilane  
IBD = Inflammatory bowel disease  
5-ASA = 5-Aminosalicylic acid  
PPAR-γ = Peroxisome proliferator-activated receptor gamma  
D.I = deionized  
ml = milliliters  
mg = milligrams  
rpm = revolutions per minute  
mmol = millimole  
μl = microliters  
μg = micogram  
°C = celsius  
M = molar  
pH = potential of hydrogen  
1H NMR = proton nuclear magnetic resonance  
MHz = megahertz  
Hz = hertz  
H = Hydrogen  
J = coupling constant  
DMSO = Dimethyl sulfoxide  
v/v = volume/volume  
Cpd = compound  
k = kilo  
MIC = minimum inhibitory concentration  
TSB = trypticase Soy Broth  
TSA = trypticase Soy Agar  
HT-29 = human colon cells  
HEK = human embryonic kidney cells
DPBS = dulbeco’s phosphate buffer solution
MTT = diphenyltetrazolium bromide
QIAGEN = effectene transfection reagent
SEM = scanning electron microscope
mV = millivolts
L-MtA = L-menthyl acrylate and styrene
D-MtA = D-menthyl acrylate and styrene
Rac –MtA = racemic menthyl acrylate and styrene
Pen G = penicillin G
DMEM = dulbeco’s minimum essential medium
EMEM = eagle minimum essential medium
PBS = phosphate-buffered saline
IC50 = half maximal inhibitory concentration
w/w = weight per weight
SDS = sodium dodecyl sulfate
DLS = dynamic light scattering
ABSTRACT

Self-stabilizing polyacrylate nanoparticle emulsions were previously investigated in the Turos laboratory, and provided a new model for delivering antibiotics via encapsulation or covalent binding of the desired bioactive compound within the polymer nanoparticles. The method used the in water, free radical emulsion polymerization of butyl acrylate/styrene mixture to form the polymer chain stabilized with a surfactant. Current research in this dissertation further explores the versatility of related nanoparticle emulsion systems. Chapter 2 provides an overview of the loading of certain therapeutic drugs, such as 5-aminosalicylic acid and derivatives thereof, for the treatment of irritable bowel syndrome. Chapter 3 explores homo-polymer nanoparticle emulsions composed of menthyl acrylate as the monomer. Thereby obviating the need for a copolymer emulsion polymerization. The homo(menthyl acrylate) nanoparticle emulsion provided greater stability compared to the previous copolymer models. The resulting homopolymer emulsion exhibited a decrease in cytotoxicity, and a 400% increase for loading of penicillin G. Chapter 4 explores novel polyacrylamide nanoparticle emulsion using only N-acrylated ciprofloxacin to form a homo-polymer polyacrylate nanoparticle emulsion, thereby requiring no additional co-monomers. The resulting emulsion has a relatively low cytotoxicity with similar bioactivity to free ciprofloxacin.
CHAPTER ONE

THE USE OF NANOPARTICLES FOR THE DELIVERY OF BIOACTIVE COMPOUNDS

1.1 Introduction to Synthesis of Nanoparticles

The topic of nanomaterials encompasses a vast variety of fields of study. Nanomaterials can be made from inorganic, organic, and hybrid materials and are 1-1000 nm in size. Some inorganic-based materials and applications include carbon nanotubes in composite materials and electronic circuit boards; nano-silver in textiles and food packaging; nano-titanium dioxide in cosmetics, creams, and paints; nano-cerium oxide as a fuel additive; nano-iron used to form smart fluids, and magnetically responsive materials.1-3

The main focus of this review will be towards the drug delivery, antibacterial, and medicinal applications of nanomaterials, which can be used for loading bioactive compounds into nanospheres, nanocapsules, and nanoparticles.

1.1.1 Nanoparticles for the Delivery of Bioactive Compounds

The loading of bioactive compounds within or on the surface of nanoparticles for delivery are typically achieved through one of three basic models as depicted in Figure 1.1. In the first model, the bioactive compound is attached to the exterior surface of the nanoparticles. In the second
model, the bioactive compound is bound to the interior surface or within the matrix of the nanoparticle. In the last model, the desired compound is encapsulated/trapped within the nanoparticle without physical attachment.\(^4\)

Figure (1.1) Three models for nanoparticle drug-loading.

1.1.2 Synthesis of Drug-Loaded Nanoparticles

The following techniques encompass the typical synthetic methods used for the formation of drug-carrying nanoparticle polymers, which include poly(alkyl cyanoacrylates), poly(styrene-butyl acrylates), poly(lactic acid), poly(lactide-glycolides), poly(glycolide), chitosan, gelatin, and sodium alginate.
1.1.2.1 Polymer Dispersion Technique

Figure (1.2) Visualization of solvent evaporation nanoparticle formation.

Figure 1.2 illustrates the general method of nanoparticle formation using the solvent evaporation method. The polymer and bioactive compound are mixed together, and dissolved in an organic solvent with a relatively low boiling point compared to water. The polymer, bioactive compound, and organic solvent mixture are further stabilized through the addition of surfactants. The organic solvent is slowly evaporated via heating, vacuum, or continuous stirring. Other polymer dispersion techniques maintain the organic solvent within the micelle without evaporation as a mode of loading hydrophobic material and drugs. The carrier solvent can be visualized as an oil droplet stabilized by the surfactant within the aqueous solution and maintained in suspension.
1.1.2.2 Monomer Polymerization Technique

Figure (1.3) Illustration of polymerization of monomers for the synthesis of nanoparticle emulsions.

Figure 1.3 illustrates a different method of nanoparticle synthesis involving the dissolution of the bioactive compound into the monomer mixture. The mixture is then suspended into an aqueous solution via the use of surfactants and emulsifiers. Polymerization of the monomers is induced and the resulting nanoparticles remain suspended within the solution. Alternatively, the bioactive compound can be incorporated into the nanoparticles via adsorption post-polymerization. The formation of the polymer inside the micelle formed by the surfactant allows for a more stable structure compared to the free monomers.
1.1.2.3 Ionic Gelation Technique

The third common method of nanoparticle formation uses the transition of a liquid polymer solution into a gel. The hydrophilic polymer (chitosan, gelatin, or sodium alginate) is mixed with stirring into a solution of sodium tripolyphosphate.\textsuperscript{10,11} The negatively charged sodium polyphosphate and the positively charged ammonium group of the hydrophilic polymer cause the

\textbf{Figure (1.4)} Ionic interaction between chitosan and sodium tripolyphosphate.
gelation to form as illustrated in Figure 1.4. The entrapment of the bioactive drug occurs through the addition of the drug to the hydrophilic polymer solution.

1.2 Polyacrylate Nanoparticles

1.2.1 Poly(Styrene-Butyl Acrylate) Nanoparticles

In 2007 the Turos laboratory provided a synthetic procedure for the formation of covalently-bound polyacrylate nanoparticle emulsions. The nanoparticle emulsion can carry and deliver bioactive compounds, specifically, β-lactams. In addition, the nanoparticle delivery vehicle affords additional shielding from degradative enzymes, and may serve as a new method for delivering anti-MRSA (methicillin-resistant *Staphylococcus aureus*) compounds. In order to form the antibiotic-conjugated polyacrylate nanoparticles, the acryloyl derivative of different β-lactams (Figure 1.5) was synthesized and used as a monomer for the nanoparticle polymerization procedure.

![Figure (1.5) O-Acrylated N-thiolated β-lactams used to prepare polyacrylate nanoparticles](image-url)
The polyacrylate nanoparticle emulsion is prepared by emulsion polymerization in water. Figure 1.6 shows the general scheme for the formation of the nanoparticle emulsion. The resulting nanoparticles had an average diameter between 30 and 50 nm. The olefin monomers are segregated into micelles via the addition of sodium dodecyl sulfate as the surfactant. The polymerization uses potassium persulfate as the radical initiator. The β-lactam bound nanoparticle emulsions exhibited a 2 to 4 fold increase in antimicrobial activity against both Staphylococcus aureus and β-lactam-resistant Staphylococcus aureus, when compared to the free non-nanoparticle compound.

\[
\text{Butyl acrylate} + \text{Styrene} + \text{Drug} \xrightarrow{\text{Sodium dodecyl sulfate 3 weight \%}} \text{Drug} \rightleftharpoons \text{Polymeraines}
\]

**Figure (1.6)** General scheme for preparing polyacrylate nanoparticle emulsions by free radical emulsion polymerization.

The Turos laboratory later that year published another paper detailing the covalent binding of various acrylated derivatives of penicillin as seen in Figure 1.7. It was also found that non-acrylated derivatives such as penicillin G were successfully encapsulated without covalent attachment to the polymer backbone (Figure 1.8).
Figure (1.7) Acrylated derivatives of penicillins used for covalent binding in nanoparticle emulsions.

Figure (1.8) Penicillin G and an esterified derivative used for encapsulation in nanoparticle emulsions.

The antibacterial assays found that the minimum inhibitory concentration values for all drug-loaded nanoparticles were similar against both *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. Kirby-Bauer assays using the penicillinase enzyme demonstrated the ability of the nanoparticle to protect the penicillin framework against hydrolytic degradation.\(^\text{12}\)
In 2009, an additional study was published by the Turos group that compared the effects of replacing sodium dodecyl sulfate in the emulsion polymerization procedure with other surfactants. Prior research determined that the cytotoxicity of some of the emulsions was due to excess surfactant (sodium dodecyl sulfate) used for emulsion polymerization, and the resulting emulsions required further purifications in order to reduce cytotoxicity. The investigation compared the effects of anionic, cationic, zwitterionic, and non-ionic surfactants on nanoparticle formation and cytotoxicity. It was determined that non-ionic surfactants reduced the cytotoxic properties of the resulting emulsions, and that all ionic surfactants exhibited an increase in toxic properties of the emulsions. The cationic surfactant was the most cytotoxic.

1.2.2 Poly(Alkyl Cyanoacrylate) Nanoparticles

The formation of nanospheres using cyanoacrylate monomers were initially reported in 1979 by Couvreur et al. and demonstrated the use of methyl cyanoacrylate and ethyl cyanoacrylate as monomers. Nanospheres were produced by dropwise addition of the cyanoacrylates into HCl solution (pH 2-3) containing Tween 20 as the surfactant.

![Figure 1.9](image_url) The structure of poly(alkyl cyanoacrylate) polymers.
The resulting nanospheres provided an ideal model for the loading of some bioactive compounds, and the field of poly(alkyl cyanoacrylate) nanoparticles has expanded tremendously since then (Figure 1.9). The concentration of surfactant directly influences the size of the nanospheres (50-300 nm). The molar masses of the resulting nanospheres were dependent on the type of monomer and surfactant used.\textsuperscript{15,16}

Poly(alkyl cyanoacrylate) nanoparticles can also form nanocapsules with the ability to load both hydrophilic or hydrophobic bioactive compounds. Water containing nanocapsules suspended in oil are able to deliver hydrophilic compounds, while oil containing nanocapsules suspended in water are able to deliver hydrophobic compounds.\textsuperscript{17}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{antibiotics.png}
\caption{Antibiotics loaded into poly(alkyl cyanoacrylate) nanoparticles.}
\end{figure}
Figure 1.10 shows a few examples of antibiotics that were loaded up to 8.1% for amoxicillin, 90% for ampicillin, 16% for ciprofloxacin, and 44.6% for moxifloxacin, all as % weight of the poly(alkyl cyanoacrylate) nanoparticles that successfully served as drug delivery vehicles.\textsuperscript{18-21}

1.3 Chiral Drug Delivery Vehicles

Chiral nanoparticles and chiral polymer materials have the potential for enantiodiscrimination.\textsuperscript{22} One such study demonstrated the ability of enantiomeric and diastereoisomeric end groups (Figure 1.11) of nanoparticles to exhibit different binding affinities towards protein targets.\textsuperscript{23}

![Figure 1.11](image1.png)

**Figure (1.11)** Examples of chiral end groups of gold nanoparticles.

Organic-based chiral nanoparticles, such as green fluorescent protein chromophore nanoparticles and poly(ethylene glycol)-modified thiolated gelatin nanoparticles, have also found uses in the fields of photonics, sensing, recognition, DNA delivery, and non-linear optics.\textsuperscript{24,25} It is rather interesting that organic chiral nanoparticles exhibit optical and electronic properties, typically
expected from inorganic-based nanoparticles. These properties are usually dependent on nanoparticle size, intermolecular interactions, and nanoparticle surface interactions.²⁶

In recent years, new models of chiral drug delivery vehicles have been more extensively researched, and may provide viable methods for delivering bioactive compounds.

1.3.1 Layered Double Hydroxides as Chiral Drug Delivery Vehicles
In 2012, the Pingxiao Wu research group developed an interesting method for preparing chiral nanoparticles for drug delivery.²⁷ In this project the unstable chiral drug L-alanyl- L-glutamine (L-(Ala-Glu)) was incorporated into the layered double hydroxides (LDHs). LDH nanocomposites are layered structures of hydroxides, metal cations, and intercalated anions. The layer sequence is typically composed of hydroxide anion/metal cation/hydroxide anion/intercalated anion/hydroxide anion/metal cation/hydroxide anion. After several experiments and computational studies, it was possible to characterize the structure of the nanoparticles and also their properties. The principal problem with the employment of (L-(Ala-Glu)) as a drug on its own is that it cannot maintain its morphology under the irradiation of UV light. LDHs provide a protection for the (L-(Ala-Glu)) avoiding the change in its morphology even under the irradiation of UV light. The LDHs appear to be useful for a wide variety of chiral drugs as a drug delivery system.

1.3.2 Molecularly Imprinted Polymers (MIP) for Chiral Drug Delivery
In 2015, a remarkable work in the field of molecular imprinted polymers (MIPs) was published Suksuwan et al.²⁸ Figure 1.12 shows that the MIPs have the ability to create templated cavities,
with the polymer surface, using the target molecule as the template. This technique mimics the natural processes which produce polymers employing natural building blocks.

**Figure (1.12) General scheme of MIPs**

In this specific study the MIPs were designed to serve as an enantioselective receptor of \((R)\)-thalidomide. When the MIPs are loaded with the bioactive compound, the final objective is the liberation of the drug into cancer cells. MIPs can be considered as an assembly of nanoparticles using covalent binding and intermolecular forces for their formation. Using different polymerization methods it was possible to control the diameters of the nanoparticles at uniform sizes of 100 nm.

One important benefit of MIPs as host of \((R)\)-thalidomide is the elimination of racemization of the chiral drug, avoiding the formation of the \((S)\) enantiomer that has toxic secondary effects.
1.3.3 Chiral Drug Delivery via Chiral Mesoporous Silica (CMS)

Chiral mesoporous silica (CMS) can be successfully synthesized employing chiral surfactants as templates. Starting with amorphous chiral silica materials, chiral directing reagents, such as chiral surfactants and chiral metal complexes, can be added to distort the chirality of the tetrahedral structure of SiO$_4$. Among the many structural characteristics that CMS hold, their helical mesoporous channels and chiral pore formation allow for enantioselective properties. These properties have been extensively studied by many research groups.\textsuperscript{29} The rate of drug release is affected by the size of the pores (the larger the pore, the more rapid is the drug delivery rate).

A chiral block copolymer can be imprinted through molecular techniques, acting as a template in which an enantioselective separation can take place. The target enantiomers have different affinities towards the chiral binding sites of this imprinted copolymer, permitting chiral recognition. These materials have been proven to be able to enantioselectively absorb chiral drugs.

In 2010, Yanhui-Yang et al. reported the synthesis of a new type of CMS materials in which the use of an enantiopure surfactant was no longer necessary.\textsuperscript{30} Conventional achiral templates and a chiral cobalt complex as a co-template were utilized to create the chiral environment in the mesoporous silica. Vibrational circular dichroism measurements proved the chirality of the CMS. These CMS materials, as carriers, were employed to control the enantioselective release of metoprolol (Figure 1.13), a chiral drug. The pore diameter and structure played a decisive role in the release profiles of the drug. Moreover, $R$- and $S$- enantiomers of metoprolol exhibited
different rates in the delivery using these chirally-imprinted CMS materials in comparison with their achiral CMS analogues (ACMS).

![Structure of (S)-metoprolol and (R)-metoprolol.](image)

**Figure (1.13)** Structure of (S)-metoprolol and (R)-metoprolol.

Five years later, in 2015, the group of Sanming Li found a method to grow CMS using biomimetic synthesis.\(^3\) In this work, B-CMS was prepared using N-palmityl-L-alanine (N-PLA) as the chiral surfactant template, 3-aminopropyltriethoxysilane (APTES) as the co-structure directing agent (CSDA) and tetraethoxysilane (TEOS) as the silica source. pH influenced the energetics of the self-assembly process of three types of chiral mesoporous silica (B-CMS1, B-CMS2 and B-CMS3). The particle length was determined by the stirring rate.

### 1.4 Antibacterial Polymers

The field of antibacterial polymers encompasses a wide variety of methods for the control of bacterial contamination. Antibiotic polymers are formed via the polymerization of the bioactive monomers, covalently binding the antibiotic to a polymer using non-bioactive co-monomers such as styrene/butyl acrylate.

Optimal antibacterial polymer activity is reliant on the molecular weight of the final polymer, with an ideal weight range of 1.4x10\(^4\) Da to 9.4x10\(^4\) Da. This effect is attributed with the
increased difficulty in diffusing through the bacterial cell wall and delivering the active
compound into the cytoplasm. Some research has demonstrated that shorter chain polymers
exhibit a decrease in bioactivity compared to similar larger chain antibacterial polymers. The
authors attributed the effects to the ability of larger chain polymers to aggregate on the bacterial
cell wall and thus allow for better adsorption of the related antibacterial compound through the
bacterial membrane. Though the shorter chain polymers would presumably provide for better
diffusion through the cell wall, larger chain polymers may have an increased interaction with the
bacterial cell wall and thus improved minimum inhibitory concentration values.\textsuperscript{32, 33}

The counter ion charge on the polymer or polymer carrying particle also attributes to the ability
to deliver the desired drug to its target. Weaker ion pairing between the bioactive compound and
the carrier polymer allows for the drug to exist closer to its free state and thus interact with the
bacteria more readily.

Though the field of antibiotic delivery via the use of polymers is vast, examples of homopolymer
based antibacterial polymers are limited, and fewer still are examples where the polymer is
solely composed of antimicrobial monomers.

1.4.1 Homopolymer Antibacterial Polymers

Figure 1.14 shows the structure of an antibacterial monomer in which the bioactivity was
compared to that of the free monomer, the homopolymer, and the copolymer made with styrene.
Both the homopolymer and the styrene-co-polymer were successfully synthesized using radical
polymerization using benzoyl peroxide as radical initiator and toluene as the solvent. However,
the biological assay against \textit{S. aureus} showed a decrease in activity of both the homopolymer and the styrene-co-polymer compared to the free monomer.\textsuperscript{34}

**Figure (1.14)** 2, 4, 4’-trichloro-2’-hydroxydiphenyl ether derivative used as a monomer in homopolymer formation.

**Figure (1.15)** 8-Hydroxyquinoline derivative used as a monomer for homopolymerization and copolymerization with N-vinylpyrrolidone.

Figure 1.15 shows another example of an acrylated derivative of a bioactive compound derivative used in homopolymer formation to test against bacteria. The acrylate monomer was
polymerized with the co-monomer N-vinylpyrrolidone. Both the homopolymer and copolymer were successfully synthesized using radical polymerization and an ethanol/buffer solution as the solvent medium, and later isolated. The bioassay found that the homopolymer and copolymer had a decrease in antibacterial activity relative to the acrylated monomers.\textsuperscript{35}

![Figure 1.16](image)

**Figure (1.16)** p-Vinylbenzyl tetramethylene sulfonium tetrafluoroborate used in the formation of an antibacterial homopolymer.

Figure 1.16 shows the monomer used for the homopolymerization of a sulfonium-based antibacterial polymer. The biological assay carried out against *S. aureus* and *E. coli* demonstrated the homopolymer had increased bioactivity compared to the free monomer. This was likely due to the increased concentration of the antibacterial moiety at the cell membrane, thus allowing the cationic moiety to freely interact with the cell membrane.\textsuperscript{36}
Figure (1.17) Thiadiazol derivative used for the formation of antibacterial homopolymers.

The thiadiazol acrylate derivative in Figure 1.17 was one of only two compounds that were successfully homopolymerized in the published study via radical polymerization using benzoyl peroxide as radical initiator and 1,4-dioxane as the solvent. The bioassay indicated the homopolymer was effective against *E. coli*, while the monomer was much less bioactive. The reason for the increase in bioactivity was not understood, and additional tests are required in order to better understand the mode of action.

Figure (1.18) Norfloxacin derivative used in the synthesis of poly(acrylated quinolone).

The acrylated quinolone in Figure 1.18 was used as a monomer in a radical polymerization technique to prepare a homopolymer of an antibiotic acryloyl derivative. Both the monomer and the homopolymer demonstrated excellent antibacterial activity with similar results against both
gram-positive (*S. aureus, B. subtilis, M. luteus*) and gram-negative (*E. coli*) bacteria. However, the testing was limited to the shake flask method of bioassay, due to the insolubility of the polymer in water. This method measures the number of bacterial cell in suspension, then the bioactive compound (suspended in phosphate buffer solution) is added to the bacterial suspension and shaken, after 24 hours of incubation the number of bacterial cells in suspension is counted and reported as % cell number reduction. In addition the formed polymer is very rigid and brittle and thus has to be compounded with other processed polymers for administration and assays.\textsuperscript{38}

### 1.5 Dynamic Light Scattering and Zeta Potential Measurement of Nanoparticles

Dynamic light scattering measurements were used to determine the size of the nanoparticles within the emulsions. The average size and surface charge of the emulsion was analyzed using a Malvern Zetasizer nano-ZS instrument. Approximately 1 ml of the nanoparticle emulsion are loaded into the Malvern disposable folded capillary cell DTS-1070. Each sample was analyzed in triplicate, and each data collection consisted of 1 run of 20 scans (for size analysis) and 3 runs of 100 scans (for zeta potential determination).

A single measurement run averages 20 scans for size measurement; the data is displayed as an intensity graph providing data for the nanoparticles within and if the samples contains multiple peaks (polydispersion) or a single peak (monodispersion). The measurement recorded is the value of the prominent peak (peak 1) within the graph. The runs are performed in triplicate and the data was overlapped and averaged as seen in Figure 1.19.
Figure (1.19) Sample graph of size data from dynamic light scattering.

Figure (1.20) Sample graph of zeta potential data from dynamic light scattering.
For zeta potential measurements the instruments performs 3 runs consisting of 100 scans each. A single measurement run averages 100 scans for zeta potential measurement; the data is displayed as an intensity graph providing data for the detected –mV of the electrical double layer. Electrical double layer values greater than 30mV are considered stable. The measurement recorded of the zeta potential reading within the graph. The runs are performed in triplicate and the data was overlapped and averaged as seen in Figure 1.20.

1.6 Exploring Poly(Styrene-Butylacrylate) Nanoparticle Emulsions

Chapter 2 explores and studies the synthesis, drug loading, and properties of poly(styrene-butylacrylate) nanoparticle emulsions. The aim is to diversify the drug loading capabilities of the nanoparticle emulsions, and expand beyond antibiotics. To study the therapeutic viability of poly(styrene-butylacrylate) nanoparticles emulsion, 5-aminosalicylic acid was loaded into the nanoparticles via emulsion polymerization. Resulting emulsions were characterized using dynamic light scattering, scanning electron microscope, *in vitro* cytotoxicity, and *in vitro* antibacterial activity.
CHAPTER TWO

POLYACRYLATE NANOPARTICLE EMULSIONS: A METHOD OF DELIVERING ANTI-INFLAMMATORY COMPOUNDS

2.1 Introduction

Inflammatory bowel disease (IBD) is a collection of human health conditions that cause inflammation in the large and small intestines. The two main IBDs are Crohn’s disease and ulcerative colitis. Others include collagenous colitis, lymphocytic colitis, ischaemic colitis, diversion colitis, Behcet’s syndrome, infective colitis, and indeterminate colitis. The site of inflammation for Crohn’s disease is in the terminal ileum, while ulcerative colitis occurs mainly in the colon and rectum. Since these conditions affect the intestinal lining they also tend to interfere with the absorption of nutrients, and the proper disposal of toxins, thus causing liver problems, arthritis, eye problems, and skin conditions. The symptoms are abdominal pain, vomiting, diarrhea, hematochezia, weight loss, arthritis, pyoderma gangrenosum, and primary sclerosing cholangitis. Typically IBDs are not fatal, however, they can reduce the quality of life severely. In addition to disturbing the normal functions of the intestine, IBDs also dramatically increase the risk for colorectal cancer. Most commonly the disease is diagnosed by performing a colonoscopy along with a biopsy of pathological lesions.\textsuperscript{39-42}
One possible reason for the development of the condition is the presence of too much hydrogen peroxide within the digestive tract, which has oxidative effects. The high concentration of hydrogen peroxide causes the colonic barrier to become weaker, allowing antigenic material to seep through the colonic barrier and cause an inflammatory reaction. This in turn leads to a release of cytokines (tissue destroyers) and additional hydrogen peroxide. Being a self-defense mechanism to rid the area of bacteria, this response causes a loop cycle because the additional hydrogen peroxide brings on additional inflammation, and so on. As the intestinal barrier becomes weaker, it becomes easier for bacteria to infiltrate.

2.2 Therapeutics for Inflammatory Conditions

Inflammatory bowel diseases such as Crohn’s disease and ulcerative colitis have been common especially among Europeans and North Americans. The physiological cause of the IBDs is not clearly understood yet. Studies indicate that certain bacteria such as salmonella, yersinia, shigella, and different strains of E.coli might be involved, as well as Helicobacter pylori and Clostridium difficile.\textsuperscript{39,40} Corticosteroids and salicylates are frequently used therapeutic agents for relieving symptoms of IBDs.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{5-Aminosalicylic acid (5-ASA), compound 1.}
\end{figure}
5-Aminosalicylic acid (5-ASA) in Figure 2.1 is an active ingredient of agents used for the long-term maintenance therapy to prevent relapses of Crohn’s disease and ulcerative colitis. 5-ASA is the choice drug for patients with mild to moderate IBD. The current average effective dose is 4.8 grams daily. In addition, studies have shown that as the amount of the therapeutic drug is increased, the response in treatment is greater. However, the side effects also increase. There has been a significant amount of research into developing additional derivatives of 5-ASA in order to increase its effectiveness. The majority of the drugs developed aim to control the release, and increase the delivery, of 5-ASA to the site of the disease; a common problem is that the further the drug moves along the gastrointestinal tract, the less effective it becomes. Thus it is important to develop a carrier for the drug that is time-released, in addition to protecting the active drug from the intestinal environment until it reaches the desired point.43

2.3 Mode of Action of 5-ASA

5-ASA is not absorbed into the body; it mainly works by providing relief at the site of the inflammation, reducing the synthesis of inflammatory mediators known as eicosanoids (Figure 2.2) and inflammatory cytokines (specifically interleukin-23).44 As an inhibitor 5-ASA functions as an antioxidant, and a free-radical eliminator. Eicosanoids analogs differ by containing various hydroxyl, epoxide, and alkene functional groups. Eicosanoids are extremely oxidative, and 5-ASA is postulated to possibly neutralize its effects. In addition, 5-ASA interacts with the receptor PPAR-γ and induces an up-regulation of its expression. The function of PPAR-γ is to reduce the inflammatory response caused by colitis.45
2.4 Preparation of Nanoparticle Emulsions

Liposomes and nanoparticles are sometimes used as drug delivery vehicles in order to increase and improve efficacy, bioavailability, and specificity of pharmaceutical compounds. Some of the more notable examples include antibiotic-encapsulated polymeric nanoparticles and liposomes, biodegradable nanospheres, and surface-coated gold nanoparticles. However, most of the research in this area has been concentrated on anti-cancer agents, and only recently, antibacterials. The Turos laboratory has developed polymer-bound drug nanoparticles for antibacterial applications, and shown that the bio-activity of the drug-containing nanoparticles can be more than the free drug. However, there has been very little focus on drug delivery for IBD therapeutic agents. In the present study we aim to synthesize the nanoparticle-bound 5-ASA nanoparticles, and to characterize them and check their bio-activity in comparison with the free drug. A derivative of 5-ASA, compound 2 (Figure 2.3), was synthesized to test the covalently-bound drug polyacrylate nanoparticle emulsions. Another derivative of 5-ASA, compound 3

*Figure (2.2) Eicosanoid core structure.*
(Figure 2.4), was synthesized to test the drug encapsulation model of polyacrylate nanoparticles emulsions.

