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14. ABSTRACT
Stimulus-induced changes in surface properties of nanoassemblies have been of interest in a variety of applications. One such change involves change in surface charge. Changes in surface charge can be conveniently assayed using interaction of the nanoassemblies with cells. We have utilized the nanoassembly-cell interaction to investigate the pH-induced changes in surface properties.
Similarly, surface functionalization of nanoparticles and host-guest properties of nanoassemblies are two critical features in the utilization of nanostructures in a variety of applications in materials, chemical, and biological

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Report Title

Stimuli Responsive Amphiphilic Assemblies

ABSTRACT

Stimulus-induced changes in surface properties of nanoassemblies have been of interest in a variety of applications. One such change involves change in surface charge. Changes in surface charge can be conveniently assayed using interaction of the nanoassemblies with cells. We have utilized the nanoassembly-cell interaction to investigate the pH-induced changes in surface properties.
Similarly, surface functionalization of nanoparticles and host-guest properties of nanoassemblies are two critical features in the utilization of nanostructures in a variety of applications in materials, chemical, and biological nanotechnology. However, simultaneously incorporating these two features in one nanoparticle design is a rather challenging task. We were interested in developing a simple approach for functionalizable polymer nanoparticles, where we stipulated that: (i) the precursor polymer is based on a random copolymer, which is synthetically accessible; (ii) the polymer self-assembles in a solvent, which can then be converted to nanoparticle in one step without the need for any additional processing; (iii) the nanoparticle contains a surface functional group, which can be further manipulated easily; (iv) the size of the nanoparticle is tunable; and (iv) the interior of the nanoparticle is capable of sequestering guest molecules.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
01/14/2011	1.00	Tejaswini S. Kale, Akamol Klaikherd, Bhooshan Popere, S. Thayumanavan. Supramolecular Assemblies of Amphiphilic Homopolymers, Langmuir, (04 2009): . doi:
10/17/2011	3.00	Feng Wang, Akamol Klaikherd, S. Thayumanavan. Temperature Sensitivity Trends and Multi-Stimuli Sensitive Behavior in Amphiphilic Oligomers, Journal of the American Chemical Society, (08 2011): 0. doi: 10.1021/ja204121a
10/17/2011	4.00	Krishna R. Raghupathi, Malar A. Azagarsamy, S. Thayumanavan. Guest-Release Control in Enzyme-Sensitive, Amphiphilic-Dendrimer-Based Nanoparticles through Photochemical Crosslinking, Chemistry - A European Journal, (10 2011): 0. doi: 10.1002/chem.201101066
10/17/2011	5.00	Rajasekharreddy Ramireddy, S. Thayumanavan, Volkan Yesilyurt. Photoregulated Release of Noncovalent Guests from Dendritic Amphiphilic Nanocontainers, Angewandte Chemie International Edition, (03 2011): 0. doi: 10.1002/anie.201006193
TOTAL:	**4**	

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Keynote Lecture, Biodendrimers Meeting, Toledo, Spain, September 5-8, 2012 (Organizers: M ‘Angeles Munoz-Fernandez)

Keynote Lecture, IUPAC International Conference on Novel Materials and their Synthesis, Xi An, China, October 14-19, 2012 (Organizers: Anning Zhou, Min Zhang & Yuping Wu, Fudan University)

Plenary Lecture, PolyTech – 2012: International Conference on Advances in Polymeric Materials & Nanotechnology, Pune, India, Dec. 15 – 17, 2012 (Organizers: R. P. Singh)

8th International Dendrimer Symposium (IDS-8), Madrid, Spain, June 23-27, 2013 (Organizers: Dr. M’Angeles Munoz-Fernandez)

Symposium on Photo-cleavable Polymers and Block Copolymers National ACS Meeting, San Diego, CA, “Title to be determined”, March 25-29, 2012 (Organizers: Drs. E. Bryan Coughlin and Patrick Theato)

Symposium on Responsive Nanostructured Materials via Self-Assembly National ACS Meeting, San Diego, CA, “Assembly and disassembly of protein-responsive polymeric nanomaterials”, March 25-29, 2012 (Organizers: Drs. Andriy Voronv and Lenoid Ionov)

