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Engineering bionanoparticles for improved biosensing and bioimaging

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The importance of bioimaging and biosensing has been clear with the onset of the COVID-19 pandemic. In addition to viral detection, detection of tumors, glucose levels, and microbes is necessary for improved disease treatment and prevention. Bionanoparticles, such as extracellular vesicles and protein nanoparticles, are ideal platforms for biosensing and bioimaging applications because of their propensity for high density surface functionalization and large loading capacity. Scaffolding large numbers of sensing modules and detection modules onto bionanoparticles allows for enhanced analyte affinity and specificity as well as signal amplification for highly sensitive detection even at low analyte concentrations. Here we demonstrate the potential of bionanoparticles for bioimaging and biosensing by highlighting recent examples in literature that utilize protein nanoparticles and extracellular vesicles to generate highly sensitive detection devices with impressive signal amplification.

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Introduction

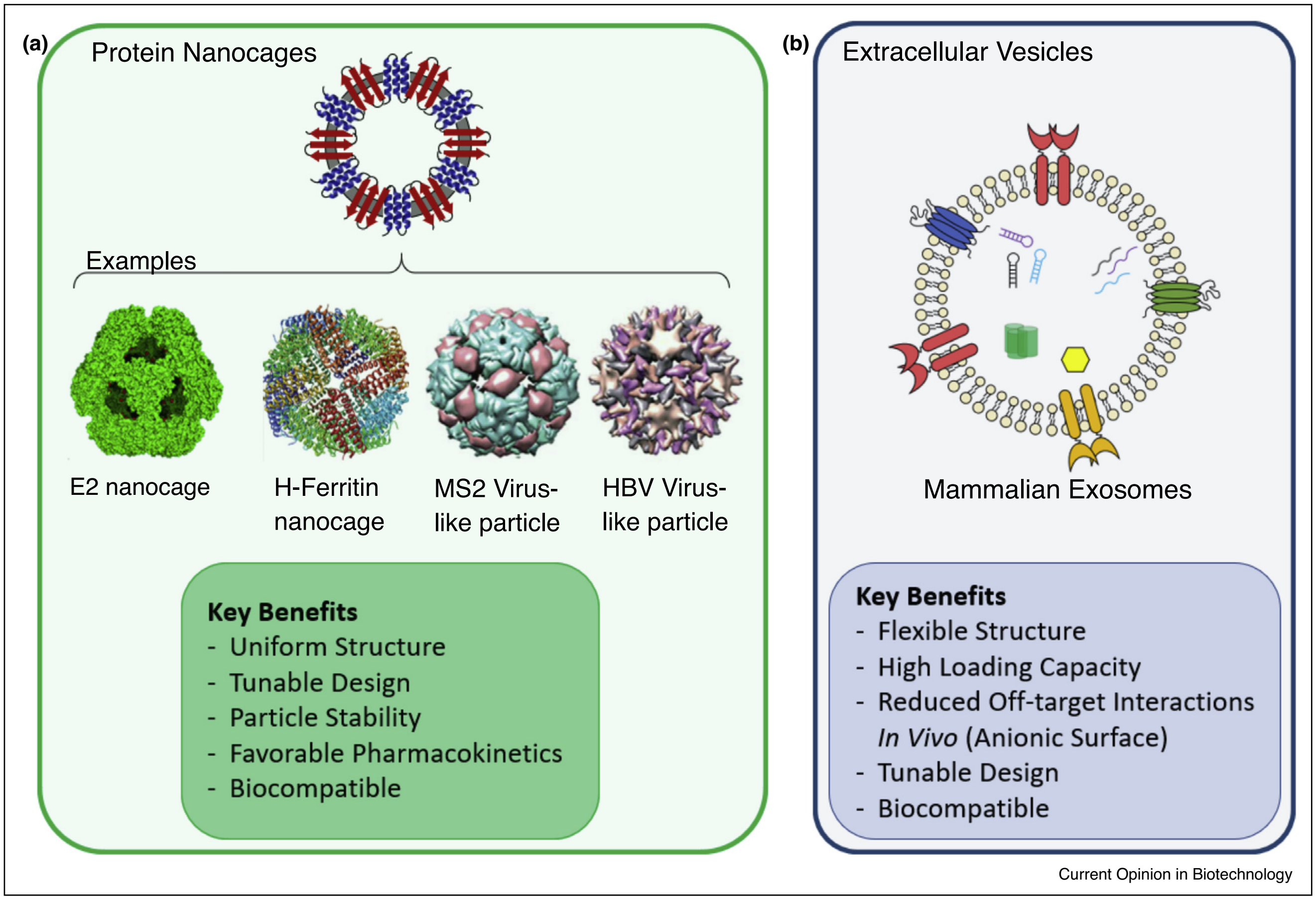
Bioimaging and biosensing facilitate our ability to investigate biological processes at a molecular level for identifying disease progression and developing next-generation therapeutics [1–3]. These processes employ sensing agents to detect and transduce an output signal upon recognizing a physiological target, with applications ranging from labelling cancer cells [4–6] to detecting analytes such as glucose or viral particles [1,4,7]. To improve the signal and reduce the detection limit, these 'signal transducers' are commonly grafted onto nanomaterials [8]. Conventional polymer nanoparticles or liposomes are usually polydisperse in size and may exhibit toxicity [9]; however, bionanoparticles naturally serve as scaffolds for cargo encapsulation [10,11] and molecular transport [12,13], making them favorable platforms for biosensing and bioimaging. Protein nanoparticles [14••,15] and extracellular vesicles (EVs) [16–18] are two naturally occurring bionanoparticles that are commonly utilized because of their amenability for genetic fusion and chemical modification and capacity as readily internalized delivery vehicles, respectively. By employing the expanding array of bioconjugation strategies, these bionanoparticles can be modified to accommodate imaging agents, analyte-processing enzymes, and targeting moieties for a wide range of bioanalytical applications [19,20].