![Compound 2](image)

**Figure (2.3)** 5-Acryloylamino-2-hydroxybenzoic acid, compound 2.

![Compound 3](image)

**Figure (2.4)** 5-Acetylamino-2-hydroxybenzoic acid, compound 3.

The basic concept is to attach the anti-inflammatory drug covalently to the nanoparticle. To do this, the first step is to add an acryloyl group to a convenient functionality on the free drug, such as the amino group. The acrylate allows the covalent bonding to the nanoparticle by a free radical polymerization. To achieve this, the drug is dissolved in compatible acrylate monomers, then a surfactant is added along with water, resulting in the formation of micelles. The size of the micelles is dependent on hydrophobic and hydrophilic interactions in the emulsion. The radical initiator is then added to cause free radical polymerization of the acrylate in the emulsified micelles. This results in a stable aqueous emulsion, containing nanoparticles of uniform size.
The general procedure for the formation of the polyacrylate nanoparticle emulsions is highlighted in Figure 2.5. A self-regulating oil bath was used to maintain a temperature of 78°C, the mixture of styrene (300 µl) and butyl acrylate (700 µl) totaling 1 ml was added to a round bottom flask. The mixture was stirred at 78°C using a 1.25 cm (300 mg) Teflon-coated magnetic stir bar at 380 rpm on a Corning PC-420D magnetic stirrer. After 30 minutes, 30 mg of sodium dodecyl sulfate and 2 ml of deionized water were added, and the stirring speed was increased to 500 rpm. Following 30 minutes, 5 mg of potassium persulfate and 2 ml of deionized water were added and the stirring speed was further increased to 750 rpm. The resulting emulsion was then decanted into a collection vial after 6 hours.

The resulting emulsions are analyzed using dynamic light scattering to obtain nanoparticle size and zeta potential. In addition, \textit{in vitro} studies are carried out for both human cell cytotoxicity and antibacterial activity.
2.5 Experimental Procedures and Results

2.5.1 Synthesis of 5-Acryloylamino-2-Hydroxybenzoic Acid

![Reaction Scheme](image)

**Figure (2.6)** One pot synthesis of compound 2.

The main problem with using 5-ASA was that it required a very polar solvent, so a procedure utilizing water was used to synthesize the N-acryloyl 5-ASA. To a round bottom flask, 5-ASA (306 mg, 2 mmol) and sodium hydroxide (160 mg, 4 mmol) were dissolved in 30 ml deionized water. It was noted that smaller amounts of water resulted in higher product yields. While stirring, the mixture was placed in an ice bath to cool to 0°C. After 20 minutes acryloyl chloride (195 µl, 2.4 mmol) was added dropwise, then the ice bath was removed, and the mixture continued to stir for one hour at room temperature (Figure 2.6).

In order to extract the N-acryloyl 5-ASA, the mixture was treated with 1M aqueous HCl, to provide a final solution pH of 1, which resulted in the precipitation of the desired compound 2. The product was then filtered, and left overnight to dry. The melting point of the product was obtained using MEL-TEMP II by Laboratory Devices.
Yielded 340 mg (82.1%) as violet colored solid. Melting point: 180-185°C. (Figure 2.7) \( ^1 \text{H} \) NMR (400 MHz, DMSO-\( d_6 \)) \( \delta \) 10.07 (s, 1 H), 8.14 (s, 1 H), 7.71 (d, \( J=8.9 \), 1 H), 6.89 (d, \( J=8.9 \) Hz, 1 H), 6.34 (dd, \( J=17.2, 2.0 \) Hz, 1 H), 6.19 (dd, \( J=17.2, 10.0 \) Hz, 1 H), 5.69 (dd, \( J=10.0, 2.0 \) Hz, 1 H).

![Figure (2.7) Proton NMR assignment for compound 2.](image)

### 2.5.2 Synthesis of 5-Acetylamino-2-Hydroxybenzoic Acid (Compound 3)

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{COOH} \quad + \quad \text{Acetyl chloride} \\
\text{OH} & \quad \text{COOH} \quad \text{Aqueous NaOH} \\
\text{Compound 1} & \quad \text{Compound 3}
\end{align*}
\]

![Figure (2.8) One pot synthesis of compound 3.](image)

To a round bottom flask, 5-ASA (306 mg, 2 mmol) and sodium hydroxide (160 mg, 4 mmol) were dissolved in deionized water. While stirring, the mixture was placed in an ice bath to cool to 0 °C. After 20 minutes, acetyl chloride (170 µl, 2.4 mmol) was added dropwise, then the ice bath was removed, and the mixture continued to stir for one hour at room temperature (Figure 2.8).
In order to extract the N-acetyl-5-ASA, the mixture was treated with 1M aqueous HCl, to provide a final solution pH of 1, which resulted in the precipitation of the desired compound 3. The resulting product was then filtered, and left overnight to dry. The melting point of the product was obtained using a MEL-TEMP II apparatus by Laboratory Devices.

Yielded 290 mg (70%) as a white solid. Melting Point: 212-220 °C. (Figure 2.9) \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 9.83 (br s, 1 H), 8.03 (br s, 1 H), 7.59 (br d, \(J=8.5\) Hz, 1 H), 6.85 (br d, \(J=8.8\) Hz, 1 H), 1.96 (s, 3 H).

![Proton NMR assignment for compound 3.](image)

**Figure (2.9)** Proton NMR assignment for compound 3.

### 2.5.3 Emulsion Polymerization Procedure

\[
\text{Butyl acrylate} + \text{Styrene} \xrightarrow{\text{Sodium dodecyl sulfate, 3% w/v}} \text{Potassium persulfate, 1% w/v} \xrightarrow{\text{H}_2\text{O, 73°C}} \text{Compound 2 covalently bound to emulsified nanoparticle}
\]

**Figure (2.10)** Formation of nanoparticle emulsions using compound 2.
The polymerization procedure used for 5-ASA is similar to the procedure used in the Turos laboratory to prepare antibacterial nanoparticles.\(^5\) The polymerization procedure used 20 mg of compound 2 as a standard amount. Nitrogen was used to flush the air out of the reaction system. Through trial and error, the optimal oil bath temperature was found to be 73°C. Compound 2 was dissolved in a 1 ml of 7:3 v/v mixture of butyl acrylate and styrene, and stirred for 20 minutes. Then sodium dodecyl sulfate (12 weight %, 120 mg) was added along with 2 ml of deionized water, and this mixture was stirred for another 20 minutes. Finally, potassium persulfate (2.5 weight %, 25 mg) was added to the mixture along with 2 ml of deionized water. The mixture was then stirred for an additional 6 hours (Figure 2.10).

![Chemical Reaction Diagram](image)

**Figure (2.11)** Formation of nanoparticle emulsions using compound 3.

Due to difficulty in loading and successfully obtaining a stable nanoparticle emulsion using 3 weight % of sodium dodecyl sulfate, various other surfactants were examined, though none proved successful in forming a stable nanoparticle emulsion. The surfactants used were anionic (sodium dodecyl sulfate), cationic (dodecyl trimethylammonium chloride), zwitterionic (3-(N,N-
dimethylmyristylammonio)-propanesulfonate), and non-ionic (Triton x100). Figure 2.11 shows a similar scheme that was used for the encapsulation of compound 3.

2.5.3.1 Nanoparticle Emulsion Data

Table (2.1) Nanoparticle emulsions using 5-ASA derivative.

<table>
<thead>
<tr>
<th>Monomer</th>
<th>Surfactant Weight %</th>
<th>Surfactant Charge</th>
<th>Radical Initiator Weight %</th>
<th>Temp (°C)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mg Cpd. 2</td>
<td>1</td>
<td>Anionic</td>
<td>0.5</td>
<td>90</td>
<td>Clear, No emulsion</td>
</tr>
<tr>
<td>30 mg Cpd. 2</td>
<td>3</td>
<td>Anionic</td>
<td>1</td>
<td>90</td>
<td>Clear, No emulsion</td>
</tr>
<tr>
<td>30 mg Cpd. 2</td>
<td>3</td>
<td>Anionic</td>
<td>2.5</td>
<td>90</td>
<td>Clear, No emulsion</td>
</tr>
<tr>
<td>30 mg Cpd. 2</td>
<td>5</td>
<td>Anionic</td>
<td>2.5</td>
<td>90</td>
<td>Clear, No emulsion</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>10</td>
<td>Anionic</td>
<td>2.5</td>
<td>65</td>
<td>Unstable</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>3</td>
<td>Zwitterionic</td>
<td>2.5</td>
<td>65</td>
<td>Unstable</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>5</td>
<td>Zwitterionic</td>
<td>2.5</td>
<td>70</td>
<td>Unstable</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>7</td>
<td>Zwitterionic</td>
<td>2.5</td>
<td>70</td>
<td>Unstable</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>10</td>
<td>Zwitterionic</td>
<td>2.5</td>
<td>70</td>
<td>Destabilized later</td>
</tr>
<tr>
<td>30 mg Cpd. 2</td>
<td>10</td>
<td>Zwitterionic</td>
<td>2.5</td>
<td>70</td>
<td>Destabilized later</td>
</tr>
<tr>
<td>20 mg 5-ASA</td>
<td>12</td>
<td>Zwitterionic</td>
<td>2.5</td>
<td>73</td>
<td>Red</td>
</tr>
<tr>
<td>20 mg 5-ASA</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Red</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>12</td>
<td>Zwitterionic</td>
<td>2.5</td>
<td>73</td>
<td>Unstable</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>12</td>
<td>Non-ionic</td>
<td>2.5</td>
<td>73</td>
<td>Unstable</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>12</td>
<td>Non-ionic</td>
<td>2.5</td>
<td>73</td>
<td>Unstable</td>
</tr>
<tr>
<td>20 mg Salicylic acid</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>20 mg Cpd. 2</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>10 mg Cpd. 2</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>30 mg Cpd. 2</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>10 mg Cpd. 3</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>20 mg Cpd. 3</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>30 mg Cpd. 3</td>
<td>12</td>
<td>Anionic</td>
<td>2.5</td>
<td>73</td>
<td>Homogeneous</td>
</tr>
</tbody>
</table>

Table 2.1 contains data from all the polymerizations that were performed, excluding repetitions, and contaminated samples. The results show that the optimal temperature for forming an emulsion using these 5-ASA derivatives was 73°C, and required 12% of the anionic surfactant
(sodium dodecyl sulfate), along with 2.5% of potassium persulfate. The only surfactant that was able to produce a homogeneous emulsion was the anionic surfactant SDS. The pH of the emulsion was measured at 2.3. However, a unique emulsion was produced when we attempted to encapsulate the free 5-ASA. The emulsion turned nearly black (actually very dark red). It is hypothesized that it was possibly the free aromatic amine that was causing this. Each compound used in the polymerization was reacted separately with 5-ASA, and the radical initiator seemed to cause the undesired side reaction (formation of the red color). To test this, we attempted a polymerization using only salicylic acid which lacks the NH₂ moiety, and it produced a homogeneous mixture. This indicated that the radical initiator and free aromatic amine of 5-ASA could not be used together.

2.5.4 Purification of Nanoparticle Emulsions

Table (2.2) Dynamic light scattering data for crude vs purified emulsions.

<table>
<thead>
<tr>
<th>Emulsion (drug, surfactant, radical initiator)</th>
<th>Average Diameter</th>
<th>Average Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg Compound 2, 12%, 2.5%</td>
<td>41 nm ± 2.8</td>
<td>-52 mV ± 4.8</td>
</tr>
<tr>
<td>10 mg Compound 2, 12%, 2.5% (purified)</td>
<td>15.6 nm ± 0.9</td>
<td>-46 mV ± 3.6</td>
</tr>
<tr>
<td>20 mg Compound 2, 12%, 2.5%</td>
<td>56 nm ± 3.2</td>
<td>-44 mV ± 3.2</td>
</tr>
<tr>
<td>20 mg Compound 2, 12%, 2.5% (purified)</td>
<td>35 nm ± 2.5</td>
<td>-49 mV ± 3.5</td>
</tr>
<tr>
<td>30 mg Compound 2, 12%, 2.5%</td>
<td>82 nm ± 6.4</td>
<td>-35 mV ± 2.4</td>
</tr>
<tr>
<td>30 mg Compound 2, 12%, 2.5% (purified)</td>
<td>75 nm ± 5.9</td>
<td>-43 mV ± 3.1</td>
</tr>
<tr>
<td>No Drug, 12%, 2.5%</td>
<td>80 nm ± 7.0</td>
<td>-52 mV ± 3.3</td>
</tr>
<tr>
<td>No Drug, 12%, 2.5% (purified)</td>
<td>28 nm ± 2.1</td>
<td>-53 mV ± 4.9</td>
</tr>
</tbody>
</table>

The purification procedure for the resulting emulsions involved centrifugation of 1 ml of the emulsion in a microcentrifuge tube at (16.1k x g) for 30 minutes. A solid pellet formed at the bottom of the conical tube, the supernatant was removed with a pipette, and placed in a dialysis
membrane tube (50K dialysis membrane, Spectrum Laboratories Inc.-Spectra/Pro 1,2,3,4,5,6,7 Regenerated cellulose Dialysis Membrane). The supernatant was dialyzed in deionized water (200 ml) for 8 hours, replacing the water every two hours. After 8 hours the emulsion was transferred into another microcentrifuge tube, and centrifuged again at (16.1k x g) for another 30 minutes. The supernatant was removed by pipette and transferred into a vial.

Table 2.2 shows the different sizes produced depending on the amount of drug added during polymerization. There was a noticeable trend that the nanoparticle increased in size as the amount of drug loading was increased. In addition, it seems that the dialysis process shrank the nanoparticles to a smaller diameter. The non-drug loaded nanoparticles were larger than those containing 10 mg of compound 2. This could be due to some hydrophobic interaction that causes the nanoparticle to form within a tighter micelle. The purified emulsions were found to exhibit similar cytotoxicity when compared to the non-purified samples. In addition, it was uncertain if the drug encapsulated model retained the drug loaded within. Thus all biological studies were carried out using the non-purified emulsions.

2.6 Biological Studies: Cytotoxicity and Antibacterial Activity

To determine if the derivatives of 5-ASA or the nanoparticle emulsions containing the drug had any change in activity against common bacteria found in the human digestive system, minimum inhibitory concentration (MIC) values were determined, and compared. In addition, a cytotoxicity study was performed in order to assess the toxicity of the analogs, the nanoparticles, and the nanoparticle components towards human cells.
2.6.1 Antibacterial Testing of 5-ASA Derivatives and Nanoparticle Emulsions

To investigate whether the nanoparticles possess antibiotic capabilities, each crude emulsion was tested against methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) and *Escherichia coli* (K12) using a 96-well plate broth assay to determine the minimum inhibitory concentration (MIC).

The original stock emulsion was diluted using Trypticase Soy Broth (TSB) solution to an initial concentration of 2.56 mg/ml of the 5-ASA derivative, then serial diluted with TSB to half the concentration each time. A volume of 10 µl of each emulsion dilution was added to the next well in series, resulting in a final concentration run of 128 µg/ml to 0.012 µg/ml. The MIC was done in triplicates for each bacterium, with penicillin G being used as a positive control and a blank of deionized water as a negative control.

To prepare the bacteria for culture, all solutions were autoclaved prior to use. The bacteria were grown overnight at 37°C on an agar plate composed of BBL TSA II Trypticase Soy Agar (TSA) and BBL Trypticase Soy Broth (TSB) in a 1:2 ratio at 4.4% concentration. A broth solution of 2.4% TSB was inoculated using the bacteria from the agar plates, and was incubated at 37°C to reach a 0.5 McFarland standard. The bacteria was then further diluted by a factor of 1000 using a broth solution of 2.4% TSB, and 190 µl of the diluted bacterial solution was transferred by micropipette into each well. The inoculated plates were incubated at 37°C for 16-20 hours and the resulting plates were observed for growth and MIC values recorded. The MIC was the lowest concentration of the antibiotic that completely inhibited bacterial growth (visually) within that series of dilutions.
2.6.1.1 Antibacterial Activity Results

![MIC Values Chart](image)

**Figure (2.12)** MIC values for compounds tested against *E.coli* and MRSA.

Figure 2.12 shows the MIC values for the different compounds that are used in making the nanoparticle, and 5-ASA and two analogs. It is interesting to see that all the components are not that effective against MRSA or *E.coli* including the empty nanoparticle. However, when the nanoparticle contains compound 2 or compound 3, there was a significant increase in the activity against MRSA. Compound 2 and compound 3 (without nanoparticle loading) are active against *E.coli*, with MIC’s of 32 mg/ml, respectively, but not active against MRSA.

2.6.2 Eukaryotic Cell Model for Cytotoxicity Testing

Human colon cells (HT-29) were recovered from storage in liquid nitrogen. For the initial recovery the cells were thawed quickly, and then suspended in McCoy’s growth medium made with 10% fetal bovine serum and 1% gentamycin (antibiotic). Once suspended, the cells were placed into a cell culture flask, and incubated under an atmosphere of 5% CO₂. The medium was
changed approximately every two days, and the cells were grown to confluence in about 4-5 days. The cells were sub-cultured (a small amount of the cell were placed into a new flask to grow) when the cells reached 80% confluence in the original culture flask. The flask was taken out of the incubator, the medium was drawn off and discarded, the cells were rinsed with 1X dulbecoo’s phosphate buffer solution (DPBS) (2-3 ml), and then trypsin was administered to cells in order to detach them from the flask wall. The cells were then suspended in the growth medium, and one third of the solution was placed into a new flask. The cells were sub-cultured at a 1:3 ratio from the original amount. Cell counting was performed to determine the number of cells present in the flask in order to determine the number of cells needed for the testing. The cells were counted using a hemocytometer glass slide, by adding 50 µl of the cell solution into 100 µl of DPBS and 50 µl of trypan (blue dye), thus giving a dilution of 4x. Cells were counted on the grid and the cell count was calculated based on dilution and number of blocks counted, with each block on the grid representing a volume of 0.1 µl.

HT-29 cells were placed into each well of a 96-well plate for preparing the in vitro cytotoxicity assay. 10,000 cells in 150 µl volume of medium were placed into each well, and incubated overnight in the CO₂ incubator. Triplicate testing was setup for each compound or emulsion with up to 6 serial dilutions for each drug. The samples were first diluted in the medium in a sterile test tube, and then serial diluted halving the concentration each time. 50 µl of each concentration was added to the 150 µl of cells, thus diluting each well an additional 4x.

A blank was also placed on the plate containing only the growth medium, and another control was setup containing cells and the growth medium. The cells were then incubated for 48 hours.
After incubation, 20 µl of a 3.75 mg/ml solution of MTT was added into each well, and then placed back into the incubator for 4 hours. Once removed from the incubator, the medium was aspirated, and 100 µl of DMSO was added to each well to dissolve the purple crystal. The plates were shaken for 5 minutes prior to placing them into the plate reader. Results were read at 595 nm, and provided as an absorbance value, which was then calculated into % inhibition by the formula (control absorption-sample absorption)/control absorption x 100%.

### 2.6.2.1 Cytotoxicity Assay Results

![Cell Inhibition Assay](image)

**Figure (2.13)** Cytotoxicity of compound 2 and compound 3.
**Figure (2.14)** Cytotoxicity of surfactant, sodium dodecyl sulfate.

**Figure (2.15)** Cytotoxicity assay of compound 2 and compound 3 loaded nanoparticle emulsions.
Figures 2.13 displays the cytotoxicity results performed for compound 2, and compound 3 at IC$_{50}$ of 750 µg/ml, and the corresponding nanoparticle emulsions. It is evident that 5-ASA derivatives exhibit low cytotoxicity to the cells. Figure 2.14 shows that the surfactant however is extremely toxic with an IC$_{50}$ of 20 µg/ml, which is probably due to destabilization of the cell membrane. Likewise, as Figure 2.15 indicates, all of the nanoparticle emulsions are toxic at an IC$_{50}$ of 25 µg/ml. As a result, both drug loaded nanoparticles exhibited high levels of cytotoxicity, deeming the emulsions unusable for anti-inflammation models.

2.7 Possible Model for Testing Inflammation (Future Studies)

As a result of the observed cytotoxicity, we were unable to carry out the anti-inflammatory nanoparticle emulsions further for biological applications. However, they would remain viable if other surfactants or other polyacrylate based nanoparticle emulsions are able to load 5-ASA derivatives without appreciable cytotoxicity. Then using the activation of PPAR-$\gamma$ we could test for the effectiveness of 5-ASA and its derivatives. This could be done with HT-29 cells transiently transfected using the Effectene transfection reagent (QIAGEN). To test PPAR-$\gamma$ activation, we could perform transfections with 500 ng of a minimal promoter construct containing two copies of PPRE obtained from the cytochrome p450 4A (2XCYP). The renilla luciferase plasmid (0.1 µg/well) would also be transfected as an internal control for monitoring transfection efficiency and for normalizing the firefly luciferase activity. Transfected cells would be incubated for 48 hours at 37°C. Stimulations would be performed for 6 hours with 5-ASA (30 mM). Total cell extracts would be prepared using the Passive Lysis Buffer (Promega). Luciferase activity would be assayed using Promega's Dual Luciferase assay system.
2.8 Scanning Electron Microscope Imaging of Nanoparticle Emulsions

HT-29 cells were grown on coverslips for this experiment. A specially coated thermonax coverslip was placed into each well of a 24 well plate. The cells were grown in each well using a similar procedure to the cytotoxicity procedure described previously. The nanoparticle emulsion was then administered 24 hours prior to the affixation procedure (dehydration of cells onto the coverslip). The plate was then removed from the incubator and the medium was aspirated. Care was taken to ensure that the cells remained on the coverslip in the wells.

The cells must be dehydrated and affixed for SEM analysis. A solution of 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer was added to each well and left for at least 1 hour. Then the sample was rinsed several times with PBS for 15 minutes, followed by addition of 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hour. Another rinse was done for 15 minutes with PBS, followed by dehydration using ethanol solutions (70% for 15 minutes, 95% for 15 minutes, 3 changes of 100% for 10 minutes each), followed by 2:1 ethanol/hexamethyldisilazane (HMDS) for 20 minutes, and then 1:2 ethanol/HMDS for 20 minutes, followed by two changes of 100% HMDS for 15 minutes each.

The sample was left in the hood to air dry overnight. The cover slip was then removed and mounted on the base, and sputter coated with gold-palladium for scanning electron microscopic imaging.
2.8.3 Scanning Electron Microscope (SEM) Imaging

**Figure (2.16)** Scanning electron microscope image of HT-29 cells.

**Figure (2.17)** Scanning electron microscope image of nanoparticle emulsion (compound 2 loaded).
Figure (2.18) Scanning electron microscope image of HT-29 cells treated with nanoparticle emulsions (compound 2 loaded).
Figure 2.16 shows the HT-29 cells look confluent and healthy. In Figure 2.17 the SEM image shows a clustering of nanoparticles. No image could be formed where the nanoparticles were not clustered or separated from each other. This was most likely due to the dehydration process causing the nanoparticles to form clusters.

Figure 2.18 shows that the cells after 24 hours of nanoparticle administration, and the cells were beginning to detach and become round. There seems to be a lot of cell components and other micelles in the background. The images provide an example of the nanoparticle emulsion causing inhibited cell growth. It is somewhat hard to see a clear image differentiating between the cell and the nanoparticle. So attempts to find an image where the nanoparticle was interacting with the human cell were not successful.

**2.10 Future Studies**

What was learned from these experiments with 5-ASA and its derivatives was that the poly(styrene-butyl acrylate) nanoparticle emulsions have a limitation in loading these particular bioactive compounds. As such an increased amount of surfactant is required in order to stabilize the micelles sufficiently to form stable polyacrylate polymers that can load the bioactive compound. However, the increase in surfactant quantities also increases the cytotoxicity towards human cells and as a result deem some bioactive compounds unusable as drug-loaded nanoparticle emulsions. Though previous research demonstrated that purification methods post-emulsification help reduce the cytotoxicity, in this case the cytotoxic levels of the nanoparticle emulsions were too great to observe any useful reduction in cytotoxicity.
Additional work may be done to find alternate polyacrylate emulsion models that allow for an increase in loading of 5-ASA analogs, and more importantly, reduce cytotoxicity of the nanoparticle emulsion. This would permit in vitro testing against anaerobic bacteria, and eukaryotic cell inflammation models. In chapter 3, studies on a new homo(menthyl acrylate) polymer nanoparticle emulsion are presented, that provide an increase in loading of compounds, while greatly reducing cytotoxicity.
CHAPTER THREE

POLYACRYLATE NANOPARTICLE EMULSIONS: USE OF MENTHYL ACRYLATE MONOMERS TO FORM CHIRAL DRUG LOADING HOMOPOLYMER NANOPARTICLE EMULSIONS

3.1 Introduction

To further advance our laboratory’s work on polyacrylate nanoparticle emulsions, the next objective was to test the possibility of replacing one of the monomers in order to introduce a chiral component to the polyacrylate nanoparticle framework. With polyacrylate nanoparticle emulsions consisting of butyl acrylate/styrene or methyl methacrylate/styrene mixtures, it was most feasible to replace the acrylate ester monomer in order to easily introduce a chiral component to the polyacrylate. There were no commercially available enantiopure chiral acrylate esters that would be cost effective and readily available in large quantities. Thus, menthol was chosen as a cheap chiral alcohol that could be converted into the acrylate ester. Both the naturally occurring L-menthol and the enantiomer D-menthol in Figure 3.1 are available commercially.
3.2 Synthesis of Menthyl Acrylate

The menthol alcohol was converted to the acrylate ester via the addition of acryloyl chloride in the presence of a base. The first goal was to try to replace the butyl acrylate monomer with the menthyl acrylate monomer to prepare polyacrylate nanoparticle emulsions having a ratio of 7:3 of the acrylate ester monomer:styrene monomer. In addition, experiments were done to alter this initial ratio to determine the optimal combination of monomers needed to form stable polyacrylate nanoparticle emulsions.

![Figure (3.2) Scheme for the synthesis of L-menthyl acrylate (L-MtA).](image)
Figure (3.3) Scheme for the synthesis of D-menthyl acrylate (D-MtA).

Figure 3.2 and Figure 3.3 show the synthetic scheme for L-menthyl acrylate and D-menthyl acrylate, respectively, and follow as such: To a round bottom flask was added 250 ml of ethyl acetate, then 5.0 g (3.2 mmol) of the desired antipode of menthol and 8.9 ml (6.4 mmol) of trimethylamine were introduced. The mixture was left stirring at 0°C for 1 hour then 3.1 ml (3.8 mmol) of acryloyl chloride was added dropwise. The ice bath was removed and the reaction was left stirring overnight.

