Author

As an Immunology major, Thomas Assali has enjoyed the interdisciplinary aspect of his research. By applying the techniques of organic synthesis to biological targets, he has had the opportunity to participate in the first steps of the drug discovery process. After graduation, Thomas hopes to continue his research and move on to a graduate or professional school, possibly working toward a Ph.D. in immunology or organic chemistry. Outside the lab, Thomas enjoys traveling and plays a number of sports; he also listens to and plays music and is a particularly enthusiastic drummer.

Zwitterionic Polymer Nanoparticles for Stable and "Tunable" Plastic Antibodies

Thomas S. Assali
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Abstract

Although natural antibodies have become useful in medicine and science, their inefficiency and high cost make them less than ideal. Synthetic polymer nanoparticles (NPs) that bind biomolecules are of great interest as "plastic antibodies." Plastic antibodies have the potential to duplicate the function of natural antibodies at a substantially lower cost, but too few studies have been done to characterize NPs for use in vivo. Previously, the Shea Laboratory developed methods for producing NPs that interact specifically with target biomolecules. By preparing a library of NPs, they synthesized an NP that can capture melittin, a 26-residue peptide from honey bee venom, and neutralize its hemolytic toxicity *in vitro* and *in vivo*. For NPs to be functional *in vivo*, they must demonstrate not only high affinity and selectivity to target proteins but also stability in different solution conditions. It is also essential to develop a complementary charged surface to the protein because proteins have complex surfaces with unique charge distributions. We introduced a zwitterion monomer in an effort to stabilize NPs over a range of pH and high salt concentrations. A series of zwitterionic NPs containing different ratios of charged monomers was prepared in an attempt to match the surfaces of target proteins. By manipulating the charge ratio, NP affinity could be "tuned" to the target peptide.

Faculty Mentor

Thomas Assali's research has made important contributions to our "plastic antibody" program. We are developing abiotic synthetic polymer alternatives to antibodies and evaluating them for protein stabilization and purification, diagnostics and as therapeutic agents. Many of these nanoparticles, however, exhibited instability at low pH and/or high salt concentrations. Thomas modified them by incorporating zwitterionic functional monomers into polymerization reaction. He then established that this enhanced nanoparticle stability and efficacy, enabling their use over a wider range of conditions. Thomas is the most recent in a long line of outstanding undergraduates associated with my laboratory; all have gone on to graduate and professional school. I believe their undergraduate research experience was a key factor in their post-graduate success.

Kenneth J. Shea
School of Physical Sciences

Key Terms

- ELISA-Mimic Assay
- Hydrogel
- Melittin
- Plastic Antibodies
- Polymer Nanoparticles
- Zwitterionic Groups

Introduction

Synthetic polymer nanoparticle (NP) technology is an emerging field of great interest in medicine and biotechnology. NPs are polymers made of monomers containing functional groups suitable for interactions with biomolecules. In particular, NPs are of great interest as "plastic antibodies." Natural antibodies, which are Y-shaped proteins made by B cells of the immune system, bind to specific antigens or biomolecules and are widely used in treating medical conditions and isolating and/or purifying biomolecules or cells of interest (Murphy, 2012; Ma et al., 2012; Maejima et al., 2012; Alberts et al., 2008). Although natural antibodies have found considerable utility as therapeutic drugs and research tools, there are some disadvantages associated with their use. While they are useful as therapeutic drugs for treating some medical conditions, non-humanized antibodies can elicit immunogenic responses in some instances. For example, serum sickness is an allergic reaction that occurs when patients' bodies reject administered antibodies purified from a non-human animal, such as a horse, to treat venomous wounds like snakebites (Murphy, 2012). The reaction can lead to shock, serious complications, or even death. Furthermore, antibody treatment regimens against autoimmune diseases such as psoriasis and rheumatoid arthritis are extremely expensive, with the average regimen costing about $10,000 per month (Samaranayake et al., 2009). Although antibodies are very useful, they are expensive to produce and purchase; thus, the search for low-cost alternatives to antibodies is an active area of research.