Symposium on Adaptive Materials through Molecular Networks, Materials Research Society Meeting, San Francisco, CA, “Responsive Polymeric Nanoassemblies” April 2-5, 2013 (Organizers: Nathan Gianneschi, Rein Ulijn, Jan van Esch, Rajesh Naik)

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Received Paper

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Received Paper

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Received Paper

TOTAL:

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Patents Awarded

Awards

Award for Outstanding Research and Creativity, University of Massachusetts (2013)
International Advisory Board, Federation of Asian Polymer Societies (2012-13)
International Advisory Board, Polytech, India (2012)
Elected Fellow, American Association for Advancement of Sciences (AAAS) (2011)

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Mallory Gordon	0.09	
Reuben Chacko	0.31	
Longyu Li	0.09	
Celia Frieler	0.20	
Kishore Raghupathi	0.09	
Hui Wang	0.44	
Priyaa Prasad	0.20	
Bin Liu	0.10	
FTE Equivalent:	**1.52**	
Total Number:	**8**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME	
Reuben Chacko	
Total Number:	**1**

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

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Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

Progress Report for the U.S. Army Research Office

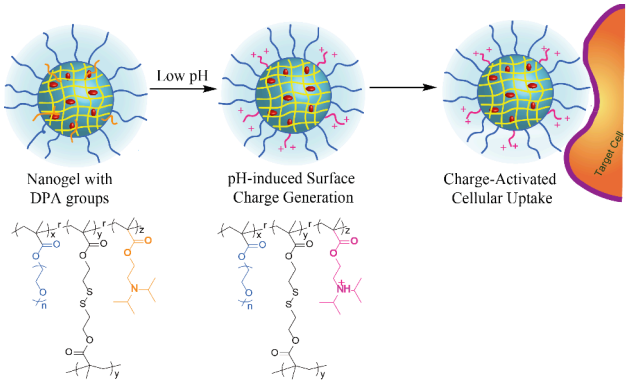
Design and Synthesis of Stimuli-Responsive Amphiphilic Nanoassemblies

Submitted by: S. Thayumanavan to Dr. Jennifer Becker (ARO); November 2013

pH-induced Surface Charge Generation in Nanogels

Stimulus-induced changes in surface properties of nanoassemblies have been of interest in a variety of applications. One such change involves change in surface charge. Changes in surface charge can be conveniently assayed using interaction of the nanoassemblies with cells. We have utilized the nanoassembly-cell interaction to investigate the pH-induced changes in surface properties. Nanocarriers that can be effectively transported across cellular membranes have potential in a variety of applications. It is well known that nanocarriers with cationic surface charge are readily internalized by the cells because of their higher affinity for the negatively charged phospholipid bilayer of cell membranes. However, the positively charged nature of carriers may also induce non-specific interactions in serum, leading to significant toxicities. In this context, stimuli-responsive charge conversional systems have been used. Typically, it is desirable that the surface charge of nanoparticles be neutral or even negatively charged under normal physiological conditions. Then, under the aberrant pH conditions, the surface charge of these environment-responsive nanoparticles become positive.

In this study, we designed a simple method to prepare polymeric nanogel containing 2-(diisopropylamine) (DPA) moieties, which generate surface charge with pH as stimulus (Scheme 1). The choice of DPA nanogel is based on its pK_b (about 6.2), which causes this functional group to be rapidly protonated upon change in the slightly acidic conditions. We show that the pH at which the charge is generated, i.e. the isoelectric point (pI) of the nanogel, can be adjusted by varying the percentage of DPA units in the nanogel, its preparation process and crosslinking density. Intracellular delivery of these nanogels was greatly enhanced in an acidic pH environment due to the surface charge generation.



Scheme 1 Schematic illustration and chemical structure of polymeric nanogels for pH-induced surface charge generation and activated cellular uptake.

Four different copolymers with varying comonomer compositions were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization reaction. Copolymers P1-P4 were synthesized by simply varying the feed ratio of the monomers during the polymerization. Copolymer P4 differs from the polymers

Table 1 Characteristics of PEGMAx-co-DPAy-co-PDSz copolymers (P1–P4).