The workup of the reaction was performed by washing once with 100 ml of deionized water, three times with 80 ml of saturated ammonium chloride solution, three times with 80 ml of saturated sodium bicarbonate solution, and once with brine. The organic layers was then dried with anhydrous sodium sulfate. The ethyl acetate was evaporated, and the resulting crude product was purified via flash column chromatography using hexane as the solvent. Hexane was evaporated, and the resulting product was obtained.
**L-menthyl acrylate (L-MtA):** Yielded 4.6 ml (4.8 g, 72%) as a clear liquid.

\[ \alpha^D_{20} = -83.1; \text{ } ^1H \text{ NMR (400 MHz, CDCl}_3\text{)} \delta 6.37 (dd, J=17.3, 1.3 Hz, 1 H), 6.09 (dd, J=17.3, 10.4 Hz, 1 H), 5.78 (dd, J=10.4, 1.3 Hz, 1 H), 4.75 (td, J=10.9, 4.4 Hz, 1 H), 2.00 (m, 1 H), 1.86 (m, 1 H), 1.69 (br d, J=2.6 Hz, 1 H), 1.66 (m, 1 H), 1.49 (m, 1 H), 1.40 (m, 1 H), 1.06 (m, 1 H), 0.99 (m, 1 H), 0.89 (m, 1 H), 0.89 (d, J=6.9 Hz, 6 H), 0.76 (d, J=6.9 Hz, 3 H).

**D-menthyl acrylate (D-MtA):** Yielded 4.9 ml (5.1 g, 76%) as a clear liquid.

\[ \alpha^D_{20} = +83.0; \text{ } ^1H \text{ NMR (400 MHz, CDCl}_3\text{)} \delta 6.36 (dd, J=17.3, 1.4 Hz, 1 H), 6.09 (dd, J=17.3, 10.4 Hz, 1 H), 5.77 (dd, J=10.4, 1.4 Hz, 1 H), 4.74 (td, J=10.9, 4.4 Hz, 1 H), 2.00 (m, 1 H), 1.85 (m, 1 H), 1.68 (br d, J=2.7 Hz, 1 H), 1.64 (m, 1 H), 1.48 (m, 1 H), 1.39 (m, 1 H), 1.05 (m, 1 H), 0.98 (m, 1 H), 0.89 (m, 1 H), 0.87 (d, J=6.9 Hz, 6 H), 0.74 (d, J=6.9 Hz, 3 H).

3.3 Preparation of Polyacrylate Nanoparticle Emulsion Using Menthyl Acrylate and Styrene

![Figure (3.4)](image)

**Figure (3.4)** Scheme for the formation of polyacrylate nanoparticle emulsion using L-menthyl acrylate and styrene as monomers.
The general procedure for the formation of the polyacrylate nanoparticle emulsions is highlighted in Figure 3.4 and Figure 3.5. This procedure was very similar to previous protocols that were developed for other acrylate ester and styrene monomer combinations within our lab. A self-regulating oil bath was used to maintain a temperature of 78°C, the mixture of styrene and menthyl acrylate totaling 1 ml were added to a round bottom flask. The mixture was stirred using a 1.25 cm (300 mg) Teflon-coated magnetic stir bar at 380 rpm on a Corning PC-420D magnetic stirrer. After 30 minutes, 30 mg of sodium dodecyl sulfate and 2 ml of deionized water were added, and the stirring speed was increased to 500 rpm. Following 30 minutes, 5 mg of potassium persulfate and 2 ml of deionized water were added and the stirring speed was increased to 750 rpm. The resulting emulsion was then decanted into a collection vial after 6 hours.

The newly formed nanoparticle emulsions contain a chiral component (menthol) attached to the polyacrylate chain. The backbone of the polyacrylate nanoparticle presumably remains racemic.
due to the radical mechanism of its formation. Various dilutions were carried out to investigate the effect of monomer ratios on stability and physical properties of the resulting nanoparticle emulsions.

3.4 Dynamic Light Scattering Analysis of Size and Zeta Potential of Emulsions

Dynamic light scattering measurements were used to determine the size of the nanoparticles within the emulsions. The average size and surface charge of the emulsion was analyzed using a Malvern Zetasizer nano-ZS instrument. To prepare the samples for the analyses, the freshly-prepared emulsion was subjected to centrifugation at 10,000 rpm for 5 minutes using an Eppendorf Centrifuge 5424. An aliquot of the liquid emulsion was then drawn and deposited into a Malvern disposable folded capillary cell DTS-1070. Each sample was analyzed in triplicate, and each data collection consisted of 1 run of 20 scans (for size analysis) and 3 runs of 100 scans (for zeta potential determination).

3.4.1 Size and Zeta Potential Results Using Dynamic Light Scattering Instrument

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Size (nm)</th>
<th>Average Zeta Potential (-mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-MtA 10%</td>
<td>73.9 ± 4.3</td>
<td>56 ± 2.9</td>
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<tr>
<td>L-MtA 20%</td>
<td>69.0 ± 3.9</td>
<td>53 ± 2.6</td>
</tr>
<tr>
<td>L-MtA 30%</td>
<td>62.2 ± 3.3</td>
<td>60 ± 4.0</td>
</tr>
<tr>
<td>L-MtA 40%</td>
<td>99.0 ± 6.2</td>
<td>56 ± 4.1</td>
</tr>
<tr>
<td>L-MtA 50%</td>
<td>60.4 ± 5.3</td>
<td>56 ± 2.3</td>
</tr>
<tr>
<td>L-MtA 60%</td>
<td>56.2 ± 5.1</td>
<td>53 ± 3.6</td>
</tr>
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Table (3.1) (Continued)

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<tr>
<th>Sample</th>
<th>Average Size (nm)</th>
<th>Average Zeta Potential (-mV)</th>
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</thead>
<tbody>
<tr>
<td>L-MtA 70%</td>
<td>58.2 ± 3.2</td>
<td>61 ± 3.8</td>
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<tr>
<td>L-MtA 80%</td>
<td>52.8 ± 3.0</td>
<td>45 ± 3.3</td>
</tr>
<tr>
<td>L-MtA 90%</td>
<td>82.4 ± 2.9</td>
<td>59 ± 3.0</td>
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<tr>
<td>L-MtA 100%</td>
<td>79.9 ± 4.2</td>
<td>56 ± 2.8</td>
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Table (3.2) Size and zeta potential for emulsions using D-menthyl acrylate and styrene.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Size (nm)</th>
<th>Average Zeta Potential (-mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-MtA 10%</td>
<td>63.5 ± 4.1</td>
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<td>D-MtA 20%</td>
<td>58.0 ± 3.8</td>
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<td>D-MtA 30%</td>
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<td>D-MtA 50%</td>
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<td>58.4 ± 3.1</td>
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<td>D-MtA 90%</td>
<td>54.0 ± 2.9</td>
<td>41 ± 3.8</td>
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<tr>
<td>D-MtA 100%</td>
<td>58.4 ± 3.1</td>
<td>51 ± 3.9</td>
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Table (3.3) Size and zeta potential for emulsions using racemic menthyl acrylate and styrene.

<table>
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<th>Sample</th>
<th>Average Size (nm)</th>
<th>Average Zeta Potential (-mV)</th>
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<tbody>
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<td>64.1 ± 2.2</td>
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<td>Rac -MtA 50%</td>
<td>66.1 ± 2.3</td>
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<td>Rac -MtA 60%</td>
<td>51.6 ± 3.1</td>
<td>34 ± 2.6</td>
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<td>Rac -MtA 70%</td>
<td>63.3 ± 2.8</td>
<td>43 ± 3.5</td>
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</table>
Table (3.3) (Continued)

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<thead>
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<th>Rac -MtA 80%</th>
<th>70.2 ± 2.4</th>
<th>42 ± 3.1</th>
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<td>Rac -MtA 90%</td>
<td>55.6 ± 2.3</td>
<td>53 ± 2.8</td>
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<tr>
<td>Rac -MtA 100%</td>
<td>57.8 ± 2.1</td>
<td>46 ± 3.9</td>
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</table>

Table 3.1-3.3 display the average size of the nanoparticles contained within the final emulsions. Table 3.1 is for emulsions formed from of L-menthyl acrylate and styrene with the percentage shown is related to the amount of L-menthyl acrylate within the organic component of the emulsion. For example, L-MtA 20% represents 20% L-menthyl acrylate and 80% styrene by weight composition of the total organic components. Table 3.2 is for emulsions formed via the use of D-menthyl acrylate. Table 3.3 is for emulsions formed using a 50/50 mixture of L-menthyl acrylate and D-menthyl acrylate.

Surprisingly, all combinations formed very stable emulsions as determined by the zeta potential values all being larger than -30 mV. In addition, all polyacrylate particles measured within the 35-67 nm range, with no specific trend. As a result, it was possible to form a stable nanoparticle emulsion using any mixture of menthyl acrylate and styrene as co-monomers up to 20% total weight of the aqueous emulsion (80% of the weight being water).

3.5 Optical Rotation of Menthyl Acrylate and Styrene Based Nanoparticle Emulsions

Using enantiopure menthol alcohols, we were able to produce enantiopure forms of the menthyl acrylates and incorporate them into the polyacrylate backbone of the nanoparticle emulsions. In order to observe if these new chiral polymer had any unique optical properties we analyzed their optical activity using an AUTOPOL IV automatic polarimeter made by Rudolph Research.
Analytical. In order to obtain this data, all samples were diluted by a factor of 1:200 in deionized water and measured at 20°C.

### 3.5.1 Optical Activity Results of Menthyl Acrylate and Styrene Based Nanoparticle Emulsions

#### Table (3.4) Optical rotation vs menthyl acrylate values for menthyl acrylate/styrene nanoparticle emulsions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Optical Rotation $\left[\alpha\right]_{D}^{20}$</th>
<th>Sample</th>
<th>Optical Rotation $\left[\alpha\right]_{D}^{20}$</th>
<th>Sample</th>
<th>Optical Rotation $\left[\alpha\right]_{D}^{20}$</th>
</tr>
</thead>
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<tr>
<td>D-MtA 10%</td>
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<td>Rac-MtA 10%</td>
<td>0.0</td>
<td>L-MtA 10%</td>
<td>-1.8</td>
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<td>D-MtA 20%</td>
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<td>Rac-MtA 20%</td>
<td>0.0</td>
<td>L-MtA 20%</td>
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<td>D-MtA 30%</td>
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<td>D-MtA 80%</td>
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<td>L-MtA 80%</td>
<td>-8.8</td>
</tr>
<tr>
<td>D-MtA 90%</td>
<td>+9.6</td>
<td>Rac-MtA 90%</td>
<td>0.0</td>
<td>L-MtA 90%</td>
<td>-9.8</td>
</tr>
<tr>
<td>D-MtA 100%</td>
<td>+10.8</td>
<td>Rac-MtA 100%</td>
<td>0.0</td>
<td>L-MtA 100%</td>
<td>-11.0</td>
</tr>
</tbody>
</table>
In order to better determine if any trend exists within the optical activity results, the data in Table 3.4 was converted to the graph in Figure 3.6. Both enantiomeric forms of the acrylate ester in the menthyl acrylate/styrene polyacrylate nanoparticles follow a similar pattern. Though the overall sinusoidal pattern is yet to be understood, it does seem that there is a consistency, and that the optical activity increased as the concentration of menthyl acrylate was also increased.

![Optical Rotation of MtA-St Nanoparticles](image)

**Figure (3.6)** Optical rotation vs for menthyl acrylate/styrene nanoparticle emulsions.

### 3.6 Discussion of Menthyl Acrylate and Styrene Based Polyacrylate Nanoparticles

Experiments to prepare stable emulsions of menthyl acrylate containing nanoparticles were successful. Most surprisingly was the ability to form stable nanoparticle emulsions using only the menthyl acrylate as the monomer. This provided the first case of being able to form a homo polymer using the nanoparticle emulsion technique researched in our laboratory. By eliminating the need for the co-monomer, and the ability to use chiral menthyl acrylate monomers to constitute 100% of the polymer within the nanoparticle framework, highly chiral environments
could be created within the emulsion. The next goal was to investigate if the menthyl acrylate emulsions could produce similar drug delivery properties compared to those observed in the butyl acrylate/styrene polyacrylate systems.

3.7 Studies on the Preparation and Properties of Poly(Menthyl Acrylate) Nanoparticles

The drug loading capabilities of the menthol-based nanoparticles was investigated using penicillin G as a test antibiotic. A central question was whether these new nanoparticles would allow for an increase in loading of the bioactive compound (in this case penicillin G). Previous nanoparticle emulsion typically allowed for a 3% loading of antibiotic by weight of the organic component of the emulsion, resulting in a maximum final concentration of 0.6% of the bioactive compound.

Figure (3.7) The emulsified polyacrylate nanoparticle protecting the loaded antibiotic against degradative enzymes.

A second question was if the newly formed polyacrylate nanoparticle emulsion would show antibacterial activity against Staphylococcus aureus (SA) (ATCC 25923) and Methicillin-
resistant Staphylococcus aureus (MRSA) (ATCC 43300). This would confirm that the	nanoparticle emulsion is able to carry and protect penicillin G from enzymatic degradation by
penicillinase, while being able to release the drug to inhibit the growth of the bacteria as depicted
in Figure 3.7.

3.7.1 Encapsulation of Penicillin G into Poly(Methyl Acrylate) Nanoparticle Emulsion

![Chemical Reaction Diagram]

Figure (3.8) Encapsulation of penicillin G into the poly(methyl acrylate) nanoparticle
emulsions.

The general procedure for the encapsulation of penicillin G into the poly(methyl acrylate)
nanoparticle emulsions is highlighted in Figure 3.8. A self-regulating oil bath was used to
maintain a temperature of 78 °C, menthyl acrylate (L or D) totaling 1 ml was added to a round
bottom flask. In order to keep the polymer composition similar for all drug loading
encapsulation, penicillin G was added as an additional % component and was not compensated
by reduction of monomer or surfactant. Penicillin G was added at concentrations of +1% to
+20%. The mixture was stirred using a 1.25 cm (300 mg) Teflon-coated magnetic stir bar at 380
rpm on a Corning PC-420D magnetic stirrer. After 30 minutes, 30 mg of sodium dodecyl sulfate
and 2 ml of deionized water were added, and the stirring speed was increased to 500 rpm. After
30 minutes, 5 mg of potassium persulfate and 2 ml of deionized water were added and the stirring speed was increased to 750 rpm. The resulting emulsion was then decanted into a collection vial after 6 hours.

3.7.1.1 Dynamic Light Scattering Analysis of Size and Zeta Potential of Emulsions

Dynamic light scattering measurements were used to determine the size of the nanoparticles within the emulsions. The average size and surface charge of the emulsion was analyzed using a Malvern Zetasizer nano-ZS instrument. To prepare the samples for the analyses, the freshly-prepared emulsion was subjected to centrifugation at 10,000 rpm for 5 min using an Eppendorf Centrifuge 5424. An aliquot of the liquid emulsion was then drawn and deposited into a Malvern disposable folded capillary cell DTS-1070. Each sample was analyzed in triplicate, and each data collection consisted of 1 run of 20 scans (for size analysis) and 3 runs of 100 scans (for zeta potential determination).

![Average Size of Nanoparticle Emulsion](image)

**Figure (3.9)** Average size of nanoparticle emulsions with increasing penicillin G loading of both L-menthyl acrylate polymers and D-menthyl acrylate polymers.
Figure (3.10) Average zeta potential for nanoparticle emulsions with increasing penicillin G loading of both L-menthyl acrylate polymers and D-menthyl acrylate polymers.

Figure 3.9 shows that both (L-menthyl acrylate and D-menthyl acrylate) homopolymer nanoparticle systems were able to achieve a significantly higher drug loading compared to the copolymer system of butyl acrylate/styrene. The poly(menthyl acrylate) emulsions can contain up to 20% penicillin G (by weight of organics), compared to only 3% for the previous butyl acrylate/styrene systems. The increased loading capability is likely indicative of additional stability within the matrix of the nanoparticles, which would deserve further exploration.

Figure 3.10 confirms the stabilities of both L-menthyl acrylate and D-menthyl acrylate polymer nanoparticle emulsions while encapsulating penicillin G, with zeta potential values above -30 mV.
3.7.2 In vitro Antibacterial Testing of Poly(Menthyl Acrylate) Nanoparticle Emulsions

To investigate whether the nanoparticles possess antibiotic capabilities, each crude emulsion was tested against *Staphylococcus aureus* (SA) (ATCC 25923) and methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) using a 96-well plate broth assay to determine the minimum inhibitory concentration (MIC).

The original stock emulsion was diluted using Trypticase Soy Broth (TBS) solution to an initial concentration of 1.28 mg/ml of the penicillin G within the emulsion, then serial diluted with TSB to half the concentration each time. A volume of 10 µl of each emulsion dilution was added to a well in series, resulting in a final concentration run of 64 µg/ml to 0.012 µg/ml. The MIC was done in triplicates for each bacterium, with penicillin G being used as a positive control and a blank of water as a negative control.

To prepare the bacteria for culture, all solutions were autoclaved prior to use. The bacteria were grown overnight at 37°C on an agar plate composed of BBL TSA II Trypticase Soy Agar (TSA) and BBL Trypticase Soy Broth (TSB) in a 1:2 ratio at 4.4% concentration. A broth solution of 2.4% TSB was inoculated using the bacteria from the agar plates, and was incubated at 37°C to reach a 0.5 McFarland standard. The bacteria was then further diluted by a factor of 1000 using a broth solution of 2.4% TSB, and 190 µl of the diluted bacterial solution was transferred by micropipette into each well. The inoculated plates were incubated at 37°C for 16-20 hours and the resulting plates were observed for growth and MIC values recorded. The MIC was the lowest concentration of the antibiotic that completely inhibited bacterial growth (visually) within that series of dilutions. Additionally, in order to confirm that the nanoparticle emulsions are able to
protect the drug from enzymatic degradation, a Kirby-Bauer assay was performed with the addition of penicillinase protein (Sigma Aldrich). One plate was tested with the penicillin G-loaded nanoparticle emulsion and penicillin G against *S. aureus*, while another plate was set up with the addition of penicillinase (100 µg) into the agar media.

**3.7.2.1 In vitro Antibacterial Testing Results**

The results obtained from the antibacterial assays demonstrated that the poly(menthyl acrylate) nanoparticle emulsions do not possess any antibacterial properties without a bioactive compound loaded into the nanoparticles. In addition, the encapsulated penicillin G nanoparticles had a MIC value of 32 µg/ml. Though it was lower antibacterial activity compared to the free penicillin G control, this result was as expected, because previous penicillin G encapsulated nanoparticles demonstrated a decrease in bioactivity also (Table 3.5).

The most important results of this assay were the MIC values for penicillin G-loaded poly(menthyl acrylate) nanoparticles when comparing activity against SA vs MRSA. The penicillin G loaded nanoparticles exhibited the same activity against both non-resistant and resistant forms of the microbe, with MIC’s of 32 mg/ml. This suggests that the nanoparticle emulsion protects penicillin from the degradative enzyme (penicillinase) that is produced by MRSA, or that is added to the culture media. In addition, the Kirby-Bauer test provided a 35 mm zone of inhibition for penicillin G against *S. aureus* without penicillinase added and a 1 mm zone of inhibition in the presence of penicillinase, while the penicillin G-loaded nanoparticles exhibited a zone of inhibition of 25 mm for both the penicillinase-free plate and that containing
added penicillinase. This further confirms the ability of the nanoparticle emulsion to protect the penicillin loaded within the nanoparticles, without reducing its bioactivity.

**Table (3.5)** MIC values of penicillin G-loaded poly(menthyl acrylate) nanoparticle emulsions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC for SA (ATCC 25923)</th>
<th>MIC for MRSA (ATCC 43300)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug D-MtA nanoparticle</td>
<td>&gt;256 µg/ml</td>
<td>&gt;256 µg/ml</td>
</tr>
<tr>
<td>No drug L-MtA nanoparticle</td>
<td>&gt;256 µg/ml</td>
<td>&gt;256 µg/ml</td>
</tr>
<tr>
<td>Pen G encapsulated D-MtA</td>
<td>32 µg/ml</td>
<td>32 µg/ml</td>
</tr>
<tr>
<td>Pen G encapsulated L-MtA</td>
<td>32 µg/ml</td>
<td>32 µg/ml</td>
</tr>
<tr>
<td>Pen G</td>
<td>0.25 µg/ml</td>
<td>16 µg/ml</td>
</tr>
</tbody>
</table>

**3.8 In vitro Cytotoxicity Studies for Poly(Menthyl Acrylate) Nanoparticle Emulsions**

In vitro cell cytotoxicity of the menthyl acrylate nanoparticle emulsion was tested on two human cell lines, human colorectal carcinoma cells HCT-116 cells, and human embryonic kidney cells HEK 293. HCT-116 cells were grown in Dulbecco’s Minimum Essential Medium (DMEM) with 10% fetal bovine serum and 0.1% penicillin/streptomycin as complete growth medium for several days at 37°C under an atmosphere of 5% CO₂ to reach confluence. HEK 293 cells were grown in Eagle Minimum Essential Medium (E MEM) with 10% fetal bovine serum and 0.1% penicillin/streptomycin as complete growth medium for several days at 37°C under an atmosphere of 5% CO₂ to reach confluence. Each cell type was then plated onto 96-well plates, at 50,000 cells per well at a volume of 150 µl with the respective complete growth medium. The
cells were counted using a hemocytometer and then incubated for 24 hours at 37°C under an atmosphere of 5% CO₂.

The test emulsion was diluted using the complete growth medium for each cell type, and added into the wells of each test plate to give a final concentration of penicillin G (loaded within the nanoparticle emulsions) of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml within a series. The testing was done in triplicate and one well in each triplicate was left untreated as the negative control for 100% growth.

The plates were further incubated and monitored for 48 hours at 37°C under an atmosphere of 5% CO₂. A 5 mg/ml solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) in sterile phosphate-buffered saline (PBS) was added to give a 10% final concentration in each well. The plates were then further incubated for 4 hours at 37°C under an atmosphere of 5% CO₂ to allow for the formation of the purple crystals of 1-(4,5-dimethylthiazol)2-yl)-3,5-diphenylformazan. The liquid was then aspirated from each well and 100 µl of dimethylsulfoxide (DMSO) was added to each well, and gently shaken for 1 min to allow for complete dissolution of the crystals. The IC₅₀ value for the assay was determined using a BioTek Synergy H1 hybrid plate reader at both 595 nm and 630 nm. The IC₅₀ was determined as the well with at least 50% cell viability compared to the untreated control cell with 100% cell growth.
### 3.8.1 Cytotoxicity Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC\textsubscript{50} for HCT-116</th>
<th>IC\textsubscript{50} for HEK-293</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug D-MtA nanoparticle</td>
<td>&gt;2 mg/ml</td>
<td>&gt;2 mg/ml</td>
</tr>
<tr>
<td>No drug L-MtA nanoparticle</td>
<td>&gt;2 mg/ml</td>
<td>&gt;2 mg/ml</td>
</tr>
<tr>
<td>Pen G encapsulated D-MtA</td>
<td>&gt;2 mg/ml</td>
<td>&gt;2 mg/ml</td>
</tr>
<tr>
<td>Pen G encapsulated L-MtA</td>
<td>&gt;2 mg/ml</td>
<td>&gt;2 mg/ml</td>
</tr>
</tbody>
</table>

The cytotoxicity assay results indicated that both the penicillin G loaded nanoparticle and the non-drug loaded nanoparticle had very low cytotoxicity against both cancerous and non-cancerous human cell lines. Results in Table 3.6 demonstrated that the new homopolymer nanoparticle emulsions would be viable for drug delivery as it may be relatively safe to administer in therapeutic doses.

### 3.9 Imaging the Poly(Menthyl Acrylate) Nanoparticle Emulsion Using Scanning Electron Microscope (SEM)

A sample of poly(menthyl acryloyl) nanoparticle emulsion was prepared for imaging using scanning electron microscopy. The samples were initially prepared by lyophilization of the emulsion, which resulted in a dry powder that could be added to the sample holder for the scanning electron microscope instrument. The samples were placed onto an aluminum-coated sample holding tape, mounted onto a copper tape, placed onto the scanning electron microscope sample holder. The initial resulting image was very difficult to discern and it was most likely due
to the non-conductive nature of the material, causing the electrons to build up on the surface of the material that distorted in the resulting image.

To try to resolve the issue, the sample was diluted 400X with deionized H$_2$O and a drop of the diluted emulsion was placed on the conductive aluminum coated sample holding tape. The sample was placed in the -80°C freeze for a few hours, then immediately lyophilized to dry the sample directly onto the sample holding tape to produce an even distribution of the material. In addition, the sample containing tape was sputter-coated with gold-palladium coating in order to increase the conductivity of the resulting sample, thus preventing or reducing the accumulation of electrons on the surface of the sample, and thus reducing distortions.

![Image](image_url)

**Figure (3.11)** Scanning electron microscope image of a cluster of nanoparticle spheres.

Figure 3.11 demonstrated that the clumping of nanoparticles occurs due to the dehydration process of the sample preparation, producing an individual sphere within the emulsion. The imaged trails appear to be artifacts of the drying process as the nanoparticles clustered.
Poly(menthyl acrylate) nanoparticles exhibited a greater insulating property than previous polyacrylate nanoparticles, and as a result they are rather difficult to image at high resolution. The samples were prepared on an aluminum-coated surface, and the samples were coated with a layer of palladium-gold to allow for better resolution. The images remained distorted, and thus the exact size determination of individual spheres was not possible (Figure 3.12).

Small improvements in the resulting images were observed when the sample was more dilute, and when the sample was directly prepared onto the samples holder rather than separately lyophilizing the sample, then loading onto the SEM sample holder. In addition, increasing the exposure time of the sample to palladium-gold plating may increase the conductivity of the sample to provide a better image.
3.10 5-ASA Derivatives Revisited

Figure (3.13) Preparation of covalently-bound compound 2 poly(L-menthyl acrylate) nanoparticle emulsions.

Figure (3.14) Preparation of encapsulated compound 3 poly(L-menthyl acrylate) nanoparticle emulsions.
5-ASA derivatives that were synthesized in chapter 2 were revisited in the hope of loading them into a nanoparticle emulsion with decreased cytotoxicity. Having observed the increase in drug loading capacity of the poly(menthyl acrylate) nanoparticle emulsions along with a decrease in cell cytotoxicity provided a new hopeful procedure for loading 5-ASA derivatives while reducing the cell cytotoxicity, which was one of the key issues preventing the application of 5-ASA loaded nanoparticle emulsions. Preliminary results were obtained by using a procedure for loading penicillin G into poly(menthyl acrylate) nanoparticles.

The general procedure for the loading of 5-ASA derivative into the poly(menthyl acrylate) nanoparticle emulsions is highlighted in Figure 3.13 for N-acryloyl-5-aminosalicylic acid and Figure 3.14 for N-acetyl-5-aminosalicylic acid. A self-regulating oil bath was used to maintain a temperature of 78°C, 1 ml of L-menthyl acrylate totaling 1 ml was added to a round bottom flask. In order to keep the polymer composition similar for all drug loading encapsulation, penicillin G was added as an additional % component and was not compensated by reduction of monomer or surfactant. Each 5-ASA derivative was added at 3% by weight (30 mg). The mixture was stirred using a 1.25 cm (300 mg) Teflon-coated magnetic stir bar at 380 rpm on a Corning PC-420D magnetic stirrer. After 30 minutes, 30 mg of sodium dodecyl sulfate and 2 ml of deionized water were added, and the stirring speed was increased to 500 rpm. After 30 minutes, 5 mg of potassium persulfate and 2 ml of deionized water were added and the stirring speed was increased to 750 rpm. The resulting emulsion was then decanted into a collection vial after 6 hours.
The covalently-bound N-acryloyl-5-ASA in poly(L-menthyl acrylate) nanoparticles measured an average diameter of 49 (± 1.9) nm and had a zeta potential of -55 (± 5.1) mV. The encapsulated N-acetyl-5-ASA in poly(L-menthyl acrylate) nanoparticles measured an average diameter of 72 (± 1.7) nm and had a zeta potential of -47 (± 3.6) mV.