Synthetic NPs, unlike natural antibodies, can be made in most laboratories and are not as costly to produce. In addition, recent advancements with NPs have demonstrated that NPs have significant potential as plastic antibodies. Because NPs are polymers made of multiple monomers with different functional groups, there are countless ways of making unique NPs. Moreover, just as natural antibodies can be tagged with fluorescent markers or magnetic beads for biomolecule visualization or isolation, NPs can be synthesized with monomers containing fluorescent or magnetized functional groups and used in exactly the same way as their natural counterparts (Wei et al., 2012; Wennmalm and Widengren, 2012; Zhang et al., 2012). The physical properties and behavior of NPs, however, have not been fully characterized and understood.

UCI's Professor Kenneth Shea and his research team are among those making advancements in NP technology. They have developed NPs that capture melittin, a 26-residue cytolytic peptide from bee venom that kills cells—especially red blood cells (RBCs)—by forming pores in cell membranes (Hoshino et al., 2010; Hoshino et al., 2012; Zeng et al., 2010; Bogaart et al., 2008). The NPs are made by dissolving monomers in water, adding an ionic surfactant, heating to 60ºC, and polymerizing by free-radical polymerization, (Figure 1). Following compositional optimization, the NPs eliminated the hemolytic activity of melittin, both *in vivo* and *in vitro* (Hoshino et al., 2010; Zeng et al., 2010), and were cleared by the liver of mice *in vivo* (Hoshino et al., 2010), demonstrating that the NPs are highly capable of acting as plastic antibodies that neutralize toxic biomolecules. Later, it was discovered that NPs have a unique temperature response to binding. Based on their monomer ratios, NPs release bound proteins if the solution temperature drops below their lower critical solution temperature (LCST), a phenomenon that was dubbed "catch and release" (Yoshimatsu et al., 2012). Temperature, however, is not the only way of enabling the NPs to catch and release bound substrates; solution pH and external magnetic fields are two other actively studied catch and release strategies (Keerl et al., 2009; Acciaro et al., 2011; Schoener et al., 2012; Barbucci et al., 2012). The catch and release property of NPs is becoming increasingly useful in drug development. Recently, scientists in Italy were able to make hydrogels from magnetic NPs that bound the chemotherapy drug doxorubicin efficiently and released it when an external magnetic field was applied to the particles (Barbucci et al., 2012). This indicates that magnetic NPs could deliver chemotherapy drugs directly to a tumor, treating cancers safely without harming healthy tissue. This sort of targeted drug delivery system is difficult to design using natural antibodies; hence, the potential for using NPs as plastic antibodies is worthy of attention.

There are many factors to consider in designing NPs for use *in vivo*, including NP stability in bodily environments and tissues and the interaction of NPs with non-target biomolecules. Each tissue has a unique microenvironment, and the bloodstream itself is filled with many inorganic and organic molecules that have the potential to denature NPs and prevent them from functioning properly. In addition, because the backbone of NP polymers and the functional groups of certain monomers are hydrophobic, there are concerns that NPs will interact with and precipitate non-target proteins *in vivo*, which could not only disable NP functioning but also increase the risk of complications. The latest reports indicate that a sulfobetaine zwitterion group (positively and negatively charged) stabilized gold NPs over a wide pH range and helped reduce nonspecific interactions of NPs with other proteins (Muro et al., 2010).