Sample code	*x/y/z*[a]	Mn(kDa)[b]	Đ [b]
P1	0.28/0.19/0.53	8.26	1.29
P2	0.28/0.36/0.36	10.2	1.34
P3	0.26/0.49/0.25	8.36	1.23
P4[c]	0.26/0.32/0.42	12.3	1.51

[a] Calculated weight ratio by 1H NMR
[b] Determined by GPC in THF using PMMA as standards
[c] The molecular weight of PEGMA is 350 g/mol

P1-P3, as P4 is synthesized by using a PEGMA with shorter length polyethyleneglycol side chain (~350 g/mol). The number-average molecular weights (Mn) and dispersity (Đ = Mw/Mn) of the copolymers were evaluated by GPC. The results are summarized in Table 1.

First, the pI indeed increased with the ratio of DPA in the copolymer. The pI of the NG1 was found to be 6.2, while those of the NG2 and NG3 were found to be 6.7 and 6.8 respectively. These results are taken to suggest that the surface charge generation is driven by the protonation-induced charge build-up within the interior of the nanogel. After a certain charge build-up, the electrostatic repulsion is likely relieved by presenting the protonated DPA units on the surface of the nanogel. This change is achieved at higher pH in the case of larger number of DPA units, because the critical charge built-up can be achieved within the nanogel interior relatively quickly. We also studied the effect of length of OEG units on the charge conversion. We found that the pI of the nanogel from NG4, which had shorter OEG units, was slightly higher than those of NG2 and NG3, even though the relative ratio of DPA units was smaller in the case of NG4.

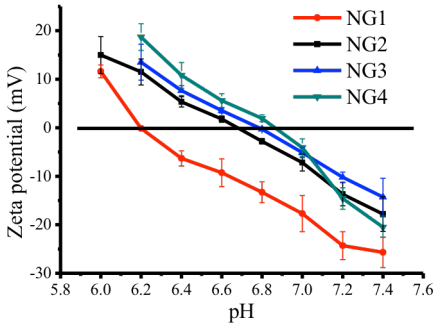


Figure 1 Change in the zeta potential of nanogels as a function of pH. NG1 - NG4 were made by using P1-P4 at pH 10. All nanogel crosslinking densities were 100%, achieved by adding 50 mol% DTT with respect to PDS groups.

Note that the crosslink density of NG4 was higher than that of NG2 and NG3 in the experiment above. The effect of crosslink density on pI was also studied by preparing three nanogels of different crosslink densities, labeled as NG1-60x, NG1-80x, and NG1-100x respectively, indicating the percent crosslink densities in each of the nanogels. As shown in Figure 2,

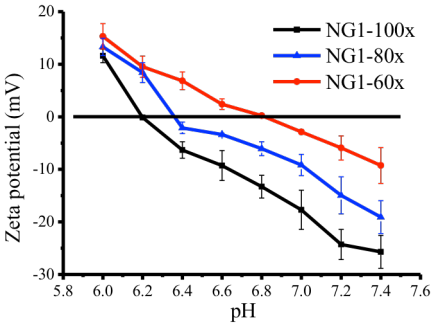


Figure 1 Change in the zeta potential of nanogels with different crosslinking density as a function of pH. All nanogels were made by using P1 at pH10, and crosslinking density was varied by changing the amount of DTT

the pI for the NG1-60x is much higher than that of NG1-80x, which was higher than that of NG1-100x. These results suggest that: (i) the pH-induced surface presentation of the DPA units, buried in the core of the nanogel when hydrophobic and unprotonated, is influenced by the flexibility of the polymer chains; and (ii) the higher pI of NG4, compared to NG2 and NG3, is not due to the crosslink density variation and is indeed due to the shorter OEG units on the surface of NG4.

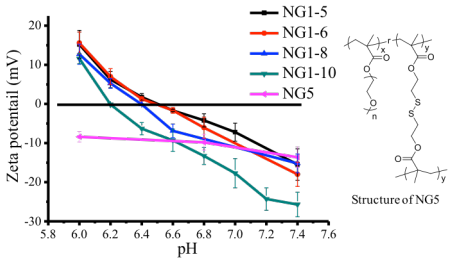


Figure 3 Change in the zeta potential of nanogels as a function of pH. Crosslinking density in all nanogels was fixed at 100%. For comparison, nanogel without DPA units (NG5) was prepared; structure of NG5 is also shown.