Protein nanoparticles range from 10 to 100 nm in size and serve numerous functions ranging from delivering cargoes, such as small molecules, ions, and nucleic acids, to docking proteins for assembling enzyme complexes [11]. Protein nanocages such as E2, ferritin, and virus-like particles (VLP) are particularly attractive because of their physical stability, monodispersity, and capacity for both interior and exterior decorations (Figure 1a) [15,21,22]. EVs, alternatively, are membrane-derived, 20–500 nm-sized particles released by all cell types, ranging from bacteria to mammalian cells [13], that encapsulate proteins, nucleic acids and other cytoplasmic biomolecules to subserve diverse functions, such as mediators for intercellular communications in mammalian cells (Figure 1b) [16,23]. EVs differentiate themselves from protein nanocages due to their structurally flexible lipid-bound architecture, allowing them to better tolerate sterically demanding modifications and exhibit higher cargo loading [24]. Furthermore, the negative surface charge from the proteoglycan-modified and glycosaminoglycan-modified membrane in mammalian EVs can prolong *in vivo* circulation [25].

Bioimaging

Bioimaging is vital for elucidating biological structures and cellular dynamics from small, intracellular scales to larger, anatomical scales to glean information about how biological systems function [4,25,27,28]. These insights are particularly useful to probe the dynamics of tumor progression by pinpointing tumor location, assessing cell phenotype, and visualizing relevant biological processes

