HHS Public Access

Author manuscript

ACS Nano. Author manuscript; available in PMC 2019 September 17.

Published in final edited form as:

ACS Nano. 2019 May 28; 13(5): 4876–4882. doi:10.1021/acsnano.9b01696.

Nanobuffering of pH-Responsive Polymers: A Known but Sometimes Overlooked Phenomenon and Its Biological Applications

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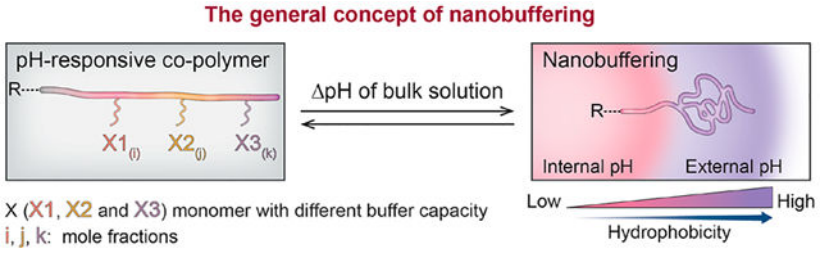
Abstract

With recent advances in polymer chemistry, materials science, and nanotechnology, pH-responsive polymers have a significant impact in a number of diverse fields. Fundamental studies of these polymers are thus highly desirable as they may lead to new insights into the rational design of pH-responsive polymers with specific effects. In this Perspective, we focus on the nanobuffering of pH-responsive polymers (NBPRP). Although researchers have known of such buffering effects for more than a century, for example, in the context of the Henderson−Hasselbalch equation, modern synthesis and analysis routes now enable us to analyze these effects on the nanometer scale. In this way, the NBPRP phenomenon was explicitly defined and described by Gauthier and colleagues in the February issue of *ACS Nano*. Here, we highlight several potential areas in which the NBPRP could enable innovative classes of biological applications. We expect deeper mechanistic understanding of nanobuffering effects induced by pH-responsive polymers to have a significant impact on the future development and applications of these polymers.

Graphical Abstract



The development of pH-responsive polymers (also known as pH-sensitive polymers) has attracted tremendous interest over the past two decades and has been widely applied in various fields, such as drug delivery,[1–4] micromechanical systems,[5,6] sensors,[5,7,8]

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The authors declare the following competing financial interest(s): O.C.F. has financial interests in Selecta Bio-sciences, Tarveda Therapeutics, Placon Therapeutics, and Seer. W.J.P. has financial interests in Seer.

theranostics,[9–11] biomimetics,[12] surface functionalization,[13,14] catalysts,[15,16] and agriculture.[17,18] In general, pH-responsive polymers are those with basic residues or ionizable acidic residues, whose ionization relies on the solution pH. They are the subset of stimuli-responsive polymers specifically triggered by environmental pH (through either accepting or releasing protons) that undergo resulting changes in physicochemical properties (*e.g.*, solubility, chain conformation, surface activity, and configuration). Diverse functional groups, such as pyridines, carboxyls, phosphates, sulfonic acids, and tertiary amines, can be found in pH-responsive polymers because they are susceptible to ionization induced by pH changes in the surrounding medium.[19]

Rationally designed pH-responsive polymers may eventually be used in a wide range of biomedical applications (Figure 1a), such as (1) controlled delivery and release of therapeutic payloads (*e.g.*, small-molecule drugs, RNAs, DNAs, and proteins) at specific pH;[20–22] (2) protein immobilization and analysis *via* manipulating protein adsorption and/or desorption on pH-responsive polymer-based brushes;[19] (3) controllable surface properties that can be reversibly switched between hydrophobicity and hydrophilicity in response to the environmental pH for applications such as microfluidic and "'lab-on-a-chip'" devices,[23,24] biochemical gates,[25,26] self-cleaning surfaces,[27] and water-repellent surfaces;[28] (4) decontamination through adjustable bacterial attraction (bacteria-adhesive) and release (bacteria-resistant) properties with respect to pH;[29] (5) cell-based diagnostics based on reversible capture and release of target cells (*e.g.*, cancer cells) *via* precise control of pH and glucose concentration;[30] and (6) pH sensors created from electrode-based devices modified with pH-responsive polymers.[31,32] However, despite extensive reports of pH-responsive polymers and their applications in various fields, the nanobuffering effect induced by these polymers has not been widely and systematically explored.