Next, we investigated the effect of nanogel preparation conditions upon its pI; more specifically the pH of the solution at which the crosslinking reaction was carried out. Nanogels were prepred at pH 5, 6, 8, and 10 using P1 and 50% DTT to obtain NG1-5, NG1-6, NG1-8, and NG1-10 respectively. We found the pI of the nanogel to be dependent on the preparative

conditions (Figure 3). For example, the pI for NG1-8 was found to be ~6.4, while the pI of the NG1-5 and NG1-10 were found to be 6.5 and 6.2 respectively. To make sure that the observed differences are not due to any adventitious effects and are indeed due to the DPA units, a structurally similar nanogel NG5, but without the DPA units, was prepared. The zeta potential of the nanogel without DPA units remained at -10 mV at whole pH range. This clearly suggests that the observed pH-dependence is indeed due to the DPA units in the nanogel.

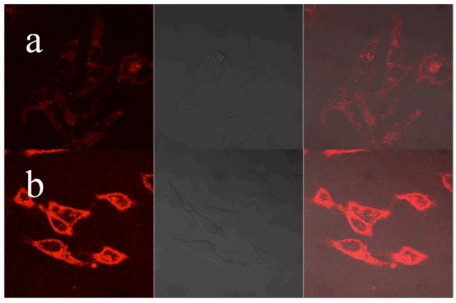


Figure 4 (a) and (b) are confocal images of NG2, containing DiI at pH 7.4 and 6.5 respectively, after incubation with HeLa cells for 2 hours.

The key motivation in altering surface charges in response to pH is the ability to demonstrate pH-dependent cellular uptake. We investigated the internalization behavior of DiI-encapsulated NG2 at the normal extracellular pH of 7.4 and pH_e of 6.5 with the HeLa cell line. Significantly different cellular uptake was observed for the DPA nanogels under these conditions. As shown in Figure 4, the red fluorescence is significantly higher at pH 6.5, compared to pH 7.4. We attributed this difference to the possibility that the DPA nanogel would become positively charged upon the protonation of DPA groups at lower pH and thus enhance the cellular internalization. To further confirm that the pH-dependent internalization is indeed due to change in surface charge, nanogels with covalently attached fluorescein were prepared and incubated with the HeLa cells for 2 hours, at both pH 6.5 and pH 7.4. From the optical microscopy images, it is clear that the cells treated with nanogels at pH 6.5 exhibit much higher cellular uptake, compared to the same nanogels at pH 7.4 (Figure 5). These results confirm that the difference in fluorescence images at two different pHs, with the DiI-encapsulated nanogels, is not due to the non-covalently encapsulated guest leakage. These results also support our assertion that the difference in cellular uptake is indeed due to the greater nanogel uptake at pH 6.5, most likely due to the difference in surface charge. These results also suggest that the current nanogel system can be used in drug delivery via both physical encapsulation and chemical conjugation.

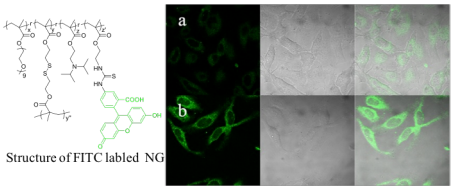


Figure 5 (a) and (b) are confocal images of fluorescein-labeled DPA nanogel at pH 7.4 and 6.5 after incubation with HeLa cells for 2 hours. The concentration of the nanogels used was 1mg/mL.

In summary, we report on a nanogel that is capable of transforming into a positively charged nanogel at a lower pH t. We have shown that: (i) the co-incorporation of DPA units to PEG-based nanogels can lead to effective pH-dependent charge generation; (ii) the pI of the DPA nanogel can be systematically tuned by varying the (a) percentage DPA units present in the nanogel, (b) percentage of DPA units that are initially exposed on the nanogel surface, and (c) crosslink density; (iii) the charge generation process can be utilized to enhance cellular uptake at lower pH_e, compared to the normal extracellular pH. It is remarkable that this charge generation event results in enhanced cellular uptake, even though the length of the OEG units are much higher than the side chains that contain the DPA moieties; and (iv) non-covalently encapsulated guest molecules are stably encapsulated at both pH and are thus effectively taken up by the cells at lower pH without any leakage.