Figure 1

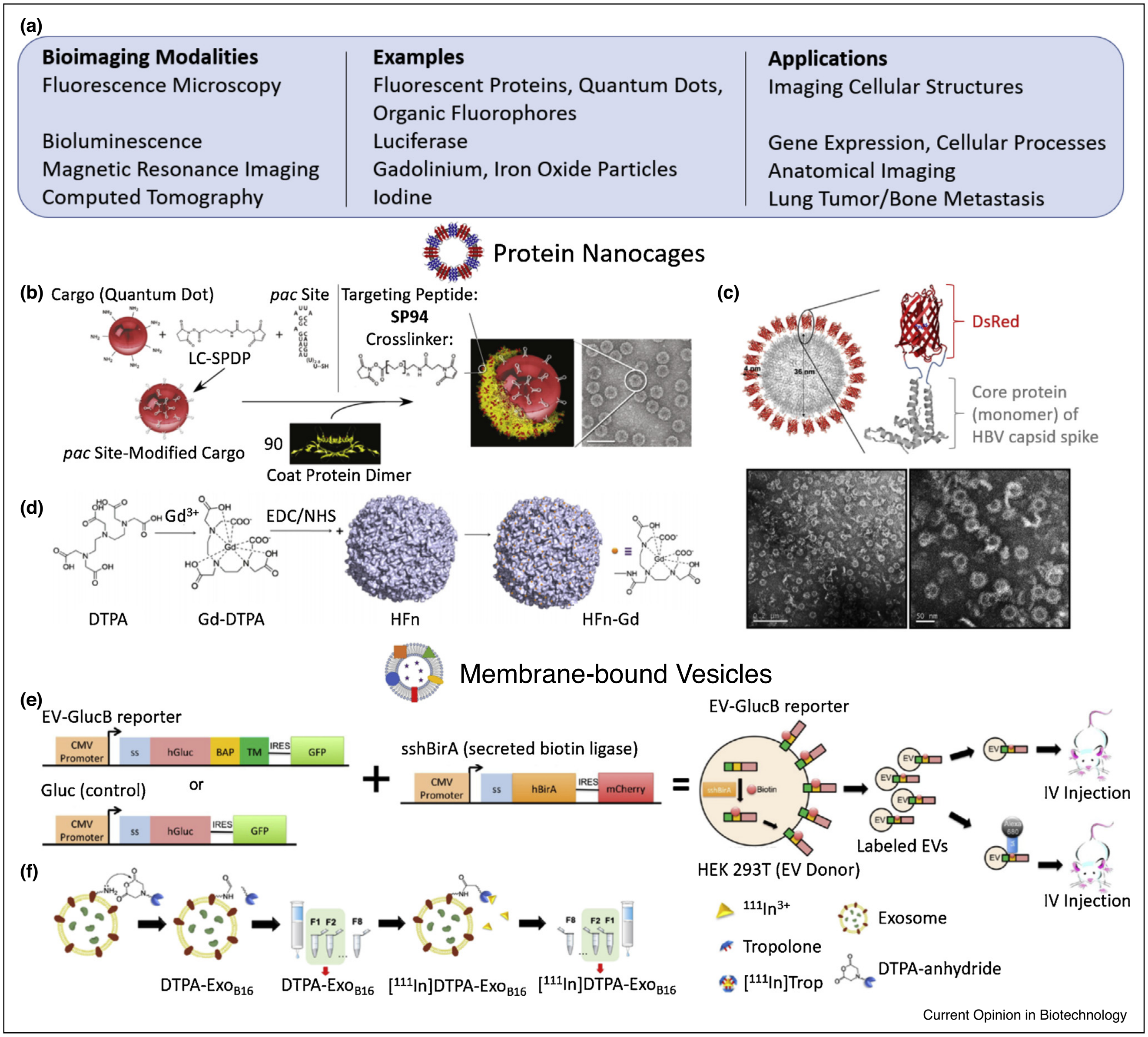


Common bionanoparticles used for drug delivery applications. **(a)** Protein nanocages are well-defined protein nanostructures that natively serve as protein scaffolds or delivery vehicles for small molecules, proteins, and nucleic acids. E2 nanocage adapted with permission from Ref. [26] Copyright 2017 American Chemical Society. H-Ferritin, MS2 VLP, HBV VLP reprinted with permission from Ref. [21] Copyright 2014 Royal Society of Chemistry **(b)** Extracellular vesicles are released from all cells — from bacteria to humans to plants — and serve to transport various cargoes, such as nucleic acids, lipids, and proteins, for intercellular signaling and communication. Both nanoparticle platforms offer advantages based on their physical and structural properties, providing options for different bioimaging and biosensing applications.