pH buffering itself was described in a general way more than a century ago, for example, in the form of the Henderson−Hasselbalch equation.[33–35] At that time, the concept was used for homogeneous bulk acid-based solutions. In the context of nanotechnology, on the other hand, surfaces are of the utmost importance. Conditions at a surface may be significantly different from those in bulk.[36] In the context of anions and cations, this phenomenon is described by classical physical chemistry, such as the Debye−Hückel theory, which notes that any charge in solution will be screened by counterions.[37] This theory applies to H^+ in aqueous solution, as well, resulting in variations of the local pH close to charged surfaces, such as one of charged polymers, on the nanoscale. This effect can be directly visualized using pH-sensitive dyes.[38,39] Taking these factors together, the "nanobuffering of pH-responsive polymers" (NBPRP) effect follows in a straightforward way: many polymers can accept or donate protons, that is, act as base or acid, according to the Henderson −Hasselbalch equation, depending on the (local) pH of the polymer surface. Positively charged (*i.e.*, protonated) and negatively charged (*i.e.*, deprotonated) polymer surfaces, however, result in local depletion (*i.e.*, increase in pH) and accumulation (*i.e.*, decrease in pH) of protons according to the Debye−Hückel screening.[39] Consider a negatively charged polymer in a "swollen" conformation. The local surface of the polymer will be screened by protons; that is, the H^+ concentration in proximity to the polymer segments is higher than that in the bulk. If the conformation of the polymer is pH-sensitive, then changes in pH can result in "shrinking" of the polymer. At this point, the polymer segments are so close to each

other (meaning closer than the Debye−Hückel screening length) that there is no bulk inside the polymer, and instead there is high H^+ concentration inside the entire volume of the polymer; that is, one can consider the polymer to be a “proton sponge”. Thus, a pH-dependent “buffering” of protons occurs. The same concept can be applied for “buffering” of molecules of different properties. For example, polarity close to a surface may be different from that in the bulk.[40] pH-dependent conformation changes of polymers may thus result in buffering or release of molecules according to their polarity, *etc.*

In the February issue of *ACS Nano*, Gauthier and co-workers systematically define and describe nanobuffering in the context of rationally designed pH-responsive polymers and report a proof-of-concept application for antibody purification.[41] Although this is not the first report of nanobuffering, Gauthier and co-workers propose a specific definition and present the requirements for generation of this nanobuffering effect. In this Perspective, we summarize the concept and requirements of the nanobuffering of pH-responsive polymers (NBPRP), according to Gauthier’s report and previous studies. We also highlight *biological* applications based on NBPRP and provide our perspective on the opportunities and challenges for NBPRP to make far-reaching impact on biomedicine and biotechnology.

Nanobuffering of pH-Responsive Polymers: Concept and Requirements.