Antibacterial testing was performed using the same method as previously reported in Section 2.6.1. The antibacterial results obtained were similar to those previously reported with 5-ASA derivatives loaded into poly(styrene-butyl acrylate) nanoparticle emulsions against methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) and *Escherichia coli* (K12). Emulsions of covalently-bound N-acryloyl-5-ASA poly(L-menthyl acrylate) nanoparticles showed an MIC value of 8 µg/ml against MRSA and 128 µg/ml against *E. coli*. Emulsions of poly(L-menthyl acrylate) nanoparticle containing encapsulated N-acetyl-5-ASA showed an MIC value of 32 µg/ml against MRSA and 128 µg/ml against *E. coli*.

Cell cytotoxicity studies were carried out as previously reported in Section 2.6.2 with HT-29 cells. The resulting IC\textsubscript{50} values for both N-acryloyl-5-ASA and N-acetyl-5-ASA loaded into the poly(L-menthyl acrylate) nanoparticle emulsions were vastly improved (IC\textsubscript{50} of 500 µg/ml) compared to the previously reported IC\textsubscript{50} of 20 µg/ml for the corresponding poly(styrene-butyl acrylate) nanoparticle emulsions.

This provides renewed interest in the application of bioactive compounds that were previously problematic to load into poly(styrene-butyl acrylate) nanoparticle emulsions. Though additional experiments are required to find the potential loading capability of 5-ASA derivatives into
poly(menthyl acrylate) nanoparticle emulsions, the preliminary results obtained in this present study provide opportunities for expansion to various other classes of bioactive compounds.

3.11 Discussion
We have observed that the substitution of the butyl acrylate monomer for the menthyl acrylate monomer allows for the construction of chiral polyacrylate nanoparticle emulsions. The use of the menthyl acrylate monomer provided the first example of forming a homopolymer nanoparticle emulsion using the polymerization emulsion technique. In addition, by synthesizing menthyl acrylate as the monomer we were able to produce enantiomerically pure nanoparticles from enantiomerically pure L-menthol and D-menthol.

The emulsions have superior drug-loading properties to those of previous polyacrylate nanoparticle emulsions. There appear to be no sizable distinctions between the all-D and all-L menthyl acrylate nanoparticles, in either their shape, diameters, surface charge, drug loading capabilities, or bioactivities. The improved antibacterial activity of the penicillin-encapsulated poly(menthyl acrylate) nanoparticle emulsions are due to their ability to encapsulate nearly 700% more bioactive compound compared to our previous butyl acrylate/styrene system, while maintaining a highly uniform and stable emulsion.

In the next and final chapter of this dissertation the possibility of removing all non-bioactive monomers from nanoparticle construction was explored. Avoiding the use of co-monomers during the emulsion polymerization procedure allows for greater amount of loading of the bioactive antibacterial monomer, producing a homopolymer nanoparticle emulsion composed solely of the antibiotic monomer.
CHAPTER FOUR

POLYACRYLATE NANOPARTICLE EMULSIONS: FORMING HOMO POLY (N-ACRYLOYLCIPROFLOXACIN) AS AN ANTIBACTERIAL POLYMER EMULSION

4.1 Introduction

As a continuation to the mixed ciprofloxacin polyacrylate nanoparticle emulsions described in chapter three, this chapter delves into tackling the issue of limited loading of bioactive compounds, and the need for a better carrier polymer to bind or encapsulate the drug for delivery. The surfactant has a limit of how many organic/hydrophobic compounds it can contain within the micelle during emulsion polymerization. As a result, the maximum amount of organic content of the final emulsion is typically in the range of 15-20% by weight. This restricts the usefulness of the nanoparticle as an effective drug carrier to 20% or less of the emulsion amount. As previously noted, polyacrylate nanoparticle emulsions can be easily prepared through radical-induced emulsion polymerization of butyl acrylate/styrene mixtures (7:3 w/w) in water at 78°C, using sodium dodecyl sulfate (SDS) as an emulsifying agent and potassium persulfate as a radical initiator (Figure 4.1).\textsuperscript{52,53}
The reactions led to the formation of a homogeneous, stable aqueous emulsion containing uniformly-sized nanoparticles of 45-50 nm in diameter. The method was successfully applied to penicillins and N-thiolated β-lactams, in which the antibacterial agents could be introduced into the nanoparticle either by non-covalent entrapment as a free drug, or covalently via their acryloyl derivative.

While these earlier nanoparticle emulsions provided increased water solubility and, in some cases, improved bioactivity of the β-lactam antibacterial agent, the polyacrylate backbone was largely comprised of the non-bioactive monomers (butyl acrylate-styrene or methyl methacrylate-styrene (20% by weight of the emulsion), and thus only 1-3% (by weight) of the nanoparticle framework was the antibacterial acrylate. Figure 4.1 shows the general scheme for the formation of the nanoparticle emulsion, and the amount of drug loading into the nanoparticle during the assembly process was limited by how much surfactant could be used, given that amounts exceeding 3 mole % of SDS caused unwanted cytotoxicity. The final crude nanoparticle emulsions contained up to 20% of solid content (a mixture of nanoparticles and a small amount of...
of non-nanoparticle polymer), and only 0.2-0.6% of active antibacterial agent inside of the nanoparticles. The resulting emulsions are typically milky in consistency and somewhat sticky when exposed to air, causing films to form when dried, and forming coagulants within syringes, micro-porous filters, and gel columns that made it very difficult to purify and use for in vivo testing.

We were able to overcome some of these issues with purification techniques that enable the removal of residual unreacted monomers and non-nanoparticle oligomers within the cloudy emulsion. We also reported on the use of other surfactant combinations to try to enhance the amount of antibiotic that could be entrapped, or to alter nanoparticle sizes, without increasing overall cytotoxicity or instability of the emulsion.\textsuperscript{5}

\textbf{Figure (4.2)} Co-monomer-based encapsulation of antibiotic into polyacrylate nanoparticle emulsions.

Figure 4.2 depicts the polyacrylate polymer that was formed that allows for the incorporation of the bioactive drug either through covalently binding to the polymer backbone or encapsulating
within the hydrophobic environment of the micelle. This in turn limits the amount of bioactive drug that can be contained by the particle.

**Figure (4.3)** Removal of co-monomers and formation of polymer via 100% of the acrylate antibiotic analog.

Figure 4.3 shows that removing all acrylates except for the acrylated bioactive drug (or other compound) for the emulsion polymerization would allow for an increase in the ability to load the desired drugs/compounds within the micelles, and thus the final concentration in the nanoparticle emulsion. If the same limit of 15-20% of organic material entrapped by the surfactant inside the micelles is maintained, then the final concentration of the drug incorporated into the nanoparticle would be considerably more than the typical 0.2%-0.6% achieved using the butyl acrylate/styrene polyacrylate nanoparticles. The use of only N-acryloylciprofloxacin as the sole monomer then would afford an advanced polyacrylate nanoparticle emulsion, which allows for the delivery of higher drug content. This would in return require much smaller volumes of the emulsion to be synthesized and used for drug delivery.
In addition, we can expect to have similar results in reducing the cytotoxicity of the resulting emulsions, as was observed for the homo poly(menthyl acrylate) nanoparticle emulsions described in chapter three. In that case, the increased drug loading of penicillin G (up to 4% of the final volume) allowed for a more diluted emulsion to be used to deliver the same amount of penicillin G compared to the previous butyl acrylate/styrene polyacrylate nanoparticle emulsions. The reduction in the concentration of sodium dodecyl sulfate required to make these emulsions provided a dramatic reduction in cytotoxicity.

The avoidance of using other monomers for the nanoparticle formation additionally removes the issue of unwanted coagulation and film formation previously observed for the poly(butyl acrylate/styrene) nanoparticle emulsions. The residual styrene and butyl acrylate and non-particle polymers that are not encapsulated within the surfactant could be removed by centrifugation and dialysis, however, the resulting emulsions after purification still continued to formed rubbery films when dehydrated, which clogged syringe needles and filtration membranes. The use of these particular monomers was problematic in this regard and not using them might eliminate the need to purify the ciprofloxacin acrylate emulsions.

In this chapter, a new approach to preparing antibiotic-bound polyacrylate nanoparticle emulsions is investigated that completely obviates the restriction of using butyl acrylate and styrene (or other co-monomers) to form the nanoparticle framework, and instead, uses the antibiotic compound itself as the sole acrylate monomer for the polymerization. This technique has never been reported and is thus an important advance in the polymer-based nanoparticle field.
For our studies, we chose ciprofloxacin as the antibiotic for the formation of the polycrylate nanoparticles. The N-acryloyl derivative of commercial ciprofloxacin hydrochloride was prepared for this purpose according to our previously reported N-acylation procedure.54,55

**4.2 Synthesis of N-Acryloylciprofloxacin**

Figure (4.4) Scheme for synthesis of N-acryloylciprofloxacin compound 9

Figure 4.4 shows the synthetic scheme for preparing N-acryloyl ciprofloxacin, and follows as such: To a round bottom flask was added 120 ml of dichloromethane, then 3.0 g (9.0 mmol) of ciprofloxacin and 1.8 ml (13.5 mmol) of triethylamine. The mixture was left stirring at 0°C for 1 hour then acryloyl chloride (1.1 ml, 13 mmol) was added dropwise. The ice bath was removed and the reaction was left stirring overnight. The dichloromethane was added dropwise to a flask of hexane (200 ml) to cause a precipitate to form. The solid was collected by filtration and allowed to air dry.

Yielded 2.90 g (83.7%) as a pale yellow solid. Melting point above 250°C. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.75 (s, 1 H), 8.03 (d, $J = 12.8$ Hz, 1 H), 7.36 (d, $J = 7.1$ Hz, 1 H), 6.60 (dd, $J = 16.8$,
10.5 Hz, 1 H), 6.35 (dd, \( J = 16.8, 1.7 \) Hz, 1 H), 5.76 (dd, \( J = 10.5, 1.7 \) Hz, 1 H), 3.86 (m, 4 H), 3.52 (br. s., 1 H), 3.33 (m, 4 H), 1.38 (d, \( J = 6.2 \) Hz, 2 H), 1.19 (br. s., 2 H).

### 4.3 Formation of Poly(N-Acryloylciprofloxacin) Nanoparticle Emulsion

#### 4.3.1 Attempted Preparation of Polyacrylate Emulsions

One of the main challenges with polymerizing the desired acryloyl analog of the bioactive drug was that most of the previous antibiotics that were acrylated and loaded into the nanoparticle emulsions were solids, and thus the liquid organic monomers of styrene and butyl acrylate could be used to pre-dissolve the small amount of the solid acrylated antibiotic. This was also the case with the poly(menthyl acrylate) nanoparticle emulsions, in that the non-bioactive monomer menthyl acrylate was a liquid that allowed for the dissolution of the solid N-acryloyl ciprofloxacin antibiotic in order to be incorporated into micelles during emulsion polymerization.

Attempts to use the same procedure for emulsion polymerization of the N-acrylated ciprofloxacin monomer failed, however. Thus it was necessary to pre-dissolve the N-acryloyl ciprofloxacin into an organic solvent that could easily be evaporated off during the polymerization process or after the formation of the emulsions.

It was considered important to use a solvent of very low cytotoxicity to aid in the dissolution of the bioactive compound, in case it would also load into the micelles along with the bioactive compound. After experimentation with various common organic solvents, including methanol, ethanol, propylene glycol, glycerol, and ethyl acetate; dichloromethane was chosen.
4.3.2 Attempted Preparation of Homo Poly(N-N-Acryloylciprofloxacin) Nanoparticle Emulsions Using Water-soluble Organic Solvents

Two liquid organic solvents were first used to aid the dissolution of N-acryloylciprofloxacin. Propylene glycol and glycerin have very low cytotoxicity and due to their hydrophobic nature would likely load into the surfactant-formed micelles, and thus potentially carry in with it the N-acryloylciprofloxacin. Though this technique would result in a co-solvent also being incorporated into the micelles, it would still possibly allow for formation of the poly(N-acryloylciprofloxacin) emulsion.

However, the resulting emulsions formed from the use of these solvents were not homogeneous. Due to glycerin’s high viscosity, it was very difficult to distribute and stir properly in the aqueous media. This led to a bilayer, preventing homogeneous mixing of the resulting emulsion. The mixture was heated up to 90°C in order to reduce the viscosity and allow for more uniform stirring and mixing with water. However, the resulting emulsions remained non-homogeneous.

Propylene glycol provided a much better carrier solvent due to its lower viscosity. It was able to form a more uniform emulsion and would require no modification in procedure compared to the typical one used to make polyacrylate nanoparticle emulsions. However, the resulting emulsions were unstable and formed a bilayer within minutes of being removed from the polymerization conditions. The DLS data did confirm multiple populations of particles within the emulsion and very low zeta potential values (-5 mV to -10 mV), which confirmed the inherent instability of the emulsions. So these attempts did not prove effective.
4.3.3 Preparation of Homo Poly(N-Acryloylciprofloxacin) Nanoparticle Emulsions Using a Water-insoluble Solvent

The other method investigated for polymerization of the solid N-acryloylciprofloxacin to be evenly distributed within the aqueous mixture was to pre-dissolve the compound in an organic solvent, and then remove the organic solvent via evaporation during the emulsion process or after the emulsion formation. It was critical to completely remove the organic solvent, because most organic solvents produce cytotoxicity.

This method was attempted using methanol, ethanol, ethyl acetate, and dichloromethane. The main problem was the poor solubility of the N-acryloylciprofloxacin in most organic solvents, except for dichloromethane. Up to 500 mg/ml of N-acryloylciprofloxacin could be dissolved into dichloromethane. However, there was a critical issue that resulted with the polymerization procedure. The typical procedure would call for the organics to be stirred at 75°C, then the surfactant and water are added in. This would cause the dichloromethane to rapidly evaporate. Thus the starting temperature was adjusted to 25°C, and water and surfactant were added to the stirring dichloromethane solution, however this resulted in an uneven distribution and clumping of the surfactant. The result was a very sticky material that separated from the water layer.

In order to solve this new issue, the surfactant and water were added first at 75°C so that the surfactant may form micelles initially, and the dichloromethane solution was added dropwise. However, this resulted in the near instant evaporation of the dichloromethane solvent, leaving clumps of solid N-acryloylciprofloxacin unincorporated into the micelles. The final adjustment
of the procedure is discussed in the following section, resulting in successful formation of the emulsion.

4.3.4 Preparation of Poly(N-Acryloylciprofloxacin) Nanoparticle Emulsions

Figure (4.5) Scheme for preparing poly(N-acryloylciprofloxacin) nanoparticle emulsions

As seen in Figure 4.5, the polyacrylate emulsions were prepared using a modified protocol of the usual nanoparticle emulsion technique used in our lab. The method to form the poly(N-acryloylciprofloxacin) emulsion required the following procedure: to a round bottom flask was added 4 ml of deionized water, which was then stirred using a 1.25 cm (300 mg) Teflon-coated magnetic stir bar at 1000 rpm on a Corning PC-420D magnetic stirrer at 30°C using a self-regulated oil bath. To this was added 30 mg of SDS. N-Acryloylciprofloxacin (500 mg) was dissolved in 1 ml of warm dichloromethane, and this solution was added dropwise to the deionized water-SDS mixture. A vent was placed on top of the flask by inserting a small stainless steel syringe needle through a rubber septum on the flask, under dry nitrogen, and the temperature of the mixture was increased at a rate of 5°C per 30 min until reaching 90°C. The mixture was left stirring overnight at this temperature, under an atmosphere of dry nitrogen. Potassium persulfate (10 mg) was added with an additional 0.5 ml of deionized water to the
stirring mixture, and left stirring for 24 hours. The stirred emulsion was then removed from the oil bath and decanted into a storage vial for analysis.

Figure (4.6) On the left, an example of a successful emulsion. On the right, two examples of unsuccessful emulsions.

Figure 4.6 shows an example of a successful emulsion (on the left), forming a uniform single layer emulsion, while previous attempted emulsions (the two on the right) show the results of an unsuccessful emulsion polymerization.

4.4 Dynamic Light Scattering (DLS) Analysis
The first question we hoped to address was if any nanoparticles were being formed in the emulsion polymerization process. For this, we used dynamic light scattering measurements. The average size and surface charge of the emulsion was analyzed on a Malvern Zetasizer nano-ZS instrument. To prepare the samples for the analyses, the freshly-made emulsion was subjected to
centrifugation at 10,000 rpm for 5 min using an Eppendorf Centrifuge 5424. An aliquot of the liquid emulsion was then drawn and deposited into a Malvern disposable folded capillary cell DTS-1070. Each sample was analyzed in triplicate, and each data collection consisted of 1 run of 20 scans (for size analysis) and 3 runs of 100 scans (for zeta potential determination). The size distribution shows a single narrow peak indicating the uniformity of the emulsion with a single population centered on average at approximately 970 nm. Similarly, surface charge measurements indicated a highly stable emulsion, with an average of -63 (± 5.6) mV.

4.4.1 Dynamic Light Scattering (DLS) Analysis Results
As Figure 4.7 demonstrates, the dynamic light scattering experiments confirmed the presence of a major population of nanoparticles in the emulsion, measuring on average approximately 970 nm in diameter. A general trend of increasing size was observed as the amount of N-acryloyciprofloxacin is increased in forming the polymer emulsions. In addition, the zeta potential measurements showed that the particles carry a high surface charge of -63 (± 5.6) mV. This indicates the long-term stability of the emulsion. It is notable that these poly(N-acryloyciprofloxacin) nanoparticles are much larger than those previously constructed with butyl acrylate-styrene co-monomers, which routinely measured 45-50 nm in diameter. The basis for this 20-fold increase in size is not apparent at this time but deserves further investigation.
4.5 In vitro Antibacterial Testing

To investigate whether the nanoparticles possess antibiotic capabilities, each crude emulsion was tested against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (K12) using a 96-well plate broth assay to determine the minimum inhibitory concentration (MIC). Each assay was done in triplicate.

The original stock emulsion was diluted using the Trypticase Soy Broth solution to an initial concentration of 1.28 mg/ml of the N-acryloylciprofloxacin, then serial diluted with TSB to half the concentration each time. A volume of 10 µl of each emulsion dilution was added to a well in series, resulting in a final concentration run of 64 µg/ml to 0.012 µg/ml. The MIC was done in triplicates for each bacterium, with ciprofloxacin hydrochloride being used as a positive control and a blank of broth medium was used as a negative control.

**Figure (4.7)** Size of emulsified nanoparticles vs the % concentration of N-acryloylciprofloxacin in the emulsions.
To prepare the bacteria for culture, all solutions were autoclaved prior to use. The bacteria were grown overnight at 37°C on an agar plate composed of BBL TSA II Trypticase Soy Agar (TSA) and BBL Trypticase Soy Broth (TSB) in a 1:2 ratio at 4.4% concentration. A broth solution of 2.4% TSB was inoculated using the bacteria from the agar plates, and incubated at 37°C to reach a 0.5 McFarland standard. The bacteria were then further diluted by a factor of 1000 using a broth solution of 2.4% TSB, and 190 µl of the diluted bacterial solution was transferred by micropipette into each well. The inoculated plates were incubated at 37°C for 16-20 hours and the resulting plates were observed for growth and MIC values recorded. The MIC was the lowest concentration of the antibiotic that completely inhibited bacterial growth (visually) within that series of dilutions.

4.5.1 Antibacterial Data for Poly (N-Acryloylciprofloxacin) Emulsions

**Table (4.1) MIC values of ciprofloxacin and ciprofloxacin emulsion vs S. aureus and E. coli.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>S.aureus (ATCC 25923)</th>
<th>E. coli (K12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Ciprofloxacin</td>
<td>0.5 µg/ml</td>
<td>0.012 µg/ml</td>
</tr>
<tr>
<td>Poly (N-acryloylciprofloxacin) emulsion</td>
<td>0.5 µg/ml</td>
<td>0.012 µg/ml</td>
</tr>
</tbody>
</table>

The in vitro antibacterial studies showed that the nanoparticle emulsion was bioactive, with an MIC of 0.5 µg/ml for *S. aureus* and 0.012 µg/ml against *E. coli*, identical to those of ciprofloxacin itself (Table 4.1). The finding that these nanoparticles show antibacterial capabilities against both
the gram-positive *S. aureus* and the gram-negative *E. coli* was surprising, given that particles of such large dimensions would not be expected to be antibacterially active.

Ciprofloxacin must enter the bacterial cell to arrive at its target, bacterial DNA gyrase. Attachment of the molecule to the polymer backbone of the nanoparticle requires it be released through hydrolysis of the amide. This occurs either outside of the cell, or within the bacterium itself if the nanoparticle can enter through the membrane. Most likely this requires enzymatic release, as the amide functionality is a difficult one to cleave otherwise. Again, this is only speculation that requires further investigation with proper controls, which are beyond the scope of this study.

### 4.6 In Vitro Cytotoxicity of the Nanoparticle Emulsions

In vitro cell cytotoxicity was tested on two human cell lines, human colorectal carcinoma cells HCT-116, and human embryonic kidney cells HEK 293. HCT-116 were grown in Dulbeco’s Minimum Essential Medium (DMEM) with 10% fetal bovine serum and 0.1% penicillin/streptomycin as a growth medium for several days at 37°C under an atmosphere of 5% CO₂ to reach confluence. HEK 293 cells were grown in Eagle Minimum Essential Medium (EMEM) with 10% fetal bovine serum and 0.1% penicillin/streptomycin as a growth medium for several days at 37°C under an atmosphere of 5% CO₂ to reach confluence. Each cell type was then plated onto 96-well plates, at 50,000 cells per well at a volume of 150 µl with the respective growth medium. The cells were counted using a hemocytometer and then incubated for 24 hours at 37°C under an atmosphere of 5% CO₂.
The test emulsion was diluted using the growth medium for each cell type, and added into the wells of each test plate to give a final concentration of N-acryloyl ciprofloxacin of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml within a series. The testing was done in triplicate and one well in each triplicate was left untreated as the negative control for 100% growth. The plates were further incubated and monitored for 48 hours at 37°C under an atmosphere of 5% CO₂. A 5 mg/ml solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) in sterile phosphate-buffered saline (PBS) was added to give a 10% final concentration in each well. The plates were then further incubated for 4 hours at 37°C under an atmosphere of 5% CO₂ to allow for the formation of the purple crystals of 1-(4,5-dimethylthiazol)2-yl)-3,5-diphenylformazan. The liquid was then aspirated from each well and 100 µl of dimethylsulfoxide (DMSO) was added to each well, and gently shaken for 1 minute to allow for complete dissolution of the crystals. The IC₅₀ value for the assay was determined using a BioTek Synergy H1 hybrid plate reader at both 595 nm and 630 nm. The IC₅₀ was determined as the well with at least 50% cell viability compared to the untreated control cell with 100% cell growth.

4.6.1 Cytotoxicity Results for Poly(N-Acryloylciprofloxacin) Nanoparticle Emulsions

The in vitro cytotoxicity results for both human colorectal carcinoma cells HCT-116 and human embryonic kidney cells HEK-293 were promising. The observed IC₅₀ was 500 µg/ml for both the HCT-116 and HEK-293 cell lines, a 1000-fold difference over the bacterial MIC value for S.aureus and greater than 40,000 for E.coli.
4.7 Imaging Nanoparticle Emulsions Using a Scanning Electron Microscope

A sample of poly(N-acryloyl ciprofloxacin) nanoparticle emulsion was prepared for imaging using scanning electron microscope. The samples was initially prepared by lyophylization of the emulsion which resulted in a dry powder that could be added to the sample holder for the scanning electron microscope instrument. The samples were placed onto an aluminum-coated sample holding tape, mounted onto a copper tape, placed onto the scanning electron microscope sample holder. The initial resulting image was very difficult to discern and it was most likely due to the non-conductive nature of the material, causing the electrons to build up on the surface of the material and distorting the image.

In order to resolve the issue, the sample was diluted 1000X with deionized water, and a drop of the diluted emulsion was placed on the conductive aluminum-coated sample holding tape. The sample was placed in the -80°C freezer for a few hours, then immediately lyophilized to dry the sample right onto the sample holding tape to produce a more even distribution of the material.

In addition, the sample-containing tape was also sputter-coated with gold-palladium in order to increase the conductivity of the resulting sample, thus preventing or reducing the accumulation of electrons on the surface of the sample, and resulting in distortions.
Figure (4.8) Scanning electron microscope image of the dried emulsion.

Figure (4.9) A zoomed in SEM image of a potential micelle within the emulsion.
As observed in Figure 4.8 and Figure 4.9, the images from the scanning electron microscope do not provide clear images of the spheres within the emulsion as were previously observed with butyl acrylate/styrene and poly(menthy acrylate) emulsions. This was thought to be the result of the material continuing to building up charge on the surface, thus giving a distorted image. Attempts to overcome this effect by ensuring a smooth and conductive surface for the sample holding tape, and sputter-coating with conductive gold-palladium coating, did not improve results. In addition, during the lyophilization process the spheres were dehydrated and deformed, thus resulting in the spheres binding to each other and not remaining separate. This led to the increase of the overall size when viewed from top down with the scanning electron microscope.

4.8 Discussion

Poly(N-acryloylicprofloxacin) nanoparticle emulsions were successfully prepared by modification of the previously reported emulsion polymerization methodology. The main difference with this new method was the need to dissolve the water-insoluble antibacterial agent in an organic solvent to permit more uniform addition into the aqueous solution, in order to form homogeneous emulsions. We found that dichloromethane provided the best combination of solubilizing the ciprofloxacin monomer and being volatile enough to evaporate from the media during emulsion polymerization at 90°C.

We also found that the increased temperature of 90°C rather than 75°C, an increased stir speed, and the addition of sodium dodecyl sulfate before the organic monomers were added, provided more optimal results. Additionally, we found it advantageous to let the reactions run for 48 hours rather than the usual 6 hours required for the butyl acrylate-styrene co-monomer systems.
These new procedures are required mainly due to the physical properties of the compounds involved, and are pushing the limits and capabilities of the existing available equipment. Higher loading of the drug could perhaps be possible if the mixture could be heated in a pressurized system that would allow for a higher temperature to be achieved without boiling off the water. In addition, a mechanical stirrer able to achieve a higher spin rate that the existing magnetic stir bar method would most likely allow for additional loading of the monomer, since it would provide more uniform distribution of large quantities of the solid monomer.

Lyophilization of the nanoparticle emulsion produced an amorphous powder that could not be reformulated back to its original emulsified state through addition of water. Moreover, the resulting powder remained insoluble in organic solvents including methanol, ethanol, dichloromethane, hexane, acetone, ethyl acetate, and dimethylformamide. We did note that extraction of the solid material with methanol, ethanol, dichloromethane, hexane, acetone, or ethyl acetate failed to show any trace of unreacted N-acryloylciprofloxacin upon evaporation and analysis by proton NMR spectroscopy. This confirms that the polymerization is complete, and thus all of the N-acryloylciprofloxacin is incorporated into the framework of the nanoparticle. Attempts to perform the emulsion polymerization procedure on the free ciprofloxacin instead of the N-acryloyl derivative led to a bilayer mixture, not an emulsion, with the layers separating within seconds after stirring was stopped. Additionally, the same procedure was attempted using N-acetyl ciprofloxacin as an analog similar in structure but without the requisite olefin. Once again, only an unstable mixture was formed, which that separated into layers with a few seconds after stirring was stopped. Therefore, the acryloyl group is a prerequisite for emulsification and subsequent nanoparticle formation.
Though the field of antibacterial polymers is well explored, and various examples exist, they typically rely on the use of other co-monomers to prepare the polymers.\textsuperscript{35-38} This study is the first involving an aqueous nanoparticle polymer emulsion being formed from a monomer that is the antibiotic agent itself. The emulsion is formed via a one pot reaction in water and the final antibiotic polymer is suspended in water. The emulsified nano-cipro particles are antimicrobially-active towards gram positive \textit{S. aureus} and gram negative \textit{E. coli}. Our laboratory hopes to further investigate the properties of the new “nanodrug” emulsions and to further expand the possibilities with other, preferably water-insoluble, antibiotics for delivery and effective treatment of drug-resistant bacterial infections.
Though several ends points have been reached with the body of work presented in this dissertation, additional points of research still remain. The project in chapter 2 can be continued through the use of poly(menthyl acrylate) nanoparticles emulsions that exhibit lower cytotoxicity than poly(styrene-butyl acrylate) nanoparticle emulsion. The poly(menthyl acrylate) nanoparticles emulsions can be further explored via covalent binding and encapsulating of various antibiotics in order to study the limits of drug loading and delivery. The chiral properties of poly(menthyl acrylate) nanoparticle emulsion can be further studied by loading enantiopure bioactive compounds into the poly(L-menthyl acrylate) vs the poly(D-menthyl acrylate) nanoparticle emulsion, and studying the difference in drug loading and drug delivery. Lastly, the poly(N-acryloylciprofloxacin) nanoparticle emulsion could be further explored through the use of alternative acryloyl derivatives of antibiotics, and used as the monomer to form homo-polyacrylate antibacterial nanoparticle emulsions.