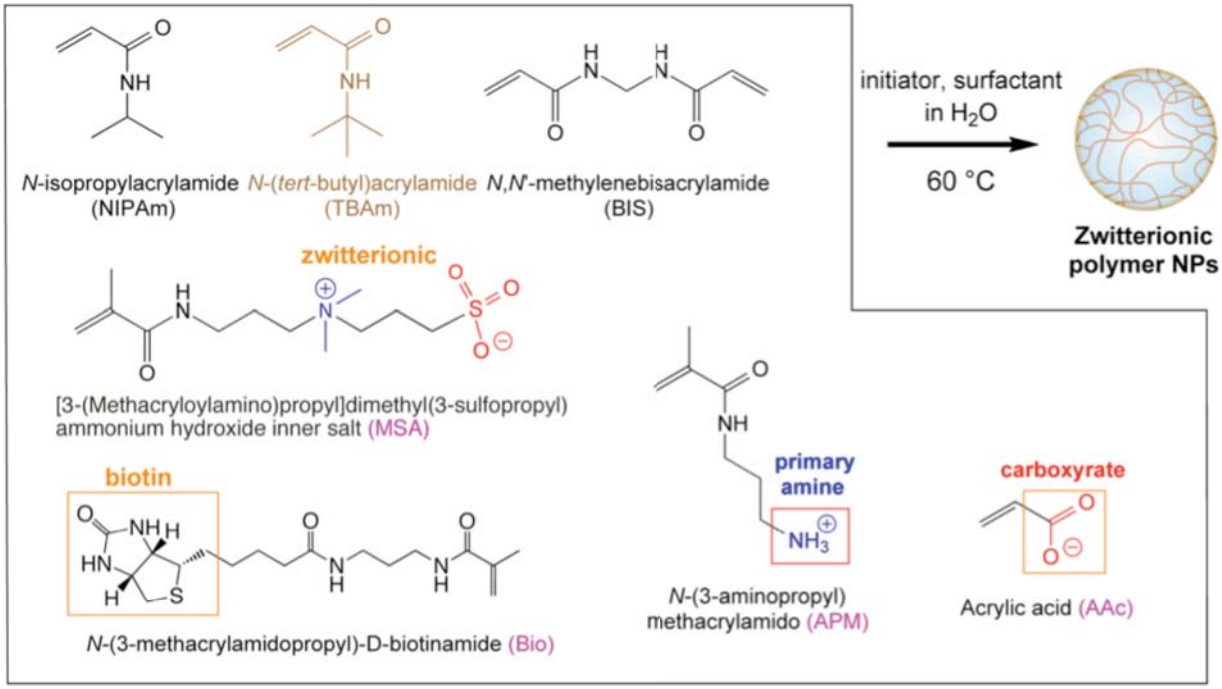


Figure 1

Monomers Used in NP Synthesis. The monomers shown were used to make NPs. They were mixed in nanopure water with a surfactant and filtered before being heated to 60°C, after which a free-radical initiator was added to begin polymerization. Non-charged monomers are always included for all syntheses, but charged monomers can be used together or individually.

In this study, we investigate the synthesis of NPs containing the zwitterion monomer MSA (see Figure 1 for full chemical name), their stability in extreme-pH and high salt solutions, and their neutralization activity of target proteins. Synthesizing NPs that can withstand the complex microenvironments *in vivo* is an essential step in the advancement of plastic antibodies. NPs have great capabilities and the potential to significantly advance the treatment of many diseases, but if they cannot withstand the intricate microenvironments inside the body then they cannot be used to improve the quality of life for people who need it.

Monomers Used in NP Synthesis. The monomers shown were used to make NPs. They were mixed in nanopure water with a surfactant and filtered before being heated to 60°C, after which a free-radical initiator was added to begin polymerization. Non-charged monomers are always included for all syntheses, but charged monomers can be used together or individually.

Materials and Methods

Chemicals

All chemicals were obtained from commercial sources: *N*-isopropylacrylamide (NIPAm), melittin (from honey-bee venom), ammonium persulfate (APS), azobisisobutyronitrile (AIBN), [3-(Methacryloylamino)propyl]dimethyl(3-sulfopropyl) ammonium hydroxide inner salt (MSA), and *N,N'*-methylenebisacrylamide (BIS) were from Sigma–Aldrich, Inc.; sodium dodecyl sulfate (SDS) and cetyltriammonium bromide (CTAB) were from Aldrich Chemical Company, Inc.; *N*-(3-aminopropyl)methacrylamide (APM) was from Polysciences Inc.; D-(+)- biotin and *N*-*t*-butylacrylamide (TBAm) were from Acros Organics; horseradish peroxidase (HRP)-conjugated avidin was from MP biomedicals; histone from calf thymus (type III−S) and fibrinogen from human plasma fraction II, type III, were from Sigma; and Immuno MicroWell 96-well plate (Maxisorp, flat-bottom, pinchbar, 400 μL) was from Nunc. The biotin monomer (N-(3-methacrylamidopropyl)-D-biotinamide; Bio) was synthesized as previously reported (Yonamine et al., 2012); and bovine red blood cells (RBCs) were from Lampire Biological Laboratories, Inc. NIPAm was recrystallized from hexane before use. Other chemicals were used as received. Water used in polymerization was distilled and then purified by using a Barnstead Nanopure Diamond system.