Functionalizable Amine-based Polymer Nanoparticles

Surface functionalization of nanoparticles and host-guest properties of nanoassemblies are two critical features in the utilization of nanostructures in a variety of applications in materials, chemical, and biological nanotechnology. However, simultaneously incorporating these two features in one nanoparticle design is a rather challenging task. We were interested in developing a simple approach for functionalizable polymer nanoparticles, where we stipulated that: (*i*) the precursor polymer is based on a random copolymer, which is synthetically accessible; (*ii*) the polymer self-assembles in a solvent, which can then be converted to nanoparticle in one step without the need for any additional processing; (*iii*) the nanoparticle contains a surface functional group, which can be further manipulated easily; (*iv*) the size of the nanoparticle is tunable; and (*iv*) the interior of the nanoparticle is capable of sequestering guest molecules.

In this work, we report on the design, synthesis, characterization, and further functionalization of amine-functionalized polymeric nanoparticles that satisfy above requirements. Random copolymer **1** was synthesized using RAFT polymerization. This amphphilic polymer form an aggregate of ~22 nm upon UV radiation at 365nm. For guest binding study, we were able to encapsulate hydrophobic dye molecules, such as DiI or DiO, using the polymer aggregates successfully and guest molecules were retained in the interiors of nanoparticles after photoinduced crosslinking.

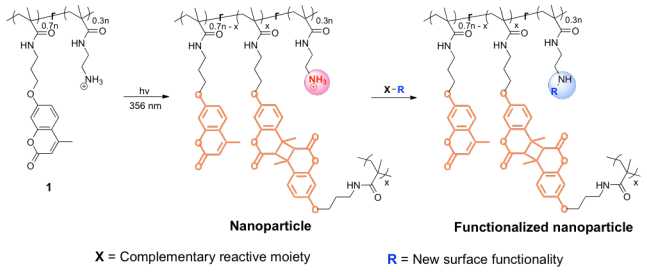


Figure 6. Synthesis of the polymer precursor and the nanoparticle.

To investigate the versatility of the amine functionality as a handle for surface functionalization of the nanoparticles, we tested a wide range of functional groups and characterized these modifications with different techniques. First, we reacted amines with an activated ester and a cyclic anhydride to provide amides with complementary surface characteristics. Reaction of the amine nanoparticles with a peg-2000 NHS ester should convert the positively charged surface of the polymer nanoparticle to a charge neutral surface, while the reaction with succinic anhydride should convert the charge to negative. Zeta potential measurements of the reactants and the products indeed confirmed such surface charge modification (Figure 7a). Second, the nanoparticles were modified by a NHS ester (of

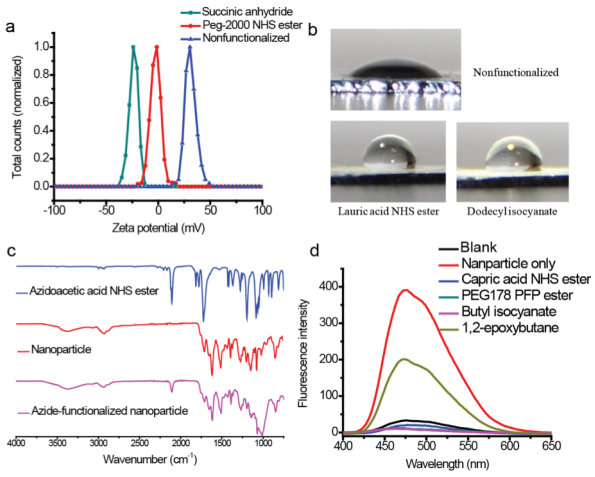


Figure 7. (a) Surface charges of nanoparticles by zeta potential (b) Contact angle measurements of unmodified nanoparticles (top) and nanoparticles modified by lauric acid NHS ester (bottom left) and dodecyl isocyanate (bottom right). (c) IR spectra of azidoacetic acid NHS ester (top), unmodified nanoparticles (middle), and nanoparticles functionalized with azidoacetic acid NHS ester (bottom). (d) Fluorescence emission intensity of nanoparticles treated with excess fluorescamine after reacting with different functional groups.