As of the publication of this dissertation, U.S Patent 09533051B2 was issued on January 2017 for the synthesis and drug delivery properties of poly(menthyl acrylate) nanoparticles. In addition, after the completion of additional data points the work included in chapter 3 is to be
submitted for publication in the spring of 2018. Research and results obtained in chapter 4 regarding poly(N-acryloylciprofloxacin) nanoparticle emulsion were submitted for publication in Nanomedicine in November of 2017, along with filing for a provisional patent.
CHAPTER SIX

EXPERIMENTAL PROCEDURES

DYNAMIC LIGHT SCATTERING DATA

NUCLEAR MAGNETIC RESONANCE SPECTRA
DLS Data For 10 mg, Compound 2 Loaded Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
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<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Z-Average (d.nm): 37.96
PDI: 0.083
Intercept: 0.929
Result quality: Good

Size Distribution by Intensity

Zeta Potential (mV): -52.4
Zeta SD (mV): 22.1
Conductivity (mS/cm): 0.0670

Mean (mV): Peak 1: -50.3, Peak 2: -31.7, Peak 3: -75.3
Area (%): Peak 1: 40.4, Peak 2: 29.3, Peak 3: 23.9
Width (mV): Peak 1: 8.32, Peak 2: 6.67, Peak 3: 8.23

Conductivity is out of range - check cell or sample
DLS Data For 20 mg, Compound 2 Loaded Nanoparticle

**Results**

<table>
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<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
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<th>Volume</th>
<th>Number</th>
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<td>0.0</td>
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<tr>
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<td>0.000</td>
<td>0.000</td>
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</table>

**Z-Average (d.nm):** 52.90  
**PdI:** 0.063  
**Intercept:** 0.930  
**Result quality:** Good  

**Size Distribution by Intensity**

**Results**

**Zeta Potential (mV):** -44.0  
**Zeta SD (mV):** 13.8  
**Conductivity (mS/cm):** 0.0597  

**Zeta potential out of range**  
**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

**Record 908: FM2-20-1-1**  
**Record 909: FM2-20-2-1**  
**Record 910: FM2-20-3-1**
DLS Data For 30 mg, Compound 2 Loaded Nanoparticle

Results

**Z-Average (d.nm):** 70.79

**PDI:** 0.168

**Intercept:** 0.808

**Result quality:** Good

![Size Distribution by Intensity Graph]

**Results**

**Zeta Potential (mV):** -35.6

**Zeta SD (mV):** 17.9

**Conductivity (mS/cm):** 0.0487

**Mean (mV):**
- Peak 1: -19.7
- Peak 2: -52.4
- Peak 3: -35.5

**Area (%):**
- Peak 1: 39.9
- Peak 2: 34.0
- Peak 3: 24.3

**Width (mV):**
- Peak 1: 8.37
- Peak 2: 9.04
- Peak 3: 4.04

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

![Zeta Potential Distribution Graph]
DLS Data For 10 mg, Compound 3 Loaded Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
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</tr>
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<td>0.000</td>
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</tbody>
</table>

**Z-Average (d.nm):** 42.10

**PDI:** 0.137

**Intercept:** 0.930

**Result quality:** Good

---

**Results**

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<th>Width (mV):</th>
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</thead>
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<td>-57.8</td>
<td>Peak 1: -68.6</td>
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<td>Peak 3: 0.000</td>
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</table>

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

---

**Zeta Potential Distribution**

**Record 933: FM3-10**

**Record 934: FM3-10-2**

**Record 935: FM3-10-3**
DLS Data For 20 mg, Compound 3 Loaded Nanoparticle

Results

Z-Average (d.nm): 57.68
PDI: 0.096
Intercept: 0.907
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -44.1
Zeta SD (mV): 20.0
Conductivity (mS/cm): 0.0437

Zeta potential out of range

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Record 918: FM3-20-1, Record 919: FM3-20-2, Record 920: FM3-20-3

Record 936: FM3-20-1, Record 937: FM3-20-2, Record 938: FM3-20-3
DLS Data For 30 mg, Compound 3 Loaded Nanoparticle

Results

Z-Average (d.nm): 84.46
PdI: 0.128
Intercept: 0.906

Result quality: Good

Size Distribution by Intensity

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
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<td>0.000</td>
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</tbody>
</table>

Results

**Zeta Potential (mV):** -35.0
**Zeta SD (mV):** 11.6
**Conductivity (mS/cm):** 0.0172

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

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<td>Total Counts</td>
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<td>Record 940: FM3-30-2</td>
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<tr>
<td>Record 941: FM3-30-3</td>
</tr>
</tbody>
</table>
DLS Data For D-Mta 10% Nanoparticle

**Results**

**Z-Average (d.nm):** 59.69

**Pdi:** 0.050

**Intercept:** 0.935

**Result quality:** Good

**Size Distribution by Intensity**

**Zeta Potential (mV):** -47.0

**Zeta SD (mV):** 17.7

**Conductivity (mS/cm):** 0.0526

**Conductivity is out of range - check cell or sample**
DLS Data For D-Mta 20% Nanoparticle

Results

Z-Average (d.nm): 53.53
PDI: 0.068
Intercept: 0.912

Result quality: Good

Size Distribution by Intensity

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<td>100</td>
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</tbody>
</table>

Zeta Potential (mV): -47.2
Zeta SD (mV): 19.0
Conductivity (mS/cm): 0.0511

Results

Mean (mV): Area (%) Width (mV):
Peak 1: -33.6 44.3 11.1
Peak 2: -51.5 34.2 4.86
Peak 3: -68.8 19.7 7.19

Conductivity is out of range - check cell or sample
DLS Data For D-Mta 30% Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Z-Average</th>
<th>PdI</th>
<th>Intercept</th>
<th>Result quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>55.78</td>
<td>0.075</td>
<td>0.919</td>
<td>Good</td>
<td></td>
</tr>
</tbody>
</table>

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-45.7</td>
<td>Peak 1: -44.0</td>
<td>81.1</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>Peak 2: -71.8</td>
<td>8.8</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>Peak 3: -8.86</td>
<td>6.0</td>
<td>5.17</td>
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</tbody>
</table>

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

<table>
<thead>
<tr>
<th>Total Counts</th>
<th>Apparent Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Record 35: D30-2</td>
<td>120000</td>
</tr>
<tr>
<td>Record 36: D30-2</td>
<td>100000</td>
</tr>
<tr>
<td>Record 37: D30-2</td>
<td>80000</td>
</tr>
</tbody>
</table>
DLS Data For D-Mta 40% Nanoparticle

**Results**

- **Z-Average (d.nm):** 50.40
- **Pdi:** 0.068
- **Intercept:** 0.907
- **Result quality:** Good

**Size Distribution by Intensity**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Intensity (Percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity</td>
<td></td>
</tr>
</tbody>
</table>

**Results**

- **Zeta Potential (mV):** -50.6
- **Zeta SD (mV):** 18.9
- **Conductivity (mS/cm):** 0.0654

**Mean (mV):**
- **Peak 1:** -61.7
- **Peak 2:** -36.9
- **Peak 3:** -96.1

**Area (%):**
- **Peak 1:** 47.9
- **Peak 2:** 47.8
- **Peak 3:** 3.8

**Width (mV):**
- **Peak 1:** 8.94
- **Peak 2:** 10.6
- **Peak 3:** 6.24

*Conductivity is out of range - check cell or sample*

**Zeta Potential Distribution**

**Total Counts**

<table>
<thead>
<tr>
<th>Counts</th>
<th>Apparent Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counts</td>
<td></td>
</tr>
</tbody>
</table>

- **Record 43: D40-1.1**
- **Record 44: D40-1.2**
- **Record 45: D40-1.3**
DLS Data For D-Mta 50% Nanoparticle

Results

Size (d.nm)... Width (d.nm)... % Intensity: Volume: Number

<table>
<thead>
<tr>
<th>Peak</th>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>54.39</td>
<td>15.20</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
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<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Z-Average (d.nm): 50.70
Pdi: 0.050
Intercept: 0.892
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -48.8
Zeta SD (mV): 20.9
Conductivity (mS/cm): 0.0447

Mean (mV): Area (%): Width (mV):
Peak 1: -51.4 84.4 15.2
Peak 2: -18.0 9.5 5.10
Peak 3: -97.5 3.2 5.62

Conductivity is out of range - check cell or sample
DLS Data For D-Mta 60% Nanoparticle

**Results**

- **Z-Average (d.nm):** 55.62
- **Pdi:** 0.184
- **Intercept:** 0.928

**Result quality:** Good

**Size Distribution by Intensity**

- **Record 66: D60-1.1**
- **Record 70: D60-2.1**
- **Record 74: D60-3.1**

**Results**

- **Zeta Potential (mV):** -50.6
- **Zeta SD (mV):** 15.8
- **Conductivity (mS/cm):** 0.0400

**Mean (mV):**
- **Peak 1:** -49.8
- **Peak 2:** -87.7
- **Peak 3:** -3.71

**Area (%):**
- **Peak 1:** 93.1
- **Peak 2:** 5.1
- **Peak 3:** 1.8

**Width (mV):**
- **Peak 1:** 12.5
- **Peak 2:** 7.84
- **Peak 3:** 4.67

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

- **Record 71: D60-2.1**
- **Record 72: D60-2.2**
- **Record 73: D60-2.3**
DLS Data For D-Mta 70% Nanoparticle

Results

Z-Average (d.nm): 46.08
Pdi: 0.144
Intercept: 0.911
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -45.0
Zeta SD (mV): 77.6
Conductivity (mS/cm): 0.0546

Mean (mV): Area (%) Width (mV):
Peak 1: -46.6 37.1 9.25
Peak 2: -74.5 16.8 9.57
Peak 3: -23.9 16.4 5.79

Conductivity is out of range - check cell or sample
DLS Data For D-Mta 80% Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>57.30</td>
<td>30.05</td>
<td>93.1</td>
<td>98.8</td>
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<tr>
<td>Peak 2:</td>
<td>2124</td>
<td>1332</td>
<td>6.9</td>
<td>1.0</td>
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<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.2</td>
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</table>

Z-Average (d.nm): 49.46  
Pdi: 0.251  
Intercept: 0.902  

Result quality: Good

Size Distribution by Intensity

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
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<tbody>
<tr>
<td>Peak 1</td>
<td>-50.8</td>
<td>75.4</td>
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<td>Peak 2</td>
<td>-26.4</td>
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<td>6.92</td>
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<td>Peak 3</td>
<td>-72.1</td>
<td>10.9</td>
<td>5.40</td>
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Conductivity is out of range - check cell or sample

Conductivity Distribution

Record 93: D80-1 1  
Record 97: D80-2 1  
Record 101: D80-3 1  
Record 102: D80-3 1  
Record 103: D80-3 2  
Record 104: D80-3 3
DLS Data For D-Mta 90% Nanoparticle

**Results**

- **Z-Average (d.nm):** 48.05
- **PDI:** 0.100
- **Intercept:** 0.922
- **Result quality:** Good

<table>
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<tr>
<th>Size (d.nm)</th>
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<th>% Intensity</th>
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<tbody>
<tr>
<td>Peak 1</td>
<td>54.02</td>
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<td>Peak 2</td>
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<tr>
<td>Peak 3</td>
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<td>0.000</td>
<td>0.0</td>
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</tbody>
</table>

**Size Distribution by Intensity**

**Results**

- **Zeta Potential (mV):** -41.6
- **Zeta SD (mV):** 18.6
- **Conductivity (mS/cm):** 0.0449

**Zeta potential out of range**

- **Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**
DLS Data For D-Mta 100% Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
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<tbody>
<tr>
<td>Peak 1:</td>
<td>58.49</td>
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<td>Peak 3:</td>
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Result quality: Good

Size Distribution by Intensity

Results

<table>
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<th>Zeta Potential (mV)</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
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<tr>
<td>-51.2</td>
<td>-55.7</td>
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<td>Zeta SD (mV)</td>
<td>-31.4</td>
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<td>7.27</td>
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<td>Conductivity (mS/cm)</td>
<td>-94.2</td>
<td>4.2</td>
<td>6.85</td>
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Conductivity is out of range - check cell or sample

Zeta Potential Distribution

111
DLS Data For L-Mta 10% Nanoparticle

**Results**

<table>
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<tr>
<th>Z-Average (d.nm)</th>
<th>66.05</th>
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<tr>
<td>PDI</td>
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<tr>
<td>Intercept</td>
<td>0.908</td>
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**Result quality:** Good

**Size Distribution by Intensity**

**Results**

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>-56.8</th>
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<tr>
<td>Zeta SD (mV)</td>
<td>17.4</td>
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<tr>
<td>Conductivity (mS/cm)</td>
<td>0.0698</td>
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**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**
DLS Data For L-Mta 20% Nanoparticle

Results

Z-Average (d.nm): 64.33
Pdi: 0.064
Intercept: 0.914
Result quality: Good

Size Distribution by Intensity

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<tr>
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<th>Intensity (Percent)</th>
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<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Intensity (Percent)</th>
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<tr>
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<td>40</td>
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<tr>
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</table>

Record 265: L20-1
Record 269: L20-2
Record 273: L20-3

Results

Zeta Potential (mV): -53.0
Zeta SD (mV): 20.6
Conductivity (mS/cm): 0.0491

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Mean (mV): Peak 1: -49.6
           Peak 2: -74.8
           Peak 3: -98.5

Area (%): Peak 1: 71.3
          Peak 2: 15.8
          Peak 3: 4.9

Width (mV): Peak 1: 11.3
           Peak 2: 5.38
           Peak 3: 10.2

Record 270: L20-2
Record 271: L20-2
Record 272: L20-2
DLS Data For L-Mta 30% Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm...)</th>
<th>Width (d.nm...)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
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<td>Peak 2</td>
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<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Result quality:** Good

**Size Distribution by Intensity**

**Results**

- **Zeta Potential (mV):** -60.2
- **Zeta SD (mV):** 22.1
- **Conductivity (mS/cm):** 0.0742

**Mean (mV):**
- Peak 1: -73.8
- Peak 2: -48.6
- Peak 3: -17.1

**Area (%):**
- Peak 1: 49.0
- Peak 2: 43.1
- Peak 3: 6.7

**Width (mV):**
- Peak 1: 13.3
- Peak 2: 7.77
- Peak 3: 10.6

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

**Record 277: L30-1 1**
**Record 281: L30-2 1**
**Record 285: L30-3 1**
DLS Data For L-Mta 40% Nanoparticle

Results

Size (d.nm)... Width (d.nm)... % Intensity : Volume : Number
Peak 1: 99.05  24.53  100.0  100.0  100.0
Peak 2: 0.000  0.000  0.0  0.0  0.0
Peak 3: 0.000  0.000  0.0  0.0  0.0

Z-Average (d.nm): 92.84
Pdi: 0.054
Intercept: 0.921
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -56.5
Zeta SD (mV): 11.5
Conductivity (mS/cm): 0.0479

Mean (mV): Area (%) Width (mV):
Peak 1: -55.8 97.5 10.2
Peak 2: -92.7 2.0 4.69
Peak 3: -103 0.5 1.94

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Apparent Zeta Potential (mV)

Record 289: L40-1 1  Record 293: L40-2 1  Record 297: L40-3 1
Record 290: L40-1 1  Record 291: L40-1 2  Record 292: L40-1 3
DLS Data For L-Mta 50% Nanoparticle

**Results**

- **Z-Average (d.nm):** 48.79
- **PDI:** 0.215
- **Intercept:** 0.854
- **Result quality:** Good

**Size Distribution by Intensity**

**Results**

<table>
<thead>
<tr>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>-61.2</td>
<td>9.02</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>-33.1</td>
<td>10.1</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>-90.4</td>
<td>4.55</td>
</tr>
</tbody>
</table>

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**
DLS Data For L-Mta 60% Nanoparticle

Result quality: Good

Result:

Size (d.nm) Width (d.nm) % intensity: Volume: Number
Peak 1: 56.20 22.12 98.2 99.7 100.0 0.0
Peak 2: 4497 878.0 1.8 0.3 0.0 0.0
Peak 3: 0.000 0.000 0.0 0.0 0.0 0.0

Z-Average (d.nm): 50.24
PDI: 0.165
Intercept: 0.880

Size Distribution by Intensity

Mean (mV): Area (%): Width (mV):
Zeta Potential (mV): -53.6
Zeta SD (mV): 20.2
Conductivity (mS/cm): 0.106
Peak 1: -51.3 45.0 6.67
Peak 2: -67.1 27.9 5.45
Peak 3: -27.8 18.2 8.87

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Record 313: L60-1 1, Record 317: L60-2 1, Record 321: L60-3 1

Record 314: L60-1 1, Record 315: L60-1 2, Record 316: L60-1 3
DLS Data For L-Mta 70% Nanoparticle

Results

Z-Average (d.nm): 53.10
PDI: 0.128
Intercept: 0.920

Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -61.7
Zeta SD (mV): 24.3
Conductivity (mS/cm): 0.0652

Zeta potential out of range

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Record 326: L70-1 1
Record 327: L70-1 2
Record 328: L70-1 3
DLS Data For L-Mta 80% Nanoparticle

Results

**Z-Average (d.nm):** 47.31
**PDI:** 0.117
**Intercept:** 0.914

Result quality: Good

Size Distribution by Intensity

Results

**Zeta Potential (mV):** -45.7
**Zeta SD (mV):** 12.4
**Conductivity (mS/cm):** 0.0355

Mean (mV): Area (%) Width (mV):
Peak 1: -38.8 70.6 4.45
Peak 2: -62.8 29.4 7.84
Peak 3: 0.00 0.0 0.00

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

**Record 943: L80-1-1**
**Record 944: L80-1-2**
**Record 945: L80-1-3**
DLS Data For L-Mta 90% Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>Width (nm)</th>
<th>% Intensity</th>
<th>Volume (%)</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>82.43</td>
<td>87.96</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Z-Average (nm): 54.00
PDI: 0.223
Intercept: 0.888

Result quality: Good

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>-58.1</td>
<td>97.1</td>
<td>15.8</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>-109</td>
<td>1.9</td>
<td>4.23</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>-121</td>
<td>1.0</td>
<td>3.77</td>
</tr>
</tbody>
</table>

Zeta Potential Distribution

Zeta potential out of range
Conductivity is out of range - check cell or sample
DLS Data For L-Mta 100% Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>79.94</td>
<td>29.63</td>
<td>97.4</td>
<td>98.8</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>4534</td>
<td>859.0</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

PDI: 0.177
Intercept: 0.891
Result quality: Good

Size Distribution by Intensity

Zeta Potential (mV): -56.3
Zeta SD (mV): 20.1
Conductivity (mS/cm): 0.0782

Mean (mV): Area (%): Width (mV):
Peak 1: -67.5 49.9 12.5
Peak 2: -34.4 24.2 5.89
Peak 3: -47.0 23.7 4.16

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Record 349: L100-1 1
Record 353: L100-2 1
Record 357: L100-3 1
Record 350: L100-1 1
Record 351: L100-1 2
Record 352: L100-1 3
DLS Data For Rac-Mta 10% Nanoparticle

Results

Z-Average (d.nm): 53.60
Pdi: 0.103
Intercept: 0.662

Result quality: Good

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1: -52.2</td>
<td>99.1</td>
<td>17.5</td>
</tr>
<tr>
<td>Peak 2: -108</td>
<td>0.7</td>
<td>2.64</td>
</tr>
<tr>
<td>Peak 3: -140</td>
<td>0.1</td>
<td>1.78</td>
</tr>
</tbody>
</table>

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Record 129: RAC10-1 1
Record 133: RAC10-2 1
Record 137: RAC10-3 1

Record 130: RAC10-1 1
Record 131: RAC10-1 2
Record 132: RAC10-1 3
DLS Data For Rac-Mta 20% Nanoparticle

Results

Z-Average (d.nm): 56.04
Pdi: 0.061
Intercept: 0.920

Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -50.0
Zeta SD (mV): 14.2
Conductivity (mS/cm): 0.0398

Mean (mV): Area (%) Width (mV):
Peak 1: -49.8 97.5 12.5
Peak 2: -93.3 1.4 3.22
Peak 3: -7.84 0.9 2.97

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Record 142: RAC20-1.1  Record 143: RAC20-1.2  Record 144: RAC20-1.3
DLS Data For Rac-Mta 30% Nanoparticle

**Results**

**Z-Average (d.nm):** 53.20  
**Pdi:** 0.107  
**Intercept:** 0.932  
**Result quality:** Good

<table>
<thead>
<tr>
<th>Peak</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>59.37</td>
<td>20.39</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Size Distribution by Intensity**

- Record 157: RAC30-1 1  
- Record 161: RAC30-2 1  
- Record 165: RAC30-3 1

**Results**

**Zeta Potential (mV):** -47.8  
**Zeta SD (mV):** 21.7  
**Conductivity (mS/cm):** 0.0395

**Mean (mV):**  
- Peak 1: -36.8  
- Peak 2: -60.2  
- Peak 3: -86.1

**Area (%):**  
- Peak 1: 58.9  
- Peak 2: 32.0  
- Peak 3: 5.7

**Width (mV):**  
- Peak 1: 12.7  
- Peak 2: 7.06  
- Peak 3: 6.12

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

- Record 162: RAC30-2 1  
- Record 163: RAC30-2 2  
- Record 164: RAC30-2 3
DLS Data For Rac-Mta 40% Nanoparticle

Results

Size (d.nm): 58.90
PdI: 0.073
Intercept: 0.929

Result quality: Good

Size Distribution by Intensity

Recnt 169: RAC40-1.1
Record 173: RAC40-2.1
Record 177: RAC40-3.1

Results

Zeta Potential (mV): -51.4
Zeta SD (mV): 22.6
Conductivity (mS/cm): 0.0480

Mean (mV): Area (%) Width (mV):
Peak 1: -45.2 68.4 10.5
Peak 2: -75.1 23.3 11.6
Peak 3: -13.1 6.9 6.38

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Record 170: RAC40-1.1
Record 171: RAC40-1.2
Record 172: RAC40-1.3
DLS Data For Rac-Mta 50% Nanoparticle

Results

Z-Average (d.nm): 60.62
PDI: 0.080
Intercept: 0.931
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -41.5
Zeta SD (mV): 35.2
Conductivity (mS/cm): 0.0354

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Mean (mV): Area (%) Width (mV):
Peak 1: -34.0 90.2 20.2
Peak 2: -130 3.2 6.23
Peak 3: -93.1 2.3 3.87
DLS Data For Rac-Mta 60% Nanoparticle

**Results**

**Size (d.nm):**
- Size 1: 47.55
- Size 2: 51.68
- Size 3: 15.16

**% Intensity:**
- Peak 1: 100.0
- Peak 2: 0.0
- Peak 3: 0.0

**Volume:**
- Peak 1: 100.0
- Peak 2: 0.0
- Peak 3: 0.0

**Number:**
- Peak 1: 100.0
- Peak 2: 0.0
- Peak 3: 0.0

**PDI:** 0.078

**Intercept:** 0.918

**Result quality:** Good

**Size Distribution by Intensity**

**Zeta Potential (mV):** -34.0

**Zeta SD (mV):** 14.9

**Conductivity (mS/cm):** 0.0403

**Mean (mV):**
- Peak 1: -32.6
- Peak 2: -66.4
- Peak 3: -149

**Area (%):**
- Peak 1: 99.7
- Peak 2: 0.2
- Peak 3: 0.1

**Width (mV):**
- Peak 1: 7.99
- Peak 2: 1.12
- Peak 3: 0.00

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

**Record:**
- Record 194: RAC60-1 1
- Record 195: RAC60-1 2
- Record 196: RAC60-1 3
- Record 193: RAC60-1 1
- Record 197: RAC60-2 1
- Record 201: RAC60-3 1
DLS Data For Rac-Mta 70% Nanoparticle

Results

Z-Average (d.nm): 57.28  
PDI: 0.151  
Intercept: 0.933  
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -43.2  
Zeta SD (mV): 50.8  
Conductivity (mS/cm): 0.0365  
Zeta potential out of range

Mean (mV):  
Area (%):  
Width (mV):  
Peak 1: -37.3  
Peak 2: -101  
Peak 3: -121  
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Record 206: RAC70-1.1  
Record 207: RAC70-1.2  
Record 208: RAC70-1.3
DLS Data For Rac-Mta 80% Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1: 70.29</td>
<td>34.96</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2: 0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3: 0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Z-Average (d.nm):** 56.91

**PDI:** 0.187

**Intercept:** 0.902

**Result quality:** Good

**Size Distribution by Intensity**

---

**Results**

**Zeta Potential (mV):** -42.6

**Zeta SD (mV):** 31.1

**Conductivity (mS/cm):** 0.0419

**Mean (mV):**
- Peak 1: -40.2
- Peak 2: -116
- Peak 3: -126

**Area (%):**
- Peak 1: 91.9
- Peak 2: 2.4
- Peak 3: 2.2

**Width (mV):**
- Peak 1: 15.9
- Peak 2: 4.28
- Peak 3: 4.26

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

---

**Record 217: RAC80-1 1**
**Record 221: RAC80-2 1**
**Record 225: RAC80-3 1**

---

129
DLS Data For Rac-Mta 90% Nanoparticle

### Results

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta Potential (mV)</td>
<td>-53.6</td>
</tr>
<tr>
<td>Zeta SD (mV)</td>
<td>26.0</td>
</tr>
<tr>
<td>Conductivity (mS/cm)</td>
<td>0.0346</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-55.9</td>
<td>38.3</td>
<td>6.25</td>
</tr>
<tr>
<td>2</td>
<td>-40.7</td>
<td>25.8</td>
<td>4.99</td>
</tr>
<tr>
<td>3</td>
<td>-24.7</td>
<td>13.7</td>
<td>7.26</td>
</tr>
</tbody>
</table>

Conductivity is out of range - check cell or sample

### Zeta Potential Distribution

![Zeta Potential Distribution Graph]

### Results

<table>
<thead>
<tr>
<th>Z-Average (d.nm)</th>
<th>50.95</th>
</tr>
</thead>
<tbody>
<tr>
<td>PdI</td>
<td>0.081</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.898</td>
</tr>
</tbody>
</table>

Result quality: Good

### Size Distribution by Intensity

![Size Distribution by Intensity Graph]
DLS Data For Rac-Mta 100% Nanoparticle

Results

Z-Average (d.nm): 50.86
PDI: 0.134
Intercept: 0.920
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -46.1
Zeta SD (mV): 32.4
Conductivity (mS/cm): 0.0432

Mean (mV): Area (%) Width (mV):
Peak 1: -43.5 69.4 11.7
Peak 2: -16.0 15.2 6.31
Peak 3: -80.5 5.6 6.79