Synthesis of NPs

NPs were prepared by first dissolving all monomers (Figure 1), except TBAm, in nanopure water with an anionic (SDS) or cationic (CTAB) surfactant. TBAm was dissolved in 1 mL of ethanol in a separate container before being added

to the monomer mixture. Water was then added to the monomer mixture to a total volume of 50 mL, and the mixture was then filtered through a no. 2 Whatman filter paper by vacuum filtration. Next, the solution was heated to 60°C while a nitrogen purge was conducted for 10 minutes. Once at 60°C, a free radical initiator (either APS or AIBN) was added and polymerization was allowed to run for three hours. NPs were then collected into cellulose dialysis tubes (Fisher Scientific) and diluted in 4 L buckets of deionized water at least eight times, with 10–12 hours in between each dilution. After dilution the NPs were placed in a final labeled container.

Dynamic Light Scattering (DLS) Analysis

NP solutions for DLS analysis were prepared by mixing 100 μL of the NP solution with 900 μL nanopure water in a 1.0 mL polystyrene cuvette. Data collected was analyzed to determine sample quality and solution dispersion.

Hemolytic Assay

Hemolytic activity of melittin was assessed by a modified hemolytic assay. RBCs were collected by centrifugation (10 min, 3000*g*) and suspended in phosphate buffered saline (PBS; 35 mM phosphate buffer, 0.15 M NaCl, pH 7.3) after being washed with PBS three times. Melittin (final concentration in RBC suspension was 1.8 mM) was pre-incubated with NPs for 30 min at 37°C in PBS. The melittin/NP mixture was then added to 100 μL of RBC solution to give a final volume of 200 μL (final erythrocyte concentration 3% v/v). The resulting suspension was incubated at 37°C for 30 min. Samples were then centrifuged at 3000*g* for 10 minutes. Release of hemoglobin was monitored by measuring the absorbance of the supernatant from 410–416 nm by UV–visible spectrometry (NanoDrop 2000c, Thermo Scientific). Melittin with no NPs was the positive control, and RBCs with no melittin or NPs were the negative control.

ELISA-Mimic Assay

The ELISA-Mimic assay was performed as specified in Yonamine *et al.* (2012). Proteins (histone or fibrinogen) were dissolved in phosphate-buffered saline (PBS; 35 mM phosphate and 150 mM NaCl, pH 7.3) to produce solutions of 500 μg/ mL each. The solutions (100 μL each) were loaded in the wells of an Immuno MicroWellTM 96-well plate (MaxisorpTM, flat-bottom, pinchbar, 400 μL) to immobilize the proteins on the plate through physical adsorption. After immobilization for 1 hr at room temperature, each well was washed twice with 250 μL of PBS buffer. NP-PBS solutions were prepared by mixing 125 μL of the NP solution with an equal amount of double concentrated PBS buffer and loaded in the wells of the protein-immobilized plate. After incubation for 1 hr at room temperature, each well was washed three times with 250 μL of PBS buffer including 0.05% of Tween 20. HRP-conjugated avidin stock solution (3 μL; 1.79 mg/mL in 0.014 M sodium phosphate, 0.1 M sodium chloride, pH 7.3, with 0.7% bovine albumin, 36% glycerol, and 0.01% thimerosal) was diluted with 21 mL of PBS buffer and the solution (200 μL each) was loaded in the wells of the plate. After incubation for 1 hr at room temperature, each well was washed three times with 250 μL of PBS buffer. The substrate solution was prepared using SIGMA*FAST*™ OPD tablet kit (Sigma- Aldrich) in the instructed manner (0.4 mg/mL *o*-phenylendiamine, 0.4 mg/mL urea hydrogen peroxide, 0.05 M phosphate-citrate, pH 5.0) and 200 μL of the solution was loaded in the each well of the plate. After incubation for 4 min at room temperature, 50 μL of 3N HCl solution was added to each well to stop the enzyme reaction. The absorbance of each well at 492 nm was measured by μQuantTM Microplate Spectrophotometer (Bio-Tek instrument, Inc.).