As NBPRP is defined as the ability of pH-responsive polymers to dissociate the local nanoscale pH from the environmental pH, the pH-responsive polymers are able to maintain a local pH range around the nanoscale systems of which they are composed, and the pH value of the nanobuffering compartments can differ from the environmental pH (Figure 1b). As demonstrated by Gauthier and coworkers, NBPRP requires intimate contact with the pH-responsive polymers (*i.e.*, the pH-responsive polymers in the immediate vicinity) in any of the following forms: electrostatic interactions, covalent bonds, hydrogen bonding, or others.[41] Although it is a nanoscale phenomenon, NBPRP can generate larger-scale impacts. For example, other constituents such as degradable linkers, drug molecules, and biomolecules (*e.g.*, therapeutic nucleic acids or proteins) in the immediate vicinity of pH-responsive polymers can be affected in terms of activity, release, conformation, or stability. In addition, the binding affinity between the appended ligands and their binding targets (*e.g.*, antigen −antibody interactions) can also be affected by NBPRP. Therefore, the implications and applications of this nanoscale phenomenon are far-reaching. It is expected that sustainable development of pH-responsive polymers may lead to increased attention to the significant nanoscale phenomenon of NBPRP, creating the potential for sustained technological advances.

Manipulating Nanobuffering of pH-Responsive Polymers for Antibody Purification.

As discussed above, Gauthier and co-workers not only systematically defined the NBPRP effect and demonstrated its ability to dissociate local (*i.e.*, in the immediate vicinity) nanoscale pH from bulk environmental pH but also applied this effect to develop a homogeneous scavenger for antibody purification.[41] Essentially, the final scavenger is a pH-responsive (co)polymer conjugated Protein-A (*i.e.*, there is intimate contact between the pH-

responsive polymer and the protein), screened from a library of six scavengers with a variety of polymer composition whose pK_a values are between 6.3 and 7.8. The specific binding site of Protein-A is the constant region of antibodies of the immunoglobulin G family (IgG) at near-neutral pH. The strength of the binding affinity depends on local pH; it is strongest between 7.4 and 7.8 and substantially weaker outside this range. As shown in Figure 2a, once the developed scavenger specifically binds to the target proteins (two human IgG molecules per scavenger in this case) at near-neutral pH, the precipitation of the scavenger −IgG complex can be induced via basification above the pK_a of the scavenger, leading to the isolation of the scavenger−IgG complex from the cell culture supernatant. There are two possible reasons for this precipitation: (1) the precipitation is caused by a change in the pH-responsive polymer's physical properties (*i.e.*, from hydrophilic to hydrophobic) under alkaline conditions; or (2) because NBPRP protects the nanoscale pH, the strong binding affinity between the scavenger and the IgG can be maintained, contributing to the co-precipitation of the scavenger and IgG.

Afterward, the precipitate (*i.e.*, the scavenger−IgG complex) is dissolved in an acidic medium, and NBPRP is terminated by adding a strong ion-pairing agent (X^-). The addition of X^- (perchlorate here) can have two possible consequences: (1) reduction of the binding affinity between the scavenger and the IgG because of the termination of the NBPRP effect (*i.e.*, the local pH is not protected and thus falls outside the optimal range), which leads to the release of IgG into the solution; or (2) the precipitation of the scavenger from solution. Therefore, the IgG can finally be purified and collected in the supernatant. In addition, the precipitated scavenger can be further recycled and dissolved at neutral pH, enabling repetition of the cycle.

This process is an excellent example of clever exploitation of NBPRP for antibody purification by dynamically controlling the binding affinity between Protein-A and antibody. Through NBPRP-mediated antibody purification methods, time-consuming and/or tedious steps, such as tangential flow filtration (reported to be a bottleneck in antibody manufacturing),[44,45] can be replaced with simple centrifugation. In addition, this report establishes lines of thought for controllably manipulating the local pH properties of nanosystems and even terminating the NBPRP effect to achieve novel or tailored applications.

Engineering the Microenvironment of Individual Enzyme(s) *via* Nanobuffering for Improved Enzyme Cascade Throughput in Biosynthesis.