Conductivity is out of range - check cell or sample
DLS Data For 1% PenG Loaded D-Mta Nanoparticle

Results

Z-Average (d.nm): 52.94
Pdi: 0.118
Intercept: 0.933

Result quality: Good

Size Distribution by Intensity

Mean (mV): Zeta Potential (mV): -62.2
Zeta SD (mV): 14.2
Conductivity (mS/cm): 0.0670

Peak 1: 59.49 21.67 100.0 100.0 100.0
Peak 2: 0.000 0.000 0.0 0.0 0.0
Peak 3: 0.000 0.000 0.0 0.0 0.0

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution
DLS Data For 2% PenG Loaded D-Mta Nanoparticle

Results

Size (d.nm) Width (d.nm) % Intensity Volume Number
Peak 1: 69.11 20.53 100.0 100.0 100.0
Peak 2: 0.000 0.000 0.0 0.0 0.0
Peak 3: 0.000 0.000 0.0 0.0 0.0

Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -59.5
Zeta SD (mV): 8.65
Conductivity (mS/cm): 0.0659

Mean (mV): Area (%) Width (mV):
Peak 1: -61.2 89.1 6.67
Peak 2: -41.7 10.9 3.98
Peak 3: 0.00 0.0 0.0

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution
DLS Data For 4% PenG Loaded D-Mta Nanoparticle

Results

**Size (d.nm)**

- **Average (d.nm):** 56.40
- **Pdi:** 0.095
- **Intercept:** 0.929

Result quality: Good

Size Distribution by Intensity

![Size Distribution by Intensity Graph](image)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>62.38</td>
<td>20.13</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
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<tr>
<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Results

**Zeta Potential (mV):** -58.9
**Zeta SD (mV):** 10.1
**Conductivity (mS/cm):** 0.0740

Conductivity is out of range - check cell or sample

Zeta potential out of range

![Zeta Potential Distribution Graph](image)
DLS Data For 5% PenG Loaded D-Mta Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d nm)</th>
<th>Width (d nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>65.45</td>
<td>21.05</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Z-Average (d nm):** 59.36

**PDI:** 0.089

**Intercept:** 0.922

**Result quality:** Good

**Size Distribution by Intensity**

**Results**

- **Zeta Potential (mV):** -62.0
- **Zeta SD (mV):** 11.2
- **Conductivity (mS/cm):** 0.0847

<table>
<thead>
<tr>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>-55.9</td>
<td>68.9</td>
</tr>
<tr>
<td>Peak 2</td>
<td>-76.1</td>
<td>31.1</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.00</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**
DLS Data For 6% PenG Loaded D-Mta Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>80.76</td>
<td>24.25</td>
<td>100.0</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

PdI: 0.083
Intercept: 0.940
Result quality: Good

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-61.0</td>
<td>Peak 1: -69.5</td>
<td>56.0</td>
<td>8.68</td>
</tr>
<tr>
<td></td>
<td>Peak 2: -49.7</td>
<td>43.1</td>
<td>7.95</td>
</tr>
<tr>
<td></td>
<td>Peak 3: -16.9</td>
<td>0.5</td>
<td>3.93</td>
</tr>
</tbody>
</table>

Zeta Potential Distribution

Conductivity is out of range - check cell or sample
DLS Data For 7% PenG Loaded D-Mta Nanoparticle

Results

**Z-Average (d.nm):** 61.43

**PDI:** 0.103

**Intercept:** 0.947

**Result quality:** Good

**Size Distribution by Intensity**

**Zeta Potential (mV):** -66.0

**Zeta SD (mV):** 29.5

**Conductivity (mS/cm):** 0.0854

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Mean (mV):**

- Peak 1: -81.4
- Peak 2: -14.3
- Peak 3: -102

**Area (%):**

- Peak 1: 72.2
- Peak 2: 22.7
- Peak 3: 3.0

**Width (mV):**

- Peak 1: 7.01
- Peak 2: 5.07
- Peak 3: 3.88

**Zeta Potential Distribution**

**Total Counts**

- 0
  - 100000
  - 200000
  - 300000

**Apparent Zeta Potential (mV):**

- 0
  - -20
  - -40
  - -60
  - -80
  - -100

- 0
  - 100
  - 200
  - 300
  - 400
  - 500

- 0
  - 100
  - 200
  - 300
  - 400
  - 500

- 0
  - 100
  - 200
  - 300
  - 400
  - 500
DLS Data For 8% PenG Loaded D-Mta Nanoparticle

Results

Z-Average (d.nm): 65.02
PdI: 0.074
Intercept: 0.944

Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -53.0
Zeta SD (mV): 12.8
Conductivity (mS/cm): 0.0621

Mean (mV): Area (%) Width (mV):
Peak 1: -45.8 59.0 8.85
Peak 2: -64.2 41.0 7.31
Peak 3: 0.00 0.00 0.00

Conductivity is out of range - check cell or sample
DLS Data For 9% PenG Loaded D-Mta Nanoparticle

---

**Results**

- **Z-Average (d.nm):** 60.93
- **PdI:** 0.152
- **Intercept:** 0.926
- **Result quality:** Good

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1: 68.28</td>
<td>26.04</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2: 0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3: 0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Size Distribution by Intensity**

---

**Results**

- **Zeta Potential (mV):** -53.0
- **Zeta SD (mV):** 12.8
- **Conductivity (mS/cm):** 0.0621

**Area (%)**

- Peak 1: 59.0
- Peak 2: 41.0
- Peak 3: 0.0

**Width (mV):**

- Peak 1: 8.85
- Peak 2: 7.31
- Peak 3: 0.0

**Conductivity is out of range - check cell or sample**

---

**Zeta Potential Distribution**

---
DLS Data For 10% PenG Loaded D-Mta Nanoparticle

Results

Z-Average (d.nm): 68.04
Pdi: 0.134
Intercept: 0.940

Result quality: Good

Size Distribution by Intensity

Mean (mV): Area (%) Width (mV):
Zeta Potential (mV): -55.2
Peak 1: -55.2 100.0 16.0
Zeta SD (mV): 16.0
Peak 2: 0.00 0.0 0.0
Conductivity (mS/cm): 0.0633
Peak 3: 0.00 0.0 0.0

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Apparent Zeta Potential (mV)
DLS Data For 11% PenG Loaded D-Mta Nanoparticle

Results

Z-Average (d.nm): 63.26
PDI: 0.063
Intercept: 0.946
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -78.4
Zeta SD (mV): 9.05
Conductivity (mS/cm): 0.102

Zeta potential out of range
Conductivity is out of range - check cell or sample
DLS Data For 12% PenG Loaded D-Mta Nanoparticle

Results

**Z-Average (d.nm):** 79.73

**PDI:** 0.068

**Intercept:** 0.931

**Result quality:** Good

![Size Distribution by Intensity](image)

**Zeta Potential (mV):** -66.1

**Zeta SD (mV):** 16.6

**Conductivity (mS/cm):** 0.0737

**Mean (mV):**
- Peak 1: -69.5
- Peak 2: -46.0
- Peak 3: -99.6

**Area (%):**
- Peak 1: 67.1
- Peak 2: 25.3
- Peak 3: 7.6

**Width (mV):**
- Peak 1: 9.64
- Peak 2: 7.36
- Peak 3: 6.24

*Zeta potential out of range*

*Conductivity is out of range - check cell or sample*
DLS Data For 13% PenG Loaded D-Mta Nanoparticle

### Size Distribution by Intensity

<table>
<thead>
<tr>
<th>Peak</th>
<th>Intensity</th>
<th>Size (d. nm)</th>
<th>Width (d. nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>63.26</td>
<td>22.84</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Result quality: Good

### Zeta Potential Distribution

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta Potential (mV): -65.9</td>
<td>Peak 1: -66.0</td>
<td>99.8</td>
<td>11.5</td>
</tr>
<tr>
<td>Zeta SD (mV): 11.6</td>
<td>Peak 2: -26.9</td>
<td>0.2</td>
<td>2.46</td>
</tr>
<tr>
<td>Conductivity (mS/cm): 0.0634</td>
<td>Peak 3: 0.00</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Zeta potential out of range

Conductivity is out of range - check cell or sample
DLS Data For 14% PenG Loaded D-Mta Nanoparticle

Results

Z-Average (d.nm): 79.73
Pdi: 0.068
Intercept: 0.931

Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -58.1
Zeta SD (mV): 15.3
Conductivity (mS/cm): 0.0596

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Mean (mV): Area (%): Width (mV):
Peak 1: -67.5 50.3 9.57
Peak 2: -47.9 48.4 9.58
Peak 3: -113 0.7 4.06
DLS Data For 15% PenG Loaded D-Mta Nanoparticle

Results

Z-Average (d.nm): 55.71
Pdi: 0.107
Intercept: 0.929
Result quality: Good

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-57.5</td>
<td>Peak 1:</td>
<td>100.0</td>
<td>13.9</td>
</tr>
<tr>
<td>Zeta SD (mV): 13.9</td>
<td>Peak 2:</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Conductivity (mS/cm): 0.0534</td>
<td>Peak 3:</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution
DLS Data For 16% PenG Loaded D-Mta Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume: Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>77.82</td>
<td>50.85</td>
<td>97.7</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>3952</td>
<td>1101</td>
<td>2.3</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Result quality:** Good

- **Z-Average (d.nm):** 57.16
- **Pdi:** 0.262
- **Intercept:** 0.903

**Size Distribution by Intensity**

**Results**

- **Zeta Potential (mV):** -60.6
- **Zeta SD (mV):** 16.3
- **Conductivity (mS/cm):** 0.0571

- **Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

**Mean (mV):**
- Peak 1: -59.7, Area (%): 95.7, Width (mV): 14.8
- Peak 2: -98.5, Area (%): 3.8, Width (mV): 4.20
- Peak 3: -6.82, Area (%): 0.5, Width (mV): 3.79
DLS Data For 17% PenG Loaded D-Mta Nanoparticle

**Results**

**Z-Average (d.nm):** 106.1
**Pdi:** 0.007
**Intercept:** 0.928
**Result quality:** Good

**Size Distribution by Intensity**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>110.6</td>
<td>25.18</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Zeta Potential (mV):** -68.7
**Zeta SD (mV):** 14.8
**Conductivity (mS/cm):** 0.0935

**Mean (mV):**
- Peak 1: -60.2 51.7 7.66
- Peak 2: -81.3 44.7 6.53
- Peak 3: -30.0 3.6 5.63

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**
DLS Data For 18% PenG Loaded D-Mta Nanoparticle

**Results**

- **Z-Average (d.nm):** 97.65
- **PDI:** 0.075
- **Intercept:** 0.936

**Result quality:** Good

**Size Distribution by Intensity**

**Results**

- **Zeta Potential (mV):** -41.6
- **Zeta SD (mV):** 9.75
- **Conductivity (mS/cm):** 0.0787

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

<table>
<thead>
<tr>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>-43.7</td>
<td>90.3</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>-19.1</td>
<td>9.7</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.00</td>
<td>0.0</td>
</tr>
</tbody>
</table>
DLS Data For 19% PenG Loaded D-Mta Nanoparticle

Results

**Z-Average (d.nm):** 61.49

**PDI:** 0.108

**Intercept:** 0.938

**Result quality:** Good

![Size Distribution by Intensity](image1)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67.33</td>
<td>20.83</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Results**

**Zeta Potential (mV):** -41.0

**Zeta SD (mV):** 14.6

**Conductivity (mS/cm):** 0.112

**Conductivity is out of range - check cell or sample**

![Zeta Potential Distribution](image2)

**Zeta Potential Distribution**

<table>
<thead>
<tr>
<th>Total Counts</th>
<th>Apparent Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40000</td>
<td>-180</td>
</tr>
<tr>
<td>30000</td>
<td>-160</td>
</tr>
<tr>
<td>20000</td>
<td>-140</td>
</tr>
<tr>
<td>10000</td>
<td>-120</td>
</tr>
<tr>
<td>0</td>
<td>-100</td>
</tr>
<tr>
<td>0</td>
<td>-80</td>
</tr>
<tr>
<td>0</td>
<td>-60</td>
</tr>
<tr>
<td>0</td>
<td>-40</td>
</tr>
<tr>
<td>0</td>
<td>-20</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
DLS Data For 20% PenG Loaded D-Mta Nanoparticle

**Results**

- **Z-Average (d.nm):** 86.40
- **Pdl:** 0.182
- **Intercept:** 0.873
- **Result quality:** Good

<table>
<thead>
<tr>
<th>Size (d.n.m)</th>
<th>Width (d.n.m)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>107.6</td>
<td>48.14</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Zeta Potential (mV):** -34.8
- **Zeta SD (mV):** 24.5
- **Conductivity (mS/cm):** 0.0981

“Zeta potential out of range”

“Conductivity is out of range - check cell or sample”

**Results**

<table>
<thead>
<tr>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>-14.2</td>
<td>47.3</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>-45.8</td>
<td>31.8</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>-76.3</td>
<td>11.4</td>
</tr>
</tbody>
</table>
DLS Data For 1% PenG Loaded L-Mta Nanoparticle

Results

Z-Average (d.nm): 56.64
PDI: 0.427
Intercept: 0.940
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -68.0
Zeta SD (mV): 12.8
Conductivity (mS/cm): 0.0587

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Mean (mV):  Area (%):  Width (mV):
Peak 1:  -63.4  58.5  8.60
Peak 2:  -79.6  37.5  4.72
Peak 3:  -35.4   4.0  4.13
DLS Data For 2% PenG Loaded L-Mta Nanoparticle

Results

Z-Average (d.nm): 66.45
Pdi: 0.250
Intercept: 0.928
Result quality: Good

Size Distribution by Intensity

Results

Zeta Potential (mV): -51.3
Zeta SD (mV): 10.8
Conductivity (mS/cm): 0.0756

Mean (mV): Area (%): Width (mV):
Peak 1: -51.7 98.3 10.1
Peak 2: -18.6 1.7 3.28
Peak 3: 0.00 0.0 0.0

Conductivity is out of range - check cell or sample
DLS Data For 3% PenG Loaded L-Mta Nanoparticle

**Results**

**Z-Average (d.nm):** 50.56  
**Pdi:** 0.158  
**Intercept:** 0.905  
**Result quality:** Good

**Size Distribution by Intensity**

**Results**

**Zeta Potential (mV):** -59.6  
**Zeta SD (mV):** 17.4  
**Conductivity (mS/cm):** 0.0572  
**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**
DLS Data For 4% PenG Loaded L-Mta Nanoparticle

**Results**

- **Z-Average (d.nm):** 65.60
- **Pdi:** 0.268
- **Intercept:** 0.924
- **Result quality:** Good

**Size Distribution by Intensity**

**Results**

- **Zeta Potential (mV):** -59.5
- **Zeta SD (mV):** 18.4
- **Conductivity (mS/cm):** 0.111

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-58.9</td>
<td>58.5</td>
<td>8.81</td>
</tr>
<tr>
<td>2</td>
<td>-43.0</td>
<td>30.5</td>
<td>4.21</td>
</tr>
<tr>
<td>3</td>
<td>-101</td>
<td>11.0</td>
<td>5.05</td>
</tr>
</tbody>
</table>

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**
DLS Data For 5% PenG Loaded L-Mta Nanoparticle

Results

Size (d.nm) Width (d.nm) % Intensity: Volume: Number
Peak 1: 109.0 95.16 94.1 96.2 100.0
Peak 2: 1223 595.5 5.9 3.8 0.0
Peak 3: 0.000 0.000 0.0 0.0 0.0

Result quality: Good

Results

Mean (mV): Area (%) Width (mV):
Zeta Potential (mV): -65.2 Peak 1: -65.2 100.0 9.65
Zeta SD (mV): 9.65 Peak 2: 0.00 0.0 0.00
Conductivity (mS/cm): 0.0565 Peak 3: 0.00 0.0 0.00

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

400000
300000
200000
100000
0
-180 -160 -140 -120 -100 -80 -60 -40 -20 0
Apparent Zeta Potential (mV)
DLS Data For 6% PenG Loaded L-Mta Nanoparticle

Results

Z-Average (d.nm): 77.82
PDI: 0.120
Intercept: 0.882
Result quality: Good

Results

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-71.4</td>
<td>55.7</td>
<td>9.99</td>
</tr>
<tr>
<td>2</td>
<td>-50.1</td>
<td>39.9</td>
<td>7.18</td>
</tr>
<tr>
<td>3</td>
<td>-26.8</td>
<td>4.4</td>
<td>5.24</td>
</tr>
</tbody>
</table>

Zeta potential out of range

Conductivity is out of range - check cell or sample
DLS Data For 7% PenG Loaded L-Mta Nanoparticle

**Results**

**Z-Average (d.nm):** 72.72

**PDI:** 0.243

**Intercept:** 0.879

**Result quality:** Good

**Size Distribution by Intensity**

- **Peak 1:** 79.94 nm, 94.8% intensity, 98.2% volume, 100.0% number
- **Peak 2:** 4322 nm, 5.2% intensity, 1.8% volume, 0.0% number
- **Peak 3:** 0.000 nm, 0.0% intensity, 0.0% volume, 0.0% number

**Zeta Potential (mV):** -54.6

**Zeta SD (mV):** 17.6

**Conductivity (mS/cm):** 0.0626

**Mean (mV):**
- **Peak 1:** -50.4 mV, 47.4% area, 6.42 mV width
- **Peak 2:** -70.4 mV, 37.6% area, 10.6 mV width
- **Peak 3:** -26.9 mV, 15.0% area, 8.72 mV width

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

- Total Counts
- Apparent Zeta Potential (mV)
DLS Data For 8% PenG Loaded L-Mta Nanoparticle

### Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>108.0</td>
<td>61.24</td>
<td>98.7</td>
<td>47.2</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>14.62</td>
<td>2.981</td>
<td>1.3</td>
<td>52.8</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Z-Average (d.nm):** 81.11  
**PDI:** 0.240  
**Intercept:** 0.829  
**Result quality:** Good

**Size Distribution by Intensity**

![Size Distribution by Intensity](image)

### Results

**Zeta Potential (mV):** -57.1  
**Zeta SD (mV):** 10.5  
**Conductivity (mS/cm):** 0.0444

**Mean (mV):**  
Peak 1: -56.7  
Peak 2: -84.7  
Peak 3: 0.00

**Area (%):**  
Peak 1: 97.8  
Peak 2: 2.2  
Peak 3: 0.0

**Width (mV):**  
Peak 1: 10.1  
Peak 2: 2.91  
Peak 3: 0.0  

**Zeta potential out of range**  
**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

![Zeta Potential Distribution](image)
DLS Data For 9% PenG Loaded L-Mta Nanoparticle

**Results**

<table>
<thead>
<tr>
<th></th>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>92.13</td>
<td>41.25</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Z-Average (d.nm):** 74.92

**PDI:** 0.174

**Intercept:** 0.915

**Result quality:** Good

**Size Distribution by Intensity**

**Results**

**Zeta Potential (mV):** -54.6

**Zeta SD (mV):** 15.5

**Conductivity (mS/cm):** 0.0594

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**
DLS Data For 10% PenG Loaded L-Mta Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Z-Average (d.nm):</th>
<th>89.83</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdl:</td>
<td>0.116</td>
</tr>
<tr>
<td>Intercept:</td>
<td>0.912</td>
</tr>
</tbody>
</table>

**Result quality:** Good

![Size Distribution by Intensity](image)

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>102.3</td>
<td>37.26</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Results**

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>-67.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta SD (mV):</td>
<td>5.84</td>
</tr>
<tr>
<td>Conductivity (mS/cm):</td>
<td>0.0840</td>
</tr>
</tbody>
</table>

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

![Zeta Potential Distribution](image)

**Mean (mV):**

<table>
<thead>
<tr>
<th>Peak 1:</th>
<th>-67.6</th>
<th>100.0</th>
<th>5.84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 2:</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
DLS Data For 11% PenG Loaded L-Mta Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Z-Average (d.nm): 81.63
- Pdl: 0.040
- Intercept: 0.941

**Result quality:** Good

**Size Distribution by Intensity**

---

**Results**

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>Mean (mV):</th>
<th>Area (%)</th>
<th>Width (mV):</th>
</tr>
</thead>
<tbody>
<tr>
<td>-68.7</td>
<td>Peak 1: -71.1</td>
<td>91.4</td>
<td>8.20</td>
</tr>
<tr>
<td></td>
<td>Peak 2: -41.7</td>
<td>8.6</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>Peak 3: 0.00</td>
<td>0.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**

---
DLS Data For 12% PenG Loaded L-Mta Nanoparticle

**Results**

**Size (d.nm...)**

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Average: 98.86</td>
<td>109.2</td>
<td>0.000</td>
</tr>
<tr>
<td>PDI: 0.086</td>
<td>34.70</td>
<td>0.000</td>
</tr>
<tr>
<td>Intercept: 0.903</td>
<td>100.0</td>
<td>0.000</td>
</tr>
<tr>
<td>% Intensity:</td>
<td>100.0</td>
<td>0.000</td>
</tr>
<tr>
<td>Volume:</td>
<td>100.0</td>
<td>0.000</td>
</tr>
<tr>
<td>Number:</td>
<td>100.0</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Result quality:** Good

---

**Size Distribution by Intensity**

---

**Results**

**Zeta Potential (mV):** -61.5

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (mV):</td>
<td>-61.5</td>
<td>0.00</td>
</tr>
<tr>
<td>Zeta SD (mV):</td>
<td>11.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Conductivity (mS/cm):</td>
<td>0.0626</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Area (%):**

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Width (mV):**

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

---

**Zeta Potential Distribution**

---
DLS Data For 13% PenG Loaded L-Mta Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>109.1</td>
<td>46.71</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Peak 1: 125.3 46.71 100.0 100.0 100.0
Peak 2: 0.000 0.000 0.0 0.0 0.0
Peak 3: 0.000 0.000 0.0 0.0 0.0

Result quality: Good

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-69.6</td>
<td>Peak 1:</td>
<td>47.6</td>
<td>6.05</td>
</tr>
<tr>
<td>Zeta SD (mV): 13.2</td>
<td>Peak 2:</td>
<td>45.5</td>
<td>7.53</td>
</tr>
<tr>
<td>Conductivity (mS/cm): 0.0594</td>
<td>Peak 3:</td>
<td>5.6</td>
<td>3.77</td>
</tr>
</tbody>
</table>

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution
DLS Data For 14% PenG Loaded L-Mta Nanoparticle

**Results**

**Size (d.nm... Width (d.nm... % Intensity : Volume : Number**

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (d.nm)</td>
<td>Width (d.nm)</td>
<td>% Intensity</td>
</tr>
<tr>
<td>86.26</td>
<td>35.08</td>
<td>100.0</td>
</tr>
<tr>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
</tr>
<tr>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Result quality: Good

**Size Distribution by Intensity**

**Results**

**Zeta Potential (mV):** -53.0

**Zeta SD (mV):** 12.8

**Conductivity (mS/cm):** 0.0621

**Mean (mV):** 59.0

**Area (%):** 8.85

**Width (mV):** 7.31

**Peak 1:** -45.8

**Peak 2:** -64.2

**Peak 3:** 0.00

Conductivity is out of range - check cell or sample

**Zeta Potential Distribution**
DLS Data For 15% PenG Loaded L-Mta Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>69.11</td>
<td>20.53</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

RESULT quality: Good

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>-53.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta SD (mV):</td>
<td>6.71</td>
</tr>
<tr>
<td>Conductivity (mS/cm):</td>
<td>0.0587</td>
</tr>
</tbody>
</table>

Mean (mV): | Area (%) | Width (mV): |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>-53.8</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>0.000</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Apparent Zeta Potential (mV)
DLS Data For 16% PenG Loaded L-Mta Nanoparticle

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>68.44</td>
<td>28.72</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Result quality:** Good

**Size Distribution by Intensity**

**Results**

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (mV):</td>
<td>-77.2</td>
<td>6.87</td>
</tr>
<tr>
<td>Peak 1:</td>
<td>49.5</td>
<td></td>
</tr>
<tr>
<td>Peak 2:</td>
<td>43.5</td>
<td>7.57</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>7.0</td>
<td>4.40</td>
</tr>
</tbody>
</table>

Zeta potential out of range

Conductivity is out of range - check cell or sample

**Zeta Potential Distribution**
DLS Data For 17% PenG Loaded L-Mta Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>62.65</td>
<td>21.90</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Result quality: Good

Size Distribution by Intensity

<table>
<thead>
<tr>
<th>Intensity (Percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean (mV)</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>-61.7</td>
<td>79.3</td>
</tr>
<tr>
<td>Peak 2</td>
<td>-76.1</td>
<td>20.7</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.00</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Apparent Zeta Potential (mV)

168
DLS Data For 18% PenG Loaded L-Mta Nanoparticle

Results

- **Z-Average (d.nm):** 82.00
- **Pdi:** 0.082
- **Intercept:** 0.930
- **Result quality:** Good

<table>
<thead>
<tr>
<th>Peak</th>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89.87</td>
<td>28.02</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Size Distribution by Intensity

- **Zeta Potential (mV):** -66.1
- **Zeta SD (mV):** 16.6
- **Conductivity (mS/cm):** 0.0737

- **Zeta potential out of range**
- **Conductivity is out of range - check cell or sample**
DLS Data For 19% PenG Loaded L-Mta Nanoparticle

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1: 86.75</td>
<td>26.26</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2: 0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3: 0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Result quality: Good

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>Mean (mV):</th>
<th>Area (%)</th>
<th>Width (mV):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta SD (mV): 13.5</td>
<td>Peak 1: -59.2</td>
<td>99.5</td>
<td>13.1</td>
</tr>
<tr>
<td>Conductivity (mS/cm): 0.0513</td>
<td>Peak 2: -11.3</td>
<td>0.5</td>
<td>3.01</td>
</tr>
<tr>
<td>Zeta potential out of range</td>
<td>Peak 3: 0.00</td>
<td>0.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Zeta Potential Distribution

Conductivity is out of range - check cell or sample
DLS Data For 20% PenG Loaded L-Mta Nanoparticle

**Results**

- **Z-Average (d.nm):** 89.83
- **PDI:** 0.116
- **Intercept:** 0.912

**Result quality:** Good

**Size Distribution by Intensity**

**Results**

- **Zeta Potential (mV):** -55.4
- **Zeta SD (mV):** 16.0
- **Conductivity (mS/cm):** 0.0572

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**
DLS Data For 3% N-Acryloyl-5-ASA in Poly(L-Menthyl Acrylate) Nanoparticle Emulsion

Results

Z-Average (d.nm): 42.81
Pdl: 0.141
Intercept: 0.936
Result quality: Good

Size Distribution by Intensity

Mean (mV): Area (%) Width (mV):
Peak 1: -54.7 100.0 7.10
Peak 2: 0.00 0.0 0.0
Peak 3: 0.00 0.0 0.0

Conductivity is out of range - check cell or sample
DLS Data For 3% N-Acetyl-5-ASA in Poly(L-Menthyl Acrylate) Nanoparticle Emulsion

**Results**

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>71.50</td>
<td>23.02</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Z-Average (d.nm):** 64.74

**Pdl:** 0.090

**Intercept:** 0.920

**Result quality:** Good

**Size Distribution by Intensity**

**Results**

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>-47.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta SD (mV):</td>
<td>23.7</td>
</tr>
<tr>
<td>Conductivity (mS/cm):</td>
<td>2.58</td>
</tr>
</tbody>
</table>