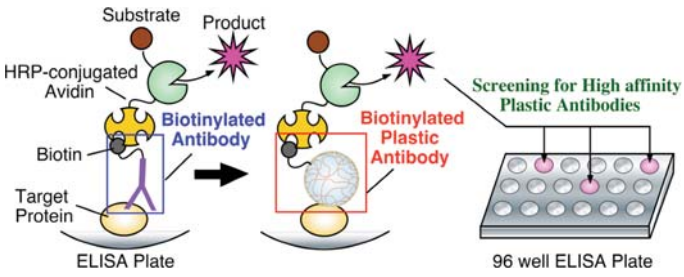


Figure 2

The ELISA-Mimic Assay. In a standard ELISA assay, biotinylated antibodies are used to visualize the presence of a target protein. In the ELISA-mimic screening protocol, a biotinylated NP is used instead of a biotinylated antibody to evaluate the affinity of the NPs to a target protein.

Results

DLS Analysis of NPs with MSA & Zwitterionic NPs

DLS analysis indicated AAc containing NPs synthesized with MSA had small variations in size (Table 1). Samples that contained a large amount of precipitate visible to the naked eye or had sediment according to DLS are labeled "aggregation." For the most part, NPs maintained a near-constant size despite extreme pH or high salt conditions. Zwitterionic NPs made with AAc, MSA, and APM were all successfully synthesized when made with the cationic surfactant CTAB (Table 2). Qualities in Table 2 are indicated by the background color of the measurement: green indicates good quality and red indicates poor quality.

Table 1

Sizes of MSA NPs. The introduction of the zwitterionic monomer MSA helped to stabilize NPs under extreme pH and high salt conditions, as shown below. The sizes of NPs in each solution condition are given in nanometers (nm). All measurements were good quality except those marked "aggregation."

AAc	MSA	H_2O	PBS	HCl	NaOH	NaCl
5%	0%	110	137	aggregation	166	aggregation
	5%	89	198	80	217	83
	10%	53	65	50	71	52

Table 2

Sizes & Qualities of Zwitterionic NPs. Some NPs containing both positively and negatively charged monomers in addition to MSA were successfully synthesized with use of CTAB surfactant. Use of SDS surfactant resulted in precipitated solutions. Values with green backgrounds indicate a good quality measurement, and red backgrounds indicate poor quality measurements. Sizes are listed in nanometers (nm).

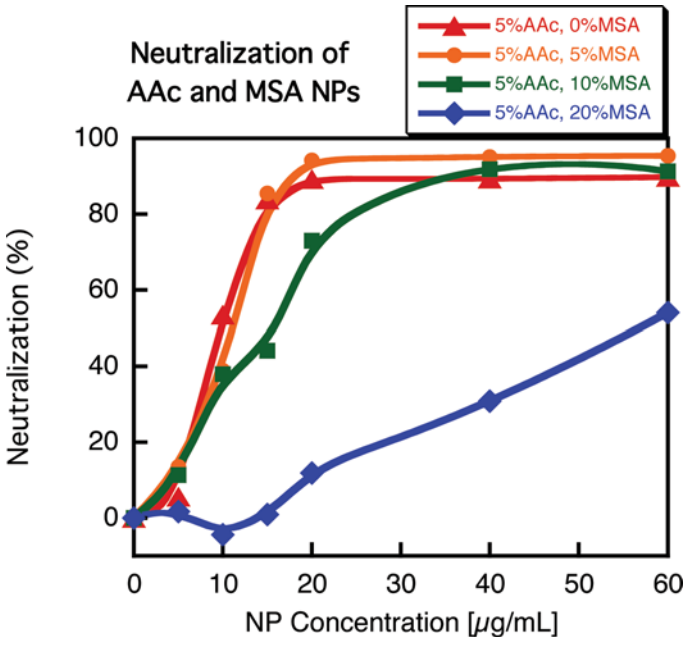
	0% APM	5% APM	7.5% APM	10% APM	20% APM	
5% MSA +5% AAc	–	99.04	7985	66.62	64.41	CTAB
	aggregation	aggregation	–	–	–	SDS

NP Neutralization Activity in the Red Blood Cell Test with MSA & Zwitterionic NPs

NPs with MSA showed a decrease in neutralization ability of melittin with increasing amounts of MSA in the red blood cell test (Figure 3). In particular, 10% and 20% MSA-containing NPs had notably lower neutralization ability of melittin, indicating lower melittin affinity; however, 5% AAc + 5% MSA showed comparable activity to 5% AAc only. Using 5% MSA, the zwitterionic NPs were prepared using different ratios of APM and AAc. The NPs with higher amounts of APM had an overall lower neutralizing activity against melittin (Figure 4), indicating a loss of affinity to melittin.