In a previous report, Hess et al. demonstrated the use of NBPRP to engineer the microenvironment (*i.e.*, local pH) of an individual enzyme for increased throughput of enzyme cascade reactions.[42] Although they did not propose this concept (*i.e.*, the NBPRP effect) outright, the authors applied this effect by conjugating a negatively charged polyelectrolyte (*i.e.*, pH-responsive polymer) to create a favorable pH level for the target enzyme in the local microenvironment. As shown in Figure 2b, cytochrome c (abbreviated cyt *c*, a small hemeprotein with peroxidase-like activity), which prefers acidic conditions for catalysis, is linked with poly(methacrylic acid) (PMAA). After conjugation with the pH-

responsive polymer PMAA, the optimal pH range of cyt *c* is effectively extended toward more alkaline conditions by 3 pH units. When the D-amino acid oxidase (DAAO, which prefers alkaline conditions for catalysis) is combined with cyt *c* in a cascade system for the generation of resorufin under alkaline conditions, the system with the cyt *c*−PMAA conjugate shows a 10-fold greater cascade throughput compared to that with unmodified cyt *c*. The application of the NBPRP effect in this case effectively and intelligently addressed the critical challenge of providing the optimum pH range for coupled enzymes in a multienzyme catalytic system. It should also be noted that in 1964 Katchalski *et al.* reported that immobilizing several proteolytic enzymes on highly charged polyanionic carriers can shift pH by 1−3 toward the alkaline,[46,47] implying that the NBPRP effect was already recognized many decades ago and sometimes also applied to control analyte-sensitive fluorophores.[48]

Nanobuffering-Mediated Fine-Scale Imaging and Perturbation for Mechanistic Investigation of Endocytic Organelle Biology.

With the goal of developing robust nanoscale tools for gaining deeper mechanistic understanding of the cellular physiological process, Gao *et al.* prepared a library of ultra-pH-sensitive (UPS) polymeric nanoparticles (NPs) with potent buffering characteristics that can clamp the pH of endocytic organelles at any operator-determined pH(4.0−7.4).[43] By using a finely tunable series of these UPS NPs, Gao and co-workers performed a variety of mechanistic investigations on multiple cell physiological processes including (1) quantifying the proton accumulation rate in endosomes, (2) gaining mechanistic understanding of coupling pH transitions to endosomal coat protein exchange, (3) clarifying the distinct pH thresholds necessary for the activation of the mammalian targets of rapamycin complex 1 (mTORC1) by free amino acids *versus* proteins, (4) characterizing on a broad scale the effects of endosomal pH transitions on cellular metabolomic profiles, and (5) functionalizing metabolic vulnerability in lung cancer cells. All of these mechanistic studies are based on the NBPRP effect of UPS NPs for clamping the pH of endocytic organelles at determined levels (Figure 2c), which had long remained a daunting challenge. Specifically, these new NPs constitute a series of pH-specific “proton sponges” for the various functional pH ranges of endocytic organelles (e.g., pH 6.0−6.5 for early endosomes, pH5.0−5.5 for late endosomes, and pH 4.0−4.5 for lysosomes, whereby these numbers are cell-dependent and rough estimates for their respective ranges). The role of the “proton sponge” for endosomal escape, for example, is under debate,[49] and better understanding will depend on NPs that can buffer the pH in highly defined ways. The UPS NPs with sharp buffer capacity are formed by a library of amphiphilic block copolymers, poly(ethylene oxide)-*b*-P(R_1-*r*-R_2), where P(R_1-*r*-R_2) represents an ionizable random copolymer. The synthesized copolymers become core−shell micelle structures via self-assembly at a high pH (*e.g.*, 7.4), whereas the UPS NPs dissociate into unimers at a low pH (*e.g.*, below the apparent pK_a of each copolymer) due to the protonation of tertiary amines. Researchers used a dual fluorescence reporter design, that is, a pH-activatable reporter (“ON” at different pH values of the endocytic organelles but “OFF” at pH 7.4) and an “always-ON” reporter (regardless of the pH environment) for simultaneous imaging with these NPs. Tracking with such pH-sensitive fluorophores enables the visualization of details in endocytic uptake.[50] This NBPRP-

mediated fine-scale imaging and perturbation strategy enables investigation of the integration of endosomal maturation with cell signaling and metabolism. Taking into account the critical role of luminal acidification during the maturation of endocytic organelles and the fact that pH-selective mechanistic consequences will affect a number of cell physiological processes (such as organelle trafficking, receptor recycling, cell survival, and protein/lipid catabolism in mammalian cells),[51,52] NBPRP-mediated perturbation strategies have unlimited potential in the exploration of cellular mechanisms.