**Mean (mV):**

- Peak 1: -39.8
- Peak 2: -66.8
- Peak 3: -16.2

**Area (%):**

- Peak 1: 44.4
- Peak 2: 37.7
- Peak 3: 11.3

**Width (mV):**

- Peak 1: 8.55
- Peak 2: 10.8
- Peak 3: 5.45

**Conductivity is out of range - check cell or sample**

**Zeta Potential Distribution**
DLS Data For 1% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

Results

Z-Average (d.nm): 2133
Pdi: 1.000
Intercept: 0.836

Result quality: Refer to quality report

Size Distribution by Intensity

Results

Zeta Potential (mV): -59.0
Zeta SD (mV): 27.6
Conductivity (mS/cm): 0.388

Zeta potential out of range

Conductivity is out of range - check cell or sample

Zeta Potential Distribution
DLS Data For 2% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

Results

<table>
<thead>
<tr>
<th>Size (d.nm...)</th>
<th>Width (d.nm...)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>856.4</td>
<td>138.0</td>
<td>83.3</td>
<td>0.1</td>
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<tr>
<td>Peak 2</td>
<td>3.966</td>
<td>0.7993</td>
<td>16.7</td>
<td>99.9</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Result quality: Refer to quality report

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV)</th>
<th>Peak 1</th>
<th>Area (%)</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-65.3</td>
<td>-93.6</td>
<td>41.9</td>
<td>12.6</td>
</tr>
<tr>
<td>Zeta SD (mV)</td>
<td>Peak 2</td>
<td>-38.0</td>
<td>8.46</td>
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<tr>
<td>32.1</td>
<td>Peak 3</td>
<td>-60.4</td>
<td>7.20</td>
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<tr>
<td>Conductivity (mS/cm)</td>
<td>Peak 1</td>
<td>22.3</td>
<td>0.371</td>
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</tbody>
</table>

Zeta potential out of range

Conductivity is out of range - check cell or sample

Zeta Potential Distribution
### Results

#### Size Distribution by Intensity

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>913.9</td>
<td>140.7</td>
<td>88.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>4.376</td>
<td>0.6091</td>
<td>11.8</td>
<td>99.8</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Result quality: Refer to quality report

#### Zeta Potential Distribution

- **Mean (mV):**
  - Peak 1: -51.8
  - Peak 2: -94.9
  - Peak 3: -74.9

- **Area (%):**
  - Peak 1: 42.2
  - Peak 2: 34.5
  - Peak 3: 15.5

- **Width (mV):**
  - Peak 1: 12.2
  - Peak 2: 12.2
  - Peak 3: 4.13

Zeta potential out of range

Conductivity is out of range - check cell or sample

---

DLS Data For 3% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion
DLS Data For 4% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

**Results**

<table>
<thead>
<tr>
<th>Z-Average (d.nm):</th>
<th>1866</th>
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<tr>
<td>PdI:</td>
<td>1.000</td>
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<tr>
<td>Intercept:</td>
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Result quality: Refer to quality report

**Size Distribution by Intensity**

**Results**

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>Mean (mV):</th>
<th>Area (%)</th>
<th>Width (mV):</th>
</tr>
</thead>
<tbody>
<tr>
<td>-62.1</td>
<td>Peak 1: -41.7</td>
<td>32.0</td>
<td>7.47</td>
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<tr>
<td>Zeta SD (mV):</td>
<td>Peak 2: -92.5</td>
<td>30.8</td>
<td>10.5</td>
</tr>
<tr>
<td>30.4</td>
<td>Peak 3: -61.8</td>
<td>24.1</td>
<td>6.93</td>
</tr>
<tr>
<td>Conductivity (mS/cm):</td>
<td>0.421</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Zeta potential out of range     Conductivity is out of range - check cell or sample

**Zeta Potential Distribution**
DLS Data For 5% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

Results

Size (d.nm) Width (d.nm) % Intensity Volume Number
Peak 1: 961.2 151.0 87.0 0.1 100.0
Peak 2: 3.454 0.5552 13.0 99.9 0.0
Peak 3: 0.000 0.000 0.0 0.0 0.0

Result quality: Refer to quality report

Size Distribution by Intensity

Results

Zeta Potential (mV): -66.2
Zeta SD (mV): 25.7
Conductivity (mS/cm): 0.519

Mean (mV): Area (%) Width (mV):
Peak 1: -55.5 48.3 9.91
Peak 2: -93.7 29.5 11.8
Peak 3: -74.7 10.8 2.97

Zeta potential out of range Conductivity is out of range - check cell or sample

Zeta Potential Distribution
DLS Data For 6% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>975.4</td>
<td>122.3</td>
<td>89.2</td>
<td>0.2</td>
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<tr>
<td>Peak 2</td>
<td>4.129</td>
<td>1.204</td>
<td>10.8</td>
<td>99.8</td>
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<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
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</table>

PDI: 0.897

Intercept: 0.841

Result quality: Refer to quality report

Size Distribution by Intensity

Results

Zeta Potential (mV): -57.5
Zeta SD (mV): 26.0
Conductivity (mS/cm): 0.539

Mean (mV): Peak 1: -50.8, Area (%): 52.3, Width (mV): 8.92
Peak 2: -73.3, 16.9, 6.08
Peak 3: -93.4, 12.2, 7.25

Zeta potential out of range
Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Total Counts

Apparent Zeta Potential (mV)
DLS Data For 7% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

### Results

- **Z-Average (d.nm):** 1943
- **PDI:** 0.780
- **Intercept:** 0.823

**Result quality:** Refer to quality report

#### Size Distribution by Intensity

- **Size (d.nm):**
  - Peak 1: 1044, 224.9, 87.4, 0.2, 100.0
  - Peak 2: 4300, 1236, 12.6, 99.8, 0.0
  - Peak 3: 0.000, 0.000, 0.0, 0.0, 0.0

#### Zeta Potential (mV):
- **Mean:** -68.7
- **SD:** 22.4
- **Conductivity (mS/cm):** 0.129

**Zeta potential out of range**

**Conductivity is out of range - check cell or sample**
DLS Data For 8% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

Results

Z-Average (d.nm): 2476
PDI: 0.891
Intercept: 0.838

Result quality: Refer to quality report

Size Distribution by Intensity

Results

Zeta Potential (mV): -69.0
Zeta SD (mV): 21.6
Conductivity (mS/cm): 0.539

Zeta potential out of range

Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Results

Mean (mV): Area (%): Width (mV):
Peak 1: -74.4 29.6 5.10
Peak 2: -57.8 28.6 6.81
Peak 3: -91.2 28.3 8.67

181
DLS Data For 9% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

Results

<table>
<thead>
<tr>
<th>Size (d.nm)</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>1095</td>
<td>223.6</td>
<td>87.4</td>
<td>0.2</td>
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<tr>
<td>Peak 2:</td>
<td>3.711</td>
<td>0.6479</td>
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<td>99.8</td>
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<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Z-Average (d.nm): 1954
Pdl: 0.914
Intercept: 0.819

Result quality: Refer to quality report

Size Distribution by Intensity

Results

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>Mean (mV):</th>
<th>Area (%)</th>
<th>Width (mV):</th>
</tr>
</thead>
<tbody>
<tr>
<td>-60.5</td>
<td>Peak 1:</td>
<td>-90.8</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>Peak 2:</td>
<td>-30.0</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>Peak 3:</td>
<td>-49.5</td>
<td>21.4</td>
</tr>
</tbody>
</table>

Zeta potential out of range Conductivity is out of range - check cell or sample

Zeta Potential Distribution

Conductivity (mS/cm): 0.533
DLS Data For 10% N-Acryloyl Ciprofloxacin Nanoparticle Emulsion

**Results**

### Size (d.nm) Distribution

<table>
<thead>
<tr>
<th>Peak</th>
<th>d.nm</th>
<th>Width (d.nm)</th>
<th>% Intensity</th>
<th>Volume</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>1161</td>
<td>244.1</td>
<td>89.5</td>
<td>0.2</td>
<td>100.0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>3.672</td>
<td>0.5581</td>
<td>10.5</td>
<td>99.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.000</td>
<td>0.000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Z-Average (d.nm):** 1693

**PDI:** 0.782

**Intercept:** 0.800

Result quality: Refer to quality report

### Size Distribution by Intensity

![Size Distribution by Intensity Graph](image)

### Zeta Potential and Conductivity

<table>
<thead>
<tr>
<th>Zeta Potential (mV):</th>
<th>Mean (mV):</th>
<th>Area (%)</th>
<th>Width (mV):</th>
</tr>
</thead>
<tbody>
<tr>
<td>-51.7</td>
<td>Peak 1: -40.0</td>
<td>48.4</td>
<td>7.96</td>
</tr>
<tr>
<td>Zeta SD (mV): 23.8</td>
<td>Peak 2: -67.9</td>
<td>18.7</td>
<td>5.87</td>
</tr>
<tr>
<td>Conductivity (mS/cm): 0.468</td>
<td>Peak 3: -56.9</td>
<td>12.8</td>
<td>3.00</td>
</tr>
</tbody>
</table>

**Conductivity is out of range - check cell or sample**

### Zeta Potential Distribution

![Zeta Potential Distribution Graph](image)
$^1$H NMR (400 MHz, CDCl$_3$) of N-acryloyl-5-ASA, (Compound 2)

Compound 2
$^1$H NMR (400 MHz, CDCl$_3$) of N-acetyl-5-ASA (Compound 3)

Compound 3
$^1$H NMR (400 MHz, CDCl$_3$) of L-menthyl acrylate (Compound 5)
$^1$H NMR (400 MHz, CDCl$_3$) of D-menthyl acrylate (Compound 7)

D-Menthyl Acrylate

Compound 7
$^1H$ NMR (400 MHz, CDCl$_3$) of N-acryloyl Ciprofloxacin (Compound 9)
LCMS of N-acryloyl Ciprofloxacin (Compound 9)
REFERENCES


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55. Cormier, R; Burda, W; Harrington, L; Edlinger, J; Kogalpalli, K; Thomas, J; Kapolka, R; Roma, G; Anderson, B; Turos, E; Shaw, L. *Bioorg Med Chem Lett*. **2012**, *22*, *20*, 6513-6520.
APPENDIX A

MENTHOL-BASED NANOPARTICLES FOR DRUG DELIVERY

BACKGROUND OF THE INVENTION

There has been increasing interest within the scientific community in the use of nanoparticles. With easily altered frameworks, these nanoparticles can be manipulated for various properties and diverse applications. One area of focus is the study of nanoparticles as a system for drug delivery. The growing antibiotic resistance of harmful microbes, such as methicillin-resistant *Staphylococcus aureus* (MRSA), has emerged as one of the dominating concerns of today’s public health system, causing scientists to look for ways to circumvent this resistance through drug delivery methods and systems.

BRIEF SUMMARY OF THE INVENTION

Aspects of the present invention provide formulations comprising poly(menthyl acrylate) nanoparticles comprising at least one active ingredient contained in a plurality of hydrophobic carriers and dispersed in an aqueous medium.

In some embodiments, the at least one active ingredient is an antibiotic, such as but not limited to, penicillin. The active ingredient can be from about 1% to about 20% (w/w) of the formulation. In some embodiments, the hydrophobic carriers are made up of a surfactant, such as but not limited to, sodium dodecyl sulfate (SDS). The hydrophobic carriers may form micelles...
dispersed in the aqueous medium such that the micelles encapsulate the poly(menthyl acrylate) nanoparticles and active ingredient(s).

In one embodiment, the poly(menthyl acrylate) nanoparticles are poly(L-menthyl acrylate) nanoparticles. In another embodiment, the poly(menthyl acrylate) nanoparticles are poly(D-menthyl acrylate) nanoparticles.

Additional aspects of the present invention provide methods of polymerization of drug loaded nanoparticles in an aqueous emulsion, comprising: adding a plurality of methyl acrylate monomers to an aqueous medium; adding an active ingredient to the aqueous medium; adding a surfactant to form the aqueous emulsion; and adding a radical initiator for alkene polymerization of the methyl acrylate monomers in the presence of the active ingredient.

In some embodiments, the menthyl acrylate monomers are selected from L-menthyl acrylate monomers, D-menthyl acrylate monomers, and a combination thereof.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** shows a diagram of the organization of nanoparticles of the invention within an aqueous bulk media of the emulsion. Note: the antibiotic is not covalently bound to the polymer backbone, allowing for easier release of the drug.

**Figure 2** illustrates acrylate monomers of the present invention.

**Figure 3** illustrates miniemulsion polymerization structures.

**Figure 4** illustrates a miniemulsion polymerization process.

**Figure 5** shows distribution of particle sizes and stability of nanoparticles of the present invention.
**Figure 6A** shows activity of penicillin and nanopenicillin particles of the present invention on an agar plate.

**Figure 7** is a graph showing that the nanoparticle emulsions have optical activity and that the specific rotation values are somewhat non-linear.

**Figure 8** shows the process of polymerization of nanoparticles from polyacrylate monomers.

**Figure 9** shows a graph of the distribution of particle sizes of various chiral nanoparticle samples of the present invention.

**Figure 10** shows a graph of the distribution of zeta potentials of various chiral nanoparticle samples of the present invention.

**Figure 11** shows a graph of the optical rotation versus concentration for the polyacrylated nanoparticle emulsions of the present invention.

**Figure 12** illustrates menthyl acrylate monomers and acrylate nanoparticle emulsions of the present invention.

**Figure 13** illustrates a method of measuring size and stability of nanoparticles utilizing dynamic light scattering.

**Figure 14** shows a graph of the size distribution of penicillin encapsulated nanoparticles in nanoparticle emulsions of the present invention.

**Figure 15** shows a graph of the zeta potentials of penicillin encapsulated nanoparticles in nanoparticle emulsions of the present invention.

**Figure 16** shows a graph of the optical activity of penicillin encapsulated nanoparticles in nanoparticle emulsions of the present invention.
Figure 17A shows emulsions of L-menthyl acrylate with drug load concentrations of 0% to 20% of Pen G by weight (shown top) and those of D-menthyl acrylate with loads ranging from 1% to 20% of Pen G by weight (shown bottom); Figure 17B shows examples of failed emulsions. Note: the D-menthyl samples were more destabilized and crashed out.

DETAILED DISCLOSURE OF THE INVENTION

The present invention provides the chemical synthesis and characterization of organic nanoparticles using menthyl acrylate as a monomer in emulsion polymerization. Either the D- or the L-stereomeric form of menthyl acrylate can be used as a means to produce homochiral polyacrylate nanoparticles in an aqueous emulsion. The present invention further provides the use of these nanoparticles to uptake chiral drug molecules (active ingredients), such as penicillin G, non-covalently, through encapsulation, at much higher concentrations compared to non-menthyl acrylate based nanoparticle formulations, as illustrated in Figure 1. Additionally, the nanoparticles can be made and/or utilized without containing an “active ingredient”.

Reference is made herein to particular features (including method steps) of the invention. Where a particular feature is disclosed in the context of a particular aspect or embodiment of the invention, that feature can also be used, to the extent possible, in combination with and/or in the context of other particular aspects and embodiments of the invention, and in the invention generally.

The term “comprises” is used herein to mean that other ingredients, components, steps, etc. are optionally present. When reference is made herein to a method comprising two or more defined steps, the steps can be carried out in any order or simultaneously (except where the context excludes that possibility), and the method can include one or more steps which are
carried out before any of the defined steps, between two of the defined steps, or after all of the defined steps (except where the context excludes that possibility).

This invention may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will convey preferred embodiments of the invention to those skilled in the art.

Aspects of the present invention provide formulations comprising poly(menthyl acrylate) nanoparticles comprising at least one active ingredient contained in a plurality of hydrophobic carriers and dispersed in an aqueous medium.

As used herein, the term "pharmaceutically active ingredient" or "active ingredient" means an ingredient in the formulation that produces a physiological effect in the user. Active ingredients include, but are not limited to, antibiotics, analgesics, anti-inflammatories, stimulants, depressants, sedatives, electrolytes, vitamins, minerals, hormones, peptides, nucleic acids, or any other pharmaceutically active substances and drugs. In some embodiments, the at least one active ingredient is an antibiotic, such as but not limited to, penicillin. The active ingredient can be from about 1% to about 20% (w/w) of the formulation; however, the concentration range may be expanded or contracted, depending on the particular active ingredient.

In some embodiments, the hydrophobic carriers are made up of a surfactant, such as but not limited to, sodium dodecyl sulfate (SDS). The hydrophobic carriers may form micelles dispersed in the aqueous medium such that the micelles encapsulate the poly(menthyl acrylate) nanoparticles and active ingredient(s). Generally, the hydrophobic carriers comprise amphiphilic properties. A micelle is formed from amphiphilic molecules. When dispersed in an aqueous solution, the hydrophilic head groups form a hydrophobic pocket composed of the hydrophobic
tail groups. One or more active ingredients and nanoparticles may be encapsulated by the micelle in the hydrophobic pocket.

In one embodiment, the poly(menthyl acrylate) nanoparticles are poly(L-menthyl acrylate) nanoparticles. In another embodiment, the poly(menthyl acrylate) nanoparticles are poly(D-menthyl acrylate) nanoparticles.

Additional aspects of the present invention provide methods of polymerization of drug loaded nanoparticles in an aqueous emulsion, comprising: adding a plurality of methyl acrylate monomers to an aqueous medium; adding an active ingredient to the aqueous medium; adding a radical initiator for alkene polymerization of the methyl acrylate monomers in the presence of the active ingredient; and adding a surfactant to form the aqueous emulsion.

Generally, a plurality of methyl acrylate monomers is dispersed in an aqueous medium. Then, the active ingredient is dispersed into the medium containing the monomers. Next, a surfactant is added to the aqueous medium to form an emulsion containing micelles encapsulating the nanopolymer-active ingredient mix. Finally, alkene polymerization is initiated to generate the nanopolymers in the presence of the active ingredient. In some embodiments, the menthyl acrylate monomers are selected from L-menthyl acrylate monomers, D-menthyl acrylate monomers, and a combination thereof. In additional embodiments, the emulsions of the present invention can also contain variable ratios of styrene as a co-monomer for polymerization.

In some embodiments, the emulsions of the present invention comprise butyl acrylate with styrene (no menthyl acrylate). In some embodiments, the emulsions of the present invention comprise L-menthyl acrylate with styrene. In some embodiments, the emulsions of the present invention comprise D-menthyl acrylate with styrene. In some embodiments, the emulsions of
the present invention comprise racemic menthyl acrylate with styrene. Additionally, the emulsions may comprise about 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 w/w% of menthyl acrylate relative to styrene.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1—Analysis studies of enantiomerically pure polyacrylate nanoparticles

Purely organic chiral nanoparticles were synthesized from polyacrylates. Polyacrylates contain a large diverse group of potential monomers, each with readily tunable physical properties. Chirality was imparted via polymerization of styrene (St) with chiral monomer, L-menthyl acrylate (L-Mta) (Figure 2). The L-Mta was synthesized as follows:
The mechanism behind the nanoparticle formation is miniemulsion polymerization. Surfactants behave as reaction vesicles to contain the polymerization (Figure 3). The chirality may be tailored by varying the chiral monomer and its concentration.

Six samples of poly (MtA-co-St) nanoparticles were synthesized using 10, 20, 30, 40, 50, and 60 v/v% of MtA relative to St (Figure 4).

As shown in Tables 1 and 2, poly(MtA-co-St) form as stable nanoparticle emulsions in water.

<table>
<thead>
<tr>
<th>Particle</th>
<th>MtA:St</th>
<th>MtA%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NanopA</td>
<td>1:9</td>
<td>10%</td>
</tr>
<tr>
<td>NanopB</td>
<td>2:8</td>
<td>20%</td>
</tr>
<tr>
<td>NanopC</td>
<td>3:7</td>
<td>30%</td>
</tr>
<tr>
<td>NanopD</td>
<td>4:6</td>
<td>40%</td>
</tr>
<tr>
<td>NanopE</td>
<td>5:5</td>
<td>50%</td>
</tr>
<tr>
<td>NanopF</td>
<td>6:4</td>
<td>60%</td>
</tr>
</tbody>
</table>

Table 1: Nanoparticle emulsions

<table>
<thead>
<tr>
<th>Zeta Potential (± mV)</th>
<th>Stability of Colloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5</td>
<td>Rapid coagulation</td>
</tr>
<tr>
<td>10-30</td>
<td>Partial stability</td>
</tr>
<tr>
<td>30-40</td>
<td>Moderate stability</td>
</tr>
<tr>
<td>40-60</td>
<td>Good stability</td>
</tr>
<tr>
<td>&gt;60</td>
<td>Excellent stability</td>
</tr>
</tbody>
</table>

Table 2: Zeta potential provide stability guidelines for colloids

Optical activity was determined by polarimetry and particle size, stability, and polydispersity determined by dynamic light scattering. The distribution of particle size and particle stability is shown in Figure 5.
The bioactivity was tested by Kirby-Bauer diffusion assay against *Staphylococcus aureus*. Figure 6 shows that both penicillin and the nanopenicillins are active (clear areas on the agar), while only the nanopenicillins are active when β-lactamase protein is added to the growth media. Figure 7 shows that the nanoparticle emulsions have optical activity and, thus, are chiral and that the specific rotation values are somewhat non-linear.

**EXAMPLE 2**—Analysis of emulsion stability and uniformity of chiral polymer nanoparticles.

In this example, the particle size and zeta potential are characterized for optically active polyacrylate nanoparticles derived from D- and L-menthyl acrylate.

D-menthyl acrylate  L-menthyl acrylate

The enantiomerically-pure acrylates of D- and L-menthol were synthesized by reaction with acryloyl chloride in the presence of triethylamine.
The chiral nanoparticles were then synthesized by emulsion polymerization of the D- and L-menthyl acrylates, using styrene as a co-monomer at 78 degrees Celsius. Potassium persulfate was used as a radical initiator and sodium dodecyl sulfate (SDS) was added as a surfactant to stabilize the nanoparticles (Figure 8). A summary of the reagents and the necessary amounts for the synthesis of a 2.5 mL emulsion sample at 3% drug load concentration is shown in Table 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menthyl acrylate</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Pen G (3% solution)</td>
<td>15 mg</td>
</tr>
<tr>
<td>SDS (3%)</td>
<td>15 mg</td>
</tr>
<tr>
<td>Potassium persulfate (1%)</td>
<td>5 mg</td>
</tr>
<tr>
<td>Water</td>
<td>2.0 mL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>2.5 mL</td>
</tr>
</tbody>
</table>

Table 3.

Six series of nanoparticle samples were prepared for physical characterization:
(1) butyl acrylate with styrene (no menthyl acrylate)
(2) L-menthyl acrylate with styrene
(3) D-menthyl acrylate with styrene
(4) racemic menthyl acrylate with styrene
Each of these four series contained ten samples with 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 w/w% of menthyl acrylate relative to styrene.

(5) nanoparticles made by polymerizing mixtures of the enantiomeric pure acrylates, in ratios from 90:10 D:L to 10:90 D:L (BE “before emulsification”) (no styrene)

(6) nanoparticles made by mixing different amounts of each enantiomeric pure nanoparticle emulsion, in ratios from 90:10 D:L to 10:90 D:L (AE “after emulsification”) (no styrene)

Each of these two series contained 9 samples with 10, 20, 30, 40, 50, 60, 70, 80, and 90 w/w% of D-menthyl acrylate relative to L-menthyl acrylate.

For each of the nanoparticle samples, dynamic light scattering was used to analyze the particle size, uniformity, and stability, in triplicates. The data for each of these are presented in Figures 9 and 10. The particle size is measured in nanometers (nm), which includes the nanoparticle as well as the surface hydration sphere. The narrowness of size distribution ensures that the nanoparticles are uniform in size. A large zeta potential value greater than 60 mV indicates the emulsion is very stable and does not precipitate (see Table 2).

This example demonstrates that chiral polyacrylate nanoparticles can be synthesized by using enantiomerically pure menthyl acrylates as monomers. There were no significant differences observed in the particle sizes or stabilities as the amounts of chiral monomers were varied. The particle sizes ranged from 40-60 nm among all series, and the zeta potentials were in the range of -30 to -50 mV, indicating high stability in aqueous emulsions.
EXAMPLE 3—Study of the optical properties of chiral polyacrylate nanoparticles synthesized from D- and L-menthyl acrylate.

In this example, chirality is added to the framework using enantiomerically-pure acrylates of D- and L-menthol as shown below, and the optical properties of the chiral nanoparticles are characterized by polarimetry and circular dichroism.

![D-menthyl acrylate and L-menthyl acrylate](image)

The enantiomerically-pure acrylates of D- and L-menthol were synthesized by reaction with acryloyl chloride in the presence of triethylamine.

![Synthesis of acrylate](image)

With the L-menthyl acrylate and D-menthyl acrylate being used as monomers, different nanoparticles were prepared using varying amounts of each chiral acrylate, from 0% to 100% by weight. The acrylate and styrene were used as co-monomers to construct the nanoparticles by free radical polymerization in aqueous solution.

Ten samples of each nanoparticle emulsion were synthesized with the varying ratios by w/w% of the chiral acrylate monomer and the styrene. Each sample was then diluted 200 times for measurements in both the polarimeter and CD.
Optical activity was determined by optical polarimetry and circular dichroism (CD). All optical rotation measurements on the polarimeter were recorded at 589 nm. A range of 195-250 nm at 1 nm intervals was used for the CD (data not shown).

A perfectly linear relationship was expected between the angle of rotation and concentration. However, as shown in Figure 11, there was a slight deviation from linearity between the 60-70 w/w% for the D and L enantiomeric particles. Also in the graph, the line labeled BE (Before Emulsion) is for nanoparticle samples containing mixtures of both enantiomeric acrylates, in ratios ranging from 10D:90L to 90D:10L, prepared by emulsion polymerization of the two chiral acrylates. The line labeled AE (After Emulsion) is for nanoparticle samples consisting of mixtures of enantiomerically pure nanoparticle emulsions (each made from the individual menthyl acrylate enantiomers) ranging from 10D:90L to 90D:10L. Neither of the BE and AE sets contained styrene but only the menthyl acrylate. These sets were synthesized to show that there is no significant difference between mixing the enantiomeric monomers then polymerizing to make the emulsions, versus mixing the pre-made chiral nanoparticle emulsions. The optical properties are similar for both samples.

This example shows that chiral polyacrylate nanoparticles can be synthesized by emulsion polymerization using enantiomerically pure menthyl acrylates as monomers. The optical activity of these chiral polyacrylated nanoparticles was analyzed using polarimetry. It was found that the optical rotation values of the nanoparticles slightly deviated from linearity for those samples having 60-70 w/w% of either D-menthyl acrylate or L-menthyl acrylate. The alternative methods used to make the BE and AE sets showed no significant differences in the optical properties of the chiral polyacrylated nanoparticles.
EXAMPLE 4—Study of the stability and uniformity of enantiomerically-pure penicillin encapsulated poly(menthyl acrylate) nanoparticle emulsions.

In order to synthesize the menthol derived polymers, L-menthol and D-menthol were individually reacted with acryloyl chloride along with triethylamine to create the single chiral unit that makes up the polymer chain. The polymer framework was prepared using potassium persulfate as the radical initiator for the alkene polymerization. To make the emulsion, the polymer is set in the presence of the surfactant sodium dodecyl sulfate (SDS) and water (Figure 12).

Nanoparticle behavior has been the subject of great scrutiny because while many methods have proven to be viable for analysis, dynamic light scattering (DLS) is one of the few that is non-destructive and employs simple methods (Livingstone, 2012). In order to analyze the nanoparticles, a beam of light is passed through the particle sample that produces a signal according to the changes in scattering intensity (Figure 13). The scattering intensity of the sample reflects the constant movement of the nanoparticles (Livingstone, 2012).

Zeta potentials are very useful tools for the measurement of stability because they can quantify the charge repulsion or attraction between the particles involved in a liquid environment (Wang et al., 2013). Since the emulsion involves two liquids of different miscibility, the zeta potential of the encapsulated nanoparticles can be measured using a zetasizer that uses dynamic light scattering technology (Sarker, 2013). Zeta potentials can be analyzed for particle uniformity and stability based on their numerical range (Table 2). Additionally, DLS can help quantify particle size.