lauric acid) and an isocyanate (dodecyl). Both modifications would change the surface nanoparticles from hydrophilic to hydrophobic. Evaluation of the nanoparticle surface hydrophobicity by contact angle showed that the unmodified nanoparticles exhibited a contact angle of 33°, while the modified nanoparticles have a contact angle of 103° and 98°, respectively (Figure 7b). Next, we sought to monitor the surface functionalization by FTIR. To this end, the nanoparticles were reacted with the NHS-ester of azidoacetic acid. The FTIR spectrum of modified nanoparticles showed the appearance of a peak at 2100 cm^{-1}, characteristic of the azido group, with the concurrent disappearance of the NHS ester peaks at 1812 cm^{-1} and 1783 cm^{-1} (Figure 7c). For directly analyze the conversion of the amino moiety on the nanoparticle surface, we used fluorescamine assay. Accordingly, nanoparticles were first reacted with molecules with different amine-reactive functional groups (pentafluorophenol (PFP) ester, NHS ester, epoxide, and isocyanate). The extent of functionalization was analyzed by comparing the fluorescence from the functionalized amine nanoparticles and the unreacted amine nanoparticle. The fluorescence from three of the functionalized nanoparticles (PFP ester, NHS ester, and isocyanate) was found to be similar to that found with the negative control, suggesting that the reaction is quantitative in these cases (Figure 7d). The reason for the inefficiency of the epoxide ring opening reaction is not clear.

Next, we sought to investigate the possibility of tuning the nanoparticle sizes. While variations such as polymer MW, concentration, and monomer ratio could afford different aggregate sizes, we were interested in a simpler variation with the same polymer. We envisioned that the pH of the solution and the ensuing variation the hydrophilic-lipophilic balance of the polymer could afford polymer aggregates with different sizes. To test our hypothesis, aqueous solutions of polymer **1** at different pH were prepared. The polymer precipitates out at pH~9, consistent with the pK_a of amine groups. We also observed that the aggregate sizes were not significantly different between pH 3.0 and 6.5. Interestingly, the greatest size differences were observed with subtle pH changes between 7.0 and 8.5, indicating that subtle changes in the degree of protonation of the amines lead to significant size differences. This is presumably due to the difference in hydrophilic lipophilic balance of the polymer at these pHs. We have utilized these differences to systematically tune the size of the nanoparticles by photochemically locking these aggregates, as shown in Figure 8a.

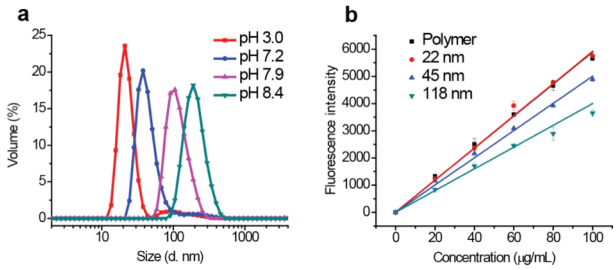


Figure 8. (a) Size distribution of nanoparticles cross-linked at different pHs in water. The DLS measurements were all done at pH 3. (b) Percentage of amine available for functionalization on different nanoparticle sizes accessed by fluorescamine assay.

Although the fluorescamine assay showed that all the accessible amines can be utilized for surface decoration, it is important to investigate the percentage of amines in the polymer nanoparticle that are inherently accessible. We hypothesized that smaller nanoparticles would have higher percentage of accessible amines, since some of these moieties might be buried in the interior of larger particles. To test our hypothesis, fluorescamine assay was carried out in 1:3 water/DMSO mixture, in which no aggregation was observed for the

non-crosslinked polymer. Therefore, the fluorescence generated from the uncrosslinked polymer is a true indicator of the amine moieties available in the polymer. Evaluation of nanoparticles of different sizes, using this as the standard, indicated that nearly all the amine moieties seem to be available at a particle size of 22 nm. However, only 85% and 65% of the amine moieties were available for functionalization in 45 nm and 118 nm particles respectively (Figure 8b). This supports the expectation that the smaller surface area of larger nanoparticles will lead to decreased availability of surface functionalities.

In summary, we have designed and characterized a versatile polymer nanoparticle platform that: (*i*) displays a versatile functional group on its surface, which can be further manipulated with a variety of complementary reactive moieties; (*ii*) is capable of non-covalently binding hydrophobic guest molecules; (*iii*) afford size tunability by simply altering the pH at which the nanoparticle is synthesized; (*iv*) has a very high percentage of the accessible surface moieties at smaller sizes. Overall, the simplicity and versatility of the surface functionalizable soft nanoparticles with host-guest capabilities will have implications in a variety of applications from materials to biology. Incorporating stimuli-responsive characteristics and expanding this method to a broader range of functional groups are among the current foci in our laboratory.