OUTLOOK

With the booming development and widespread application of pH-responsive polymers, their associated nanoscale phenomena are arousing worldwide interest. Such phenomena offer fundamental insight into their mechanisms and open up new approaches to solving previously unaddressed problems. In the February issue of *ACS Nano*, Gauthier and colleagues define and describe nanobuffering by pH-responsive polymers, a local nanoscale phenomenon with a global effect, and demonstrate the feasibility of manipulating and terminating the NBPRP effect for antibody purification. Avoiding tedious and lengthy purification processes involving affinity columns and tangential flow filtration, the newly developed NBPRP-based scavenger could be applied *via* the robust and simple centrifugation technique. Considering the increasing prevalence of monoclonal antibodies (mAbs) in therapeutic approaches, we expect that strategies based on the NBPRP effect might make the manufacturing process more efficient at significantly lower cost. The mAb market is expected to reach US$130−200 billion in 2022;[53] thus, the application of NBPRP-based strategies may be exciting and profitable.

We may also see applications of the NBPRP effect in enzyme cascades to improve throughput. Although multienzyme catalysis has great potential in biotransformation, biosynthesis, and biodiagnostics, coupled enzymes must work together in the same environment, despite disparate enzyme characteristics such as pH dependence, temperature dependence, kinetic parameters, *etc*. It is much more challenging—but crucial—to match the optimum pH range for coupled enzymes. Encouragingly, by exploiting the NBPRP effect (*e.g.*, simple interaction with a chosen pH-responsive polymer), each enzyme in a multienzyme catalytic system can be engineered with a specific and optimal pH, which can be expected to maximize catalytic activity in multistep “one-pot” biotransformations. In addition, the NBPRP effect also has great potential in clamping the pH of endocytic organelles for biological mechanistic studies. Current reagents and tools used to manipulate luminal acidification usually perturb a wide range of pH-dependent cellular activities because of their membrane permeability, which causes nonspecific compounded effects on multiple acidic organelles (*e.g.*, Golgi). By developing ultra-pH-sensitive polymeric nanoparticles that enter cells exclusively via endocytosis, we can achieve finely tuned buffering of luminal pH through the NBPRP effect at desired levels in endocytic organelles without disrupting the cell or organelle membranes. Therefore, we expect that new NBPRP-based NP tools could have the potential to benefit basic biological studies, especially in growth regulatory signaling pathways and cell metabolism.

As mentioned above, the physical chemistry of the NBPRP effect is, in principle, well-known, but its description and application cannot be found so far under a common name or keyword, which complicates literature retrieval and accurate depiction of the current state of research. It is necessary to perform more detailed studies to verify the exact nanoscale distance/range of the NBPRP effect in each study. For example, the use of pH-sensitive fluorophores together with spacers may offer the first approach in this direction.[39] Addressing the following questions should also be helpful: (1) what factors affect the distance/range and precision (or value) of nanobuffering-controlled local pH (some possibilities are polymer length, functional groups, or surrounding ions) and (2) how do we characterize the nanobuffering capabilities of different pH-responsive polymers? Finally, the NBPRP effect can greatly enable unexplored applications that would benefit from controlling pH on a local scale, and we expect to see more studies involving the NBPRP effect in the near future.