Melittin Neutralization Activity of AAc and MSA NPs in the Red Blood Cell Test. Several NPs with greater percentages of MSA were prepared and their neutralization activity of melittin was assessed. As shown above, increasing the amount of MSA incorporated in the NP decreases melittin's neutralization ability. This indicates that increasing the levels of MSA lowers NP affinity to melittin.

Melittin Neutralization Activity of Zwitterionic NPs including AAc and APM in the Red Blood Cell Test. NPs



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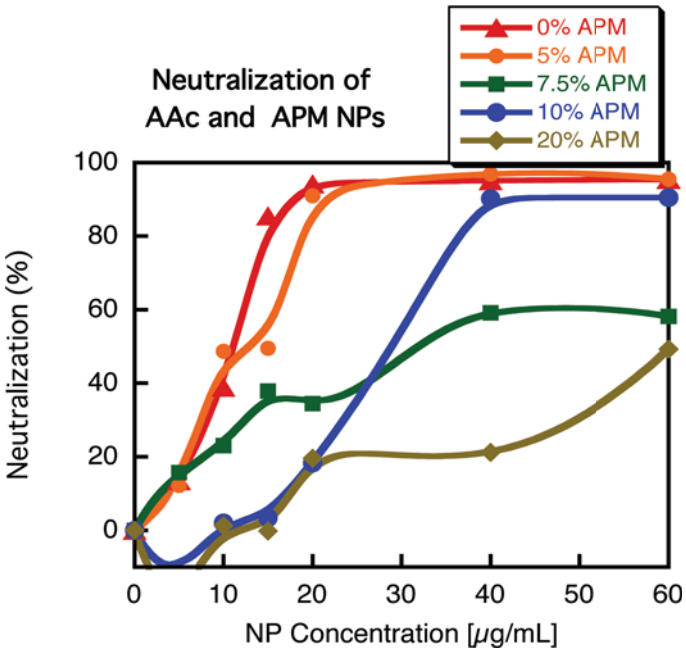

Figure 4

Melittin Neutralization Activity of Zwitterionic NPs including AAc and APM in the Red Blood Cell Test. NPs were synthesized with 5% MSA and the cationic surfactant CTAB, except for the NP with 0% APM (this is the 5% AAc + 5% MSA NP from Figure 3, and was made with SDS surfactant). As the data shows, increasing the amount of the positively- charged monomer APM decreases the neutralization of melittin, a positively-charged peptide.

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Evaluation of NP Affinity by the ELISA-Mimic Assay

NPs with different ratios of AAc and APM, each with a constant 5% MSA, were prepared. A biotin monomer (1%) was also included in the polymerization. The unique affinities to positively charged histone and negatively charged fibrinogen are evaluated by the ELISA-mimic screen (Figure 5). The patterns of affinity to histone and fibrinogen were clearly different. AAc-incorporated NPs had strong affinity to histone while the APM NP without AAc showed no interaction with histone. On the other hand, only 5% APM/15% AAc NP showed high affinity to fibrinogen.

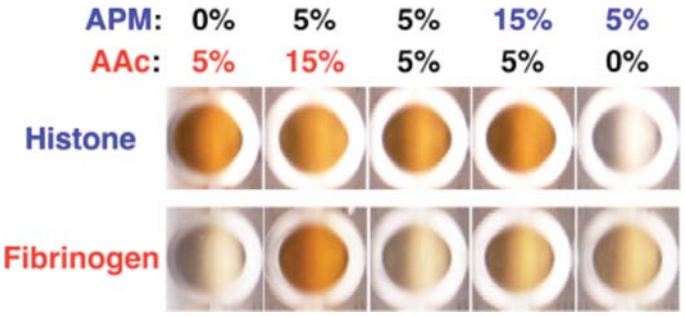

Figure 5

ELISA-Mimic Assay Results. Negative charges are indicated in red and positive charges in blue. APM and histone are positively charged and AAc and fibrinogen are both negatively charged. NPs with net positive charge showed weak affinity to histone but good affinity to fibrinogen, while overall negatively-charged NPs showed strong affinity to histone but weak affinity to fibrinogen, demonstrating that NP affinity can be tuned, or adjusted, to each protein.