Each sample was prepared by encapsulating different percentages of Penicillin G within the polymer framework. The different samples were loaded starting with 1% Pencillin G (by weight)
and the percentage of the antibiotic was increased by 1% in each new sample all the way up to 20% (Table 4). Each particle size including the emulsion was calculated by taking the average of 3 sample sizes in nanometers (nm). The zeta potential is the average of triplicate samples, in millivolts (mV). Furthermore, to ensure chirality was maintained the optical rotation of the nanoparticles was measured through polarimetry.

<table>
<thead>
<tr>
<th>L-MENTHYL</th>
<th>D-MENTHYL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of PenG</td>
<td>Zeta Potential (mV)</td>
</tr>
<tr>
<td>1%</td>
<td>-59.26</td>
</tr>
<tr>
<td>2%</td>
<td>-48.14</td>
</tr>
<tr>
<td>3%</td>
<td>-60.03</td>
</tr>
<tr>
<td>4%</td>
<td>-48.46</td>
</tr>
<tr>
<td>5%</td>
<td>-50.49</td>
</tr>
<tr>
<td>6%</td>
<td>-49.49</td>
</tr>
<tr>
<td>7%</td>
<td>-44.88</td>
</tr>
<tr>
<td>8%</td>
<td>-45.96</td>
</tr>
<tr>
<td>9%</td>
<td>-45.54</td>
</tr>
<tr>
<td>10%</td>
<td>-45.82</td>
</tr>
<tr>
<td>11%</td>
<td>-47.11</td>
</tr>
<tr>
<td>12%</td>
<td>-47.77</td>
</tr>
<tr>
<td>13%</td>
<td>-47.43</td>
</tr>
<tr>
<td>14%</td>
<td>-49.13</td>
</tr>
<tr>
<td>15%</td>
<td>-47.7</td>
</tr>
<tr>
<td>16%</td>
<td>-41.2</td>
</tr>
<tr>
<td>17%</td>
<td>-62.43</td>
</tr>
<tr>
<td>18%*</td>
<td>-28.53</td>
</tr>
<tr>
<td>19%**</td>
<td>-31.73</td>
</tr>
<tr>
<td>20%</td>
<td>-24.37</td>
</tr>
</tbody>
</table>

Table 4. *Data average included from partial emulsion **Data averageset includes samples with no emulsion

As illustrated in Figure 14, most of the nanoparticles were less than 100nm. When comparing the D- and L-methyl acrylate systems, the nanoparticle emulsions prepared using L-methyl acrylate were more consistent in size, even when the percentage of Penicillin G was higher. Alternatively, the size of the D-methyl-derived particles fluctuated in comparison, yet at the highest percentage of PenG, the nanoparticle was well within the range of the L-menthol sample prepared with the same percentage of PenG.
Zeta Potentials for both D- and L-menthyl nanoparticle emulsions (Figure 15) showed consistent uniformity and stability with most zeta potentials being at least moderately stable. The zeta potentials for both D- and L-menthyl were as high as -24 mV but peaked at ~-63 mV on the nanoparticle made with L-menthyl. D-menthyl, however, did still show high uniformity with a particle having a value of ~-100 mV.

<table>
<thead>
<tr>
<th>PenG %</th>
<th>L-1: Avg</th>
<th>L-2: Avg</th>
<th>D-1: Avg</th>
<th>D-2: Avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>-0.041</td>
<td>0.049</td>
<td>0.049</td>
</tr>
<tr>
<td>2</td>
<td>-0.033</td>
<td>-0.032</td>
<td>0.04</td>
<td>0.056</td>
</tr>
<tr>
<td>3</td>
<td>-0.038</td>
<td>-0.021</td>
<td>0.044</td>
<td>0.055</td>
</tr>
<tr>
<td>4</td>
<td>-0.04</td>
<td>-0.023</td>
<td>0.04</td>
<td>0.056</td>
</tr>
<tr>
<td>5</td>
<td>-0.038</td>
<td>-0.033</td>
<td>0.042</td>
<td>0.0533</td>
</tr>
<tr>
<td>6</td>
<td>-0.027</td>
<td>-0.038</td>
<td>0.049</td>
<td>0.0527</td>
</tr>
<tr>
<td>7</td>
<td>-0.019</td>
<td>-0.036</td>
<td>0.038</td>
<td>0.058</td>
</tr>
<tr>
<td>8</td>
<td>-0.035</td>
<td>-0.041</td>
<td>0.046</td>
<td>0.051</td>
</tr>
<tr>
<td>9</td>
<td>-0.003</td>
<td>-0.0423</td>
<td>0.045</td>
<td>0.049</td>
</tr>
<tr>
<td>10</td>
<td>-0.04</td>
<td>-0.0417</td>
<td>0.041</td>
<td>0.063</td>
</tr>
<tr>
<td>11</td>
<td>-0.038</td>
<td>-0.0417</td>
<td>0.015</td>
<td>0.012</td>
</tr>
<tr>
<td>12</td>
<td>-0.041</td>
<td>-0.036</td>
<td>0.033</td>
<td>0.008</td>
</tr>
<tr>
<td>13</td>
<td>-0.038</td>
<td>-0.038</td>
<td>0.0143</td>
<td>0.011</td>
</tr>
<tr>
<td>14</td>
<td>-0.031</td>
<td>-0.04</td>
<td>0.0137</td>
<td>0.03</td>
</tr>
<tr>
<td>15</td>
<td>-0.038</td>
<td>-0.037</td>
<td>0.015</td>
<td>0.019</td>
</tr>
<tr>
<td>16</td>
<td>-0.029</td>
<td>-0.028</td>
<td>0.007</td>
<td>0.055</td>
</tr>
<tr>
<td>17</td>
<td>-0.037</td>
<td>-0.006</td>
<td>0.027</td>
<td>0.058</td>
</tr>
<tr>
<td>18</td>
<td>-0.034</td>
<td>-0.037</td>
<td>0.033</td>
<td>0.062</td>
</tr>
<tr>
<td>19</td>
<td>-0.036</td>
<td>-0.041</td>
<td>0.014</td>
<td>0.015</td>
</tr>
<tr>
<td>20</td>
<td>-0.028</td>
<td>0.016</td>
<td>0.053</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.

The optical rotation values of the nanoparticles were more consistent for the L-menthyl samples, which did not show a significant drop in activity at PenG concentrations of 11-15% (Figure 16). However, the average rotation values for the D-menthyl samples were greater in magnitude than that of the L-menthyl samples (Table 5).

Since both D- and L-menthyl acrylate nanoparticles had zeta potentials ranging from ~-24mV to ~-100mV, the synthesized nanoparticle emulsions were consistently uniform and stable.
in the aqueous media. Even while the percentage of PenG increased, the uniformity of the particle remained consistent and particles such as the D-menthyl that contained 17% PenG had an excellent zeta potential close to -100mV. While most of the tested samples appeared as single layers, one nanoparticle polymer was not emulsified D-menthyl with 19% Pen G. Additionally, some samples had partial emulsions, but the zeta potential of the nanoemulsion layer still supported uniformity. Based on the data acquired and visible uniformity of the emulsions, L-menthyl acrylate nanoparticles were more uniform. Varying zeta potentials among both menthyl enantiomers could be the subject of future study, however, based on collected data, both D- and L-menthyl acrylate nanoparticle emulsions act as viable systems for drug delivery.

The resultant nanoparticle emulsions were found to be stable both in size and zeta potential when analyzed via dynamic light scattering (DLS). There is also a definite hue to be noted as the concentration of the drug load increases in the system (Figure 17A). Additionally, it was noted that D-menthyl acrylate emulsions were more likely to destabilize than L-menthyl acrylate emulsions (Figure 17B).

This study demonstrated the successful synthesis of antibiotic carrying nanoparticle emulsions using a polyacrylate backbone system composed of enantiomerically pure D- and L-menthyl acrylates of up to 20% by weight. Characterization of size and stability via DLS analysis found these particles to be 100 nm in diameter, and having zeta potentials of roughly -50 mV for both enantiomers. The polarimetry measurements for optical rotation were found to be rather inconsistent across the D-Menthyl samples, with a significant drop in activity between drug load concentrations of 11-15%, and the optical activity of the L-Menthyl samples were notably smaller in magnitude than its enantiomeric counterpart. This could be due to the large dilution factor at which these samples are prepared, which introduces a large margin of error. It is also
possible that the varying lengths of the polymer present in each sample had an effect on the optical activity. There were some minor destabilizing effects observed in the D-Menthy samples in comparison to the L-Menthy samples.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.
REFERENCES


Wang, N, Hsu, C, Zhu, L, Tseng, S, & Hsu, J n.d., 'Influence of metal oxide nanoparticles concentration on their zeta potential', *Journal Of Colloid And Interface Science*, 407, pp. 22-28, Science Citation Index, EBSCOhost.
We claim:

1. A formulation comprising poly(menthyl acrylate) nanoparticles comprising at least one active ingredient contained in a plurality of hydrophobic carriers dispersed in an aqueous medium.

2. The formulation of claim 1, wherein the at least one active ingredient is an antibiotic.

3. The formulation of claim 1, wherein the at least one active ingredient is penicillin.

4. The formulation of claim 1, wherein the at least one active ingredient is about 1% to about 20% (w/w) of the formulation.

5. The formulation of claim 1, wherein the plurality of hydrophobic carriers are a surfactant.

6. The formulation of claim 1, wherein the plurality of hydrophobic carriers form micelles dispersed in the aqueous medium.

7. The formulation of claim 1, wherein the poly(menthyl acrylate) nanoparticles are poly(L-menthyl acrylate) nanoparticles.

8. The formulation of claim 1, wherein the poly(menthyl acrylate) nanoparticles are poly(D-menthyl acrylate) nanoparticles.

9. A method of polymerization of drug loaded nanoparticles in an aqueous emulsion, comprising:

   adding a plurality of methyl acrylate monomers to an aqueous medium;

   adding an active ingredient to the aqueous medium;
adding a radical initiator for alkene polymerization of the methyl acrylate monomers in
the presence of the active ingredient; and

adding a surfactant to form the aqueous emulsion.

10. The method of claim 9, wherein the menthyl acrylate monomers are L-menthyl
acrylate monomers.

11. The method of claim 9, wherein the menthyl acrylate monomers are D-menthyl
acrylate monomers.

12. The method of claim 9, wherein the menthyl acrylate monomers are selected from L-
menthyl acrylate monomers, D-menthyl acrylate monomers, and a combination thereof.
ABSTRACT OF THE DISCLOSURE

The subject invention pertains to formulations comprising poly(menthyl acrylate) nanoparticles comprising at least one active ingredient contained in a plurality of hydrophobic carriers and dispersed in an aqueous medium. The subject invention further pertains to methods of polymerization of drug loaded nanoparticles made up of methyl acrylate monomers in an aqueous emulsion.
Figure 1
Figure 4
Figure 5

<table>
<thead>
<tr>
<th>Particle</th>
<th>Zeta potential</th>
<th>Conductance x$10^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NanopA</td>
<td>-64.3mV</td>
<td>5.04 uS/cm</td>
</tr>
<tr>
<td>NanopB</td>
<td>-63.4mV</td>
<td>5.96 uS/cm</td>
</tr>
<tr>
<td>NanopC</td>
<td>-64.1mV</td>
<td>6.14 uS/cm</td>
</tr>
<tr>
<td>NanopD</td>
<td>-65.5mV</td>
<td>6.04 uS/cm</td>
</tr>
<tr>
<td>NanopE</td>
<td>-52.4mV</td>
<td>7.36 uS/cm</td>
</tr>
<tr>
<td>NanopF</td>
<td>-59.2mV</td>
<td>6.96 uS/cm</td>
</tr>
</tbody>
</table>
Figure 7

Specific rotation [alpha]

Specific rotation vs MtA mmol
\[ f(x) = -0.026175623x - 0.0805; R^2 = 0.9945 \]
### Step 1: Initial Addition
D- or L-Menthyl Acrylate & Pen G (0% - 20% by weight)

20 minutes

### Step 2: Surfactant Addition
Sodium Dodecyl Sulfate (3% by weight) & Water

20 minutes

### Step 3: Radical Initiator
Potassium Persulfate (1% by weight) & Water

6 hours

Nanoparticle Emulsion

---

Figure 8
Figure 10
Figure 11
Figure 12

Figure 13
Figure 14

Size of Nanoparticle Emulsions

% of PenG

Size (nm)

L Menthyl
D Menthyl
Figure 15

% of PenG

Polarimeter Data

Optical Rotation

Percentage Concentration of PenG in Sample

Emulsions
APPENDIX B

CIPROFLOXACIN-BASED POLYACRYLATE NANOPARTICLE FOR ANTIBACTERIAL APPLICATIONS

Abstract

We investigate for the first time a method for forming polyacrylate nanoparticles using N-acryloyl ciprofloxacin for its construction. The procedure entails a free radical induced emulsion polymerization of the ciprofloxacin acrylate as the sole monomer in water to produce a stable emulsion containing nanoparticles of highly uniform size and morphology with an average diameter of 970 nm and average surface charge of -63 mV. The nanoparticles were found to be capable antibacterials with minimum inhibitory concentration values against *Staphylococcus aureus* and *Escherichia coli* comparable to that of free ciprofloxacin, with essentially no observable cytotoxicity.

Keywords: ciprofloxacin, homopolymer, emulsion, nanoparticle, drug delivery, antibacterial polymer
**Background**

Previous experiments in our laboratory have demonstrated the ability to form aqueous polyacrylate nanoparticle emulsions for the purpose of water-solubilizing and encasing certain antibacterial compounds, as a means to improve their stability and antibiotic activity especially towards multi-drug resistant strains of bacteria. These nanoparticle emulsions were prepared through radical-induced emulsion polymerization of butyl acrylate/styrene mixtures (7:3 w/w) in water at 60°C, using sodium dodecyl sulfate (SDS) as an emulsifying agent and potassium persulfate as a radical initiator (Figure 1).\(^1\)\(^{-4}\) The reactions led to the formation of a homogeneous, stable aqueous emulsions containing uniformly-sized nanoparticles of 45-50 nm in diameter. The method was successfully applied to penicillins and N-thiolated β-lactams, and such that the antibacterial agents could be introduced into the nanoparticle either by non-covalent entrapment as a free drug, or covalently via their acryloyl derivative. The antibiotic-containing nanoparticles show promising in vitro activity against pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).

\(\text{(INSERT FIG1.DOCX)}\)

While these earlier nanoparticle emulsions provided increased water solubility and, in some cases, improved bioactivity of the β-lactam antibacterial agent, the polyacrylate backbone was largely comprised of non-bioactive monomers (butyl acrylate-styrene or methyl methacrylate-styrene), and only 1-3 % (by weight) of the antibacterial acrylate in the nanoparticle. The amount of drug loading into the nanoparticle during the assembly process was limited by how much surfactant could be used, given that amounts exceeding 3 % (by weight) of SDS caused discernable cytotoxicity. The nanoparticle emulsions contained up to 20% of solid content (a mixture comprising of nanoparticles and a small amount of non-surfactated polymers), 80%
water, and 0.2-1% of active antibacterial agent inside of the nanoparticles. The resulting emulsions were typically milky in consistency and somewhat sticky when exposed to air, causing films to rapidly form when dried, and unwanted coagulation within syringes, micro-porous filters, and gel columns that made it very difficult to purify and use for in vivo testing. We were able to overcome some of these issues with purification techniques that enable the removal of residual unreacted monomers and non-nanoparticle oligomers within the emulsion. We also reported on the use of other surfactant combinations to try to enhance the amount of antibiotic that could be entrapped, or to alter nanoparticle sizes, without increasing overall cytotoxicity or instability of the emulsion.  

In this report, we describe an altogether new approach to preparing antibiotic-bound polyacrylate nanoparticle emulsions that completely obviates the restriction of using butyl acrylate and styrene (or other monomers) to form the nanoparticle framework, and these limitations in their properties. The new procedure uses the antibiotic compound itself as the sole acrylate monomer for the polymerization. This technique has never been reported and is thus an important advance in the polymer-based nanoparticle field.

Methods

For our studies, we chose ciprofloxacin as the antibiotic for the formation of the polyacrylate nanoparticles. The N-acryloyl derivative of commercial ciprofloxacin hydrochloride was prepared for this purpose according to our previously reported acylation procedure.  

(INSERT FIG2.DOCX)

Figure 2 shows the synthetic scheme for N-acryloylciprofloxacin, and follows as such: To a round bottom flask was added 120 ml of dichloromethane, then 3.0 g (9.0 mmol) of ciprofloxacin and 1.9 ml (13.5 mmol) of triethylamine. The mixture was left stirring at 0°C for 1
hour then acryloyl chloride (1.1 ml, 13 mmol) was added dropwise. The ice bath was removed and the reaction was left stirring overnight. The solution was added dropwise to a flask of containing hexane (60-80 ml) and the resulting solid was collected by filtration and allowed to air dry.

Yielded 2.90 g (83.7%) as a pale yellow solid. Melting point above 250°C. $^1$H NMR (400 MHz, CDCl$_3$) δ 1.18 (br. s., 2 H) 1.38 (d, J=6.6 Hz, 2 H) 3.33 (m, 4 H) 3.51 (br. s., 1 H) 3.47 (m, 1 H) 3.86 (m, 4 H) 5.76 (dd, J=10.5, 1.7 Hz, 1 H) 6.35 (dd, J=16.8, 1.7 Hz, 1 H) 6.59 (dd, J=16.8, 10.5 Hz, 1 H) 7.35 (d, J=7.1 Hz, 1 H) 8.03 (d, J=12.8 Hz, 1 H) 8.75 (s, 1 H)

(The polyacrylate emulsions were prepared using a modified protocol to that we previously reported.$^{1,2}$ As seen in Figure 3 the method for forming the poly(N-acryloylciprofloxacin) emulsion requires the following procedure: to a round bottom flask was added 4 ml of deionized water, which was then stirred using a 1.25 cm (300 mg) Teflon-coated magnetic stir bar at 1000 rpm on a Corning PC-420D magnetic stirrer at 30°C using a self-regulated oil bath. To this was added 30 mg of SDS. N-Acryloylciprofloxacin (500 mg) was dissolved in 1 ml of warm dichloromethane, and this solution was added dropwise to the DI water-SDS mixture. A vent was placed on top of the flask by inserting a small stainless steel syringe needle through a rubber septum on the flask, under dry nitrogen, and the temperature of the mixture was increased at a rate of 5°C per 30 min until reaching 90°C. The mixture was left stirring overnight at this temperature, under an atmosphere of dry nitrogen. Potassium persulfate (10 mg) was added with an additional 0.5 ml of deionized water to the stirring mixture and left stirring for an additional 24 hours. The stirred emulsion was then removed from the oil bath and decanted into a storage vial for analysis.
The first question we hoped to address was whether any nanoparticles were being formed in the emulsion polymerization process. For this, we used dynamic light scattering analysis to evaluate the average size and surface charge of the emulsion using a Malvern Zetasizer nano-ZS instrument. To prepare the samples for the analyses, the freshly-prepared emulsion was subjected to centrifugation at 10,000 rpm for 5 min using an Eppendorf Centrifuge 5424. An aliquot of the liquid emulsion was then drawn and deposited into a Malvern disposable folded capillary cell DTS-1070. Each sample was analyzed in triplicate, and each data collection consisted of 1 run of 100 scans (for size analysis) and 3 runs of 100 scans (for zeta potential determination). The size distribution shows a single narrow peak indicating the uniformity of the emulsion with a single population centered on average at approximately 970 nm. Similarly, surface charge measurements indicated a highly stable emulsion, with an average reading of -63 (± 5.6) mV.

To investigate whether the nanoparticles possess antibiotic capabilities, each crude emulsion was tested against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (K12) using a 96-well plate broth assay to determine the minimum inhibitory concentration (MIC). The original stock emulsion was diluted using the above Trypticase Soy Broth solution to an initial concentration of 1.28 mg/ml of the N-acryloylciprofloxacin, then serial diluted with TSB to half the concentration each time. A volume of 10 µl of each emulsion dilution was added to a well in series, resulting in a final concentration run of 64 µg/ml to 0.012 µg/ml. The MIC was done in triplicates for each bacterium, with ciprofloxacin hydrochloride being used as a positive control and a blank broth used as a negative control.

To prepare the bacteria for culture, all solutions were autoclaved prior to use. The bacteria were grown overnight at 37°C on an agar plate composed of BBL TSA II Trypticase Soy Agar (TSA) and BBL Trypticase Soy Broth (TSB) in a 1:2 ratio at 4.4% concentration. A broth solution of
2.4% TSB was inoculated using the bacteria from the agar plates, and was incubated at 37°C to reach a 0.5 McFarland standard. The bacteria was then further diluted by a factor of 1000 using a broth solution of 2.4% TSB, and 190 µl of the diluted bacterial solution was transferred by micropipette into each well. The inoculated plates were incubated at 37°C for 16-20 hours and the resulting plates were observed for growth and MIC values recorded. The MIC was the lowest concentration of the antibiotic that completely inhibited bacterial growth (visually) within that series of dilutions.

In vitro cell cytotoxicity was tested on two human cell lines, human colorectal carcinoma cells HCT-116, and human embryonic kidney cells HEK 293. HCT-116 were grown in Dulbeco’s Minimum Essential Medium (DMEM) with 10% fetal bovine serum and 0.1% penicillin/streptomycin as complete growth medium for several days at 37°C and 5% CO₂ to reach confluence. HEK 293 cells were grown in Eagle Minimum Essential Medium (EMEM) with 10% fetal bovine serum and 0.1% penicillin/streptomycin as complete growth medium for several days at 37°C and 5% CO₂ to reach confluence. Each cell type was then plated onto 96 well plates, at 50,000 cells per well at a volume of 150 ul with the respective complete growth medium. The cells were counted using a hemocytometer and then incubated for 24 hours at 37°C under an atmosphere of 5% CO₂.

The test emulsion was diluted using the complete growth medium for each cell type, and added into the wells of each test plate to give a final concentration of N-acryloylciprofloxacin of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml within a series. The testing was done in triplicate and one well in each triplicate was left untreated as the negative control for 100% growth. The plates were further incubated and monitored for 48 hours at 37°C under an atmosphere of 5% CO₂. A 5 mg/ml solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-
diphenyltetrazolium bromide (MTT) in sterile phosphate-buffered saline (PBS) was added to give a 10% final concentration in each well. The plates were then further incubated for 4 hours at 37°C under an atmosphere of 5% CO₂ to allow for the formation of the purple crystals of 1-(4,5-dimethylthiazol)2-yl)-3,5-diphenylformazan. The liquid was then aspirated from each well and 100 µl of dimethylsulfoxide (DMSO) was added to each well, and gently shaken for 1 min to allow for complete dissolution of the crystals. The IC₅₀ value for the assay was determined using a BioTek Synergy H1 hybrid plate reader at both 595 nm and 630 nm. The IC₅₀ was determined as the well with at least 50% cell viability compared to the untreated control cell with 100% cell growth.

Results

Poly(N-acryloyl ciprofloxacin) nanoparticle emulsions were successfully prepared by modification of the previously reported emulsion polymerization methodology. The main difference with this new procedure was the pre-solubilization of the water-insoluble antibacterial agent in an organic solvent to permit more uniform addition into the aqueous solution, in order to form homogeneous emulsions. We found that dichloromethane provided the best combination of solubilizing the ciprofloxacin monomer and being volatile enough to evaporate from the media during emulsion polymerization at 90°C.

We also found that the increased temperature (90°C rather than 75°C), stir speed (1100 rpm rather than 750 rpm), and the addition of sodium dodecyl sulfate before the monomers were added provided more optimal results. Additionally, we found it advantageous to let the reactions run for 48 hours rather than the usual 6 hours required for the butyl acrylate-styrene co-monomer systems.
As Figure 4 demonstrates, the dynamic light scattering confirmed the presence of a major population of nanoparticles in the emulsion, measuring approximately 970 nm in diameter, with a general trend of increasing size as the amount of N-acryloyl ciprofloxacin is increased from 1% to 10% in forming the polymer emulsions. In addition, the zeta potential measurements show that the particles carry a rather high surface charge of -63 (± 5.6) mV, indicative of the stability of the emulsion. It is notable that these homo(ciprofloxacin acryloyl) nanoparticles are much larger than those previously constructed with butyl acrylate-styrene co-monomers, which routinely measured 45-50 nm in diameter. The basis for this 20-fold increase in size is not apparent at this time.

(INSERT FIG4.DOCX)

The in vitro antibacterial studies showed that the nanoparticle emulsion was strongly bioactive, with an MIC of 0.5 µg/ml for *S. aureus* and 0.012 µg/ml against *E. coli*. These values are identical to those of ciprofloxacin itself. The finding that these nanoparticles show antibacterial capabilities at all against both the gram-positive *S. aureus* and the gram-negative *E. coli* was surprising, given the large dimensions of the particles, and the fact that the active antibacterial agent is chemically attached to the nanoparticle matrix.

Ciprofloxacin must enter the bacterial cell to arrive at its target, bacterial DNA gyrase, and bind within the gyrase tertiary structure.

Attachment of ciprofloxacin to the polymer backbone of the nanoparticle presumably requires hydrolysis of the amide linkage, prior to interaction with DNA gyrase. This occurs either outside of the cell, or within the bacterium itself if the nanoparticle can enter. Most likely this requires enzymatic involvement, as the amide functionality is a difficult one to cleave otherwise. The details of if and how this occurs within bacterial cells requires further investigation that is
beyond the immediate scope of this study. The in vitro cytotoxicity results for both human colorectal carcinoma cells HCT-116 and human embryonic kidney cells HEK-293 were also highly promising. The observed IC$_{50}$ was 500 µg/ml for both cell lines, a 1000-fold difference over the bacterial MIC value for *S. aureus* and greater than 40,000 fold for *E. coli*. Lyophilization of the nanoparticle emulsion produced an amorphous powder that could not be reformulated back to its original emulsified state through addition of water. Moreover, the resulting powder remained insoluble in organic solvents including methanol, ethanol, dichloromethane, hexane, acetone, ethyl acetate, and dimethylformamide. We did note that extraction of the solid material with methanol, ethanol, dichloromethane, hexane, acetone, and ethyl acetate failed to show any trace of unreacted N-acryloylciprofloxacin upon evaporation and analysis by proton NMR spectroscopy. This confirms that the polymerization is complete, and thus all of the ciprofloxacin is incorporated into the framework of the nanoparticle. Attempts to perform the emulsion polymerization procedure on the free ciprofloxacin instead of the N-acryloyl derivative led to a bilayer mixture, not an emulsion, with the layers separating within seconds. Additionally we attempted the same procedure using N-acetylciprofloxacin as an analog similar in structure but without the requisite olefin. Once again, we obtained only an unstable mixture that separated into layers within a few seconds. Therefore, the acryloyl group is a prerequisite for emulsification, and for nanoparticle formation.

Though the field of antibacterial polymers is well explored, typically they rely on the use of co-monomers in organic media.$^{8-10}$ This is the first case of an aqueous nanoparticle polymer emulsion being formed from a single monomer that carries the antibiotic agent itself. We hope to further investigate the properties of these and other types of antibiotic nanoparticle emulsions to expand the methodology for delivery and effective treatment of drug-resistant infections.
References


**Figure Legends**

**Figure 1**
Scheme for dimer based polymerization emulsions.

**Figure 2**
Scheme for synthesis of N-acryloylciprofloxacin.

**Figure 3**
Scheme for poly(N-acryloylciprofloxacin) polymerization emulsions.

**Figure 4**
Size of nanoparticles in emulsion vs the % concentration of N-acryloyleiprofloxacin.
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( Turos, E; Mahzamani, F; Bachman, A; Flores, K. “Menthol Based Nanoparticle for Drug Delivery”. U.S Patent 09533051B2, Jan, 2017)

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