ACKNOWLEDGMENTS

This work was supported by the U.S. METAvivor Early Career Investigator Award (W.T.), U.S. National Institutes of Health Grants HL127464 (O.C.F.) and CA200900 (J.S.), the U.S. Department of Defense Lung Cancer Research Program W81XWH1810392 (J.S.), and the German Research Society (Cluster of Excellence “Advanced Imaging of Matter” of the Deutsche Forschungsgemeinschaft (DFG)—EXC 2056—project ID 390715994 to W.J.P.).

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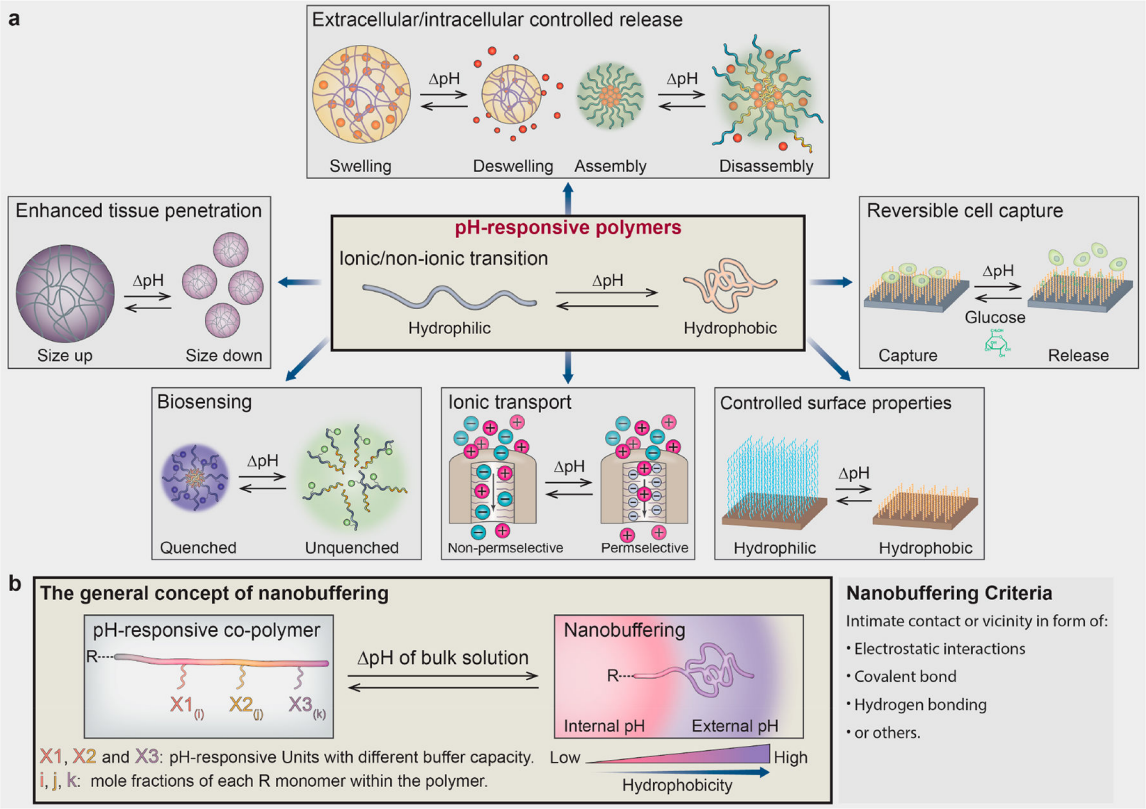

Figure 1.

(a) Various applications of pH-responsive polymers and their mechanisms. (b) General concept of the nanobuffering of pH-responsive polymers (NBPRP).