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Discussion

NP Stabilization with MSA

NPs synthesized with only AAc functional monomers were stable under most conditions but could not withstand low pH and/or high salt concentrations (Table 1). We expected the addition of MSA groups to stabilize NPs under all conditions, and indeed it did, as evidenced by the fact that the NPs exhibited only small variations in size and stability even in low pH and high salt conditions. This result can be attributed to the zwitterionic structure of MSA that keeps NPs charged and hydrated in both strongly acidic and basic conditions. We were very pleased with the results of MSA incorporation because NP stability *in vivo* can now be guaranteed by synthesizing NPs with MSA. The resulting NPs can handle different microenvironments *in vivo* and will now be synthesizable. Also, it appears that the addition of increasing amounts of MSA into the NPs results in smaller particle sizes and better size preservation under different solution conditions, possibly due to increasing stability in water, as seen with the 5% AAc/10% MSA NP in Table 1.

Hemolytic Assay of MSA NPs

The NPs made with 10% and 20% MSA had markedly lower neutralization activity against melittin in the red blood cell test compared to NPs with 5% or no MSA. This result was attributed to the loss in hydrophobicity of NPs by the incorporation of the hydrophilic MSA. Hydrophobicity is essential for high affinity with melittin. However, the 5% AAc/5% MSA NP had comparable activity to the 5% AAc only NP, and even had slightly higher neutralization capacity; therefore, we concluded that 5% MSA was an optimal amount for use in making NPs that are not only stable under a variety of solution conditions but that also maintain their high affinity towards our target peptide, melittin. This finding is very important because it demonstrates that stabilizing NPs across varying solution conditions does not affect NP affinity to the target biomolecule, confirming their potential to replace natural antibodies with plastic antibodies.

Hemolytic Assay of Zwitterionic NPs

The zwitterionic NPs with AAc and APM (and MSA) showed similar patterns of efficacy in the red blood cell test, except it was noted that increasing positive charge by the addition of APM resulted in loss of melittin affinity. Melittin, a short peptide, contains positively charged residues and therefore is positively-charged. Like-charged NPs would be expected to have little to no affinity to it because of electrostatic repulsion. The AAc and APM NPs demonstrate this trend, as NP neutralization of melittin falls

markedly with the increase of APM, especially in NPs with 10% and 20% APM. The 10% APM NP reached its peak neutralization at 40 μg/mL, unlike the 7.5% and 20% APM. However, we believe every NP solution eventually saturates at high concentration, possibly because of non-specific binding, and that all the melittin becomes bound, regardless of the particle affinity; perhaps this explains what happened with the 10% APM NP. Nevertheless, the trend clearly shows that NP affinity to melittin is easily affected by the ratio of positive and negative charges in NPs. This confirms our reasoning that NP affinity can be tuned to the target biomolecule by adjusting the percentage of functional monomers, the charge ratio in this case. This will be important for future designs where NP affinity to the target needs to be enhanced while nonspecific binding of NPs to other proteins needs to be suppressed.

ELISA-Mimic Assays of Zwitterionic NPs

The zwitterionic NPs with AAc and APM (and MSA) had clear patterns of affinity to histone and fibrinogen. We expected APM NPs with more positive charge to bind more strongly to fibrinogen and AAc NPs with more negative charge to bind more strongly to histone. The 15% APM/5% AAc NP from the ELISA-mimic assay showed unusually high affinity towards histone, pI (isoelectric point) 10.8 (Nelson and Cox, 2008), despite the high APM content, but this could be because the AAc monomer was better incorporated into the polymer, which allowed a significant portion of the NP to bind histone despite the presence of a positive charge. In the case of fibrinogen, with pI between 5.5-5.95 (Triantaphyllopoulos and Triantaphyllopoulos, 1967), the 5% APM/15% AAc NP showed strong affinity while more positively charged NPs did not show significant affinity. These results indicate there is an optimal balance between negative and positive charges for making a complementary surface to a target protein. We concluded the addition of MSA not only stabilized NPs under harsh solution conditions but allowed us to tune NP affinity to target proteins simply by manipulating the ratio of positively and negatively charged monomers in the NP. As stated previously, this is important in future designs where NP stability and binding to the target biomolecule need to be enhanced while simultaneously suppressing nonspecific interactions to other biomolecules.

Acknowledgements

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