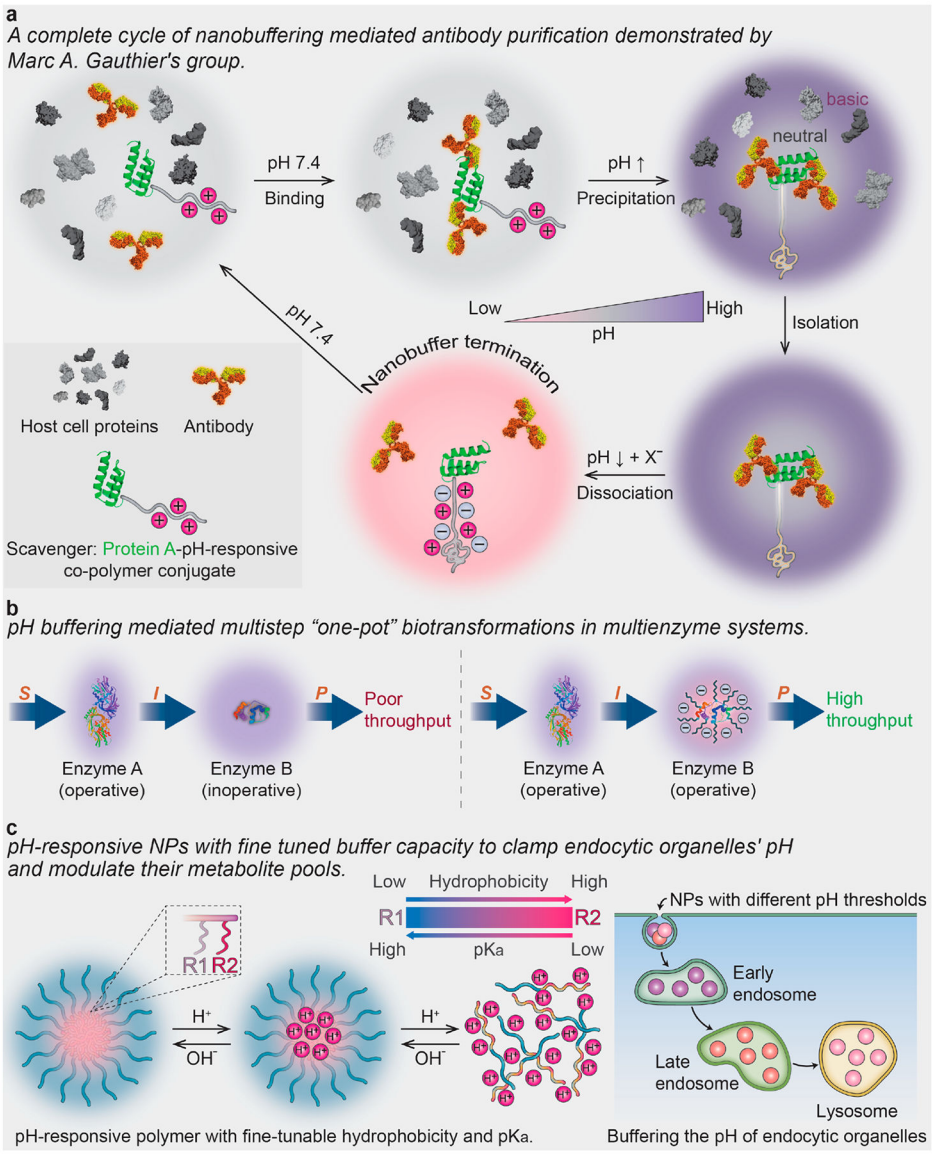

Figure 2.

(a) Schematic illustration of a complete cycle of nanobuffering of pH-responsive polymers (NBPRP)-mediated antibody purification reported by Gauthier's group. Reproduced from ref 41. Copyright 2019 American Chemical Society. (b) NBPRP-mediated enzyme cascade for increased throughput in biosynthesis reported by Hess's group. Reproduced from ref 42. Copyright 2017 American Chemical Society. (c) NBPRP-mediated fine-scale imaging and perturbation for mechanistic investigation of endocytic organelle biology reported by Gao's group. Reproduced from ref 43. Copyright 2015 Macmillan Publishers Limited.