via the composition of cells and other biomarkers within the tumor microenvironment [4]. To achieve this, different bioimaging modalities are utilized as determined by their respective spatial and temporal resolution [29–31]. Protein nanocages are particularly attractive as they can be modified with multiple contrast agents and targeting moieties for orthogonal detection and imaging of different cellular markers (Figure 2a) [32–35,36••]. To demonstrate how these nanocages can be controllably modified on both the interior and exterior to form multifunctional nanoparticles, Ashley *et al.* demonstrated the versatility of MS2 bacteriophage virus-like particles (VLPs) for theranostic applications. MS2 VLPs were functionalized with hepatocellular carcinoma cell (HCC)-targeting peptides, quantum dots, small-molecule fluorophores, and other therapeutic agents for HCC-specific detection and destruction (Figure 2b) [37]. For VLP surface modification, Alexa Fluor 555 dyes (AF555) were chemically conjugated onto exterior amine groups and for interior modifications, Qdot 585 ITK amino (PEG) quantum dots were encapsulated via conjugation to a capsid-specific RNA aptamer (*pac* site). The MS2 capsids were further conjugated with 60 HCC-targeting peptides (SP94) to impart 45 000-fold higher avidity to Hep3B HCC cells compared to control hepatocytes, which resulted in increased Hep3B uptake of targeted, quantum dot-loaded MS2 VLPs. The controlled modification of both the interior and exterior of MS2 VLPs highlight their versatility as a bioanalytical platform, demonstrating how they can be easily tailored with bioactive molecules to target various cell types.

Fluorescent proteins are used extensively for bioimaging due to their biocompatibility and potential genetic fusion to protein nanoparticles for a simple, one-pot synthesis (Figure 2c), but may denature during *in vivo* circulation

Figure 2



Bioimaging with bionanoparticles. **(a)** Examples of various types of bioimaging modalities and their typical applications are provided. **(b)** MS2 virus-like particles are functionalized with SP94 targeting peptides and organic fluorophores and loaded with quantum dots for cancer cell imaging. Adapted with permission from Ref. [37] Copyright 2011 American Chemical Society **(c)** DsRed is genetically fused at the exterior of Hepatitis B VLPs to assess *in vivo* biodistribution via fluorescence in mice. Adapted with permission from Ref. [38] Copyright 2012 Elsevier **(d)** Gd-DTPA complexes are conjugated on human ferritin nanocages to investigate *in vivo* biodistribution in tumor-bearing mice models via MRI. Adapted with permission from Ref. [39•] Copyright 2020 American Chemical Society **(e)** Gluc and Alexa Fluor 680 are simultaneously incorporated to exosomes and delivered to glioma-bearing mice to assess biodistribution using both bioluminescence and FMT. Adapted with permission from Ref. [42] Copyright 2014 American Chemical Society **(f)** Exosomes are loaded with [^{111}In] DTPA complexes for investigating tumor accumulation via SPECT/CT in melanoma bearing mice. Adapted from Ref. [43••] under Creative Commons Attribution license.

from serum protein adsorption. Yoo *et al.* demonstrated how incorporation of the DsRed fluorescent protein in the solvent-exposed loop of the hepatitis B virus (HBV) VLP monomer enhanced protein stability, thereby forming red fluorescent capsid nanoparticles (rFCNP) [38]. rFCNPs demonstrated superior fluorescence when incubated in 50% fetal bovine serum for five hours, retaining 80% of the initial fluorescence level compared to the 10% relative fluorescence of free DsRed. In mice, this enhanced fluorescence stability and circulation half-life yielded fluorescence at sampling times of 16 and 24 hour post-injection, time points when free DsRed was undetectable.

While the HBV VLP in this example was designed to stabilize and deliver DsRed for bioimaging, it can be easily modified to incorporate other cargoes such as therapeutic proteins. Furthermore, conjugating targeting moieties or cell-penetrating peptides onto HBV can greatly enhance the localization and internalization of the fluorescent signal within targeted cells.

Protein nanocages are also utilized to reduce adverse effects of toxic bioimaging modalities, such as gadolinium — used in magnetic resonance imaging, by localizing them on a nanoparticle and simultaneously facilitating target-specific *in vivo* localization. Cai *et al.* chemically conjugated diethylenetriamine pentaacetate (DTPA)-chelated gadolinium (Gd-DTPA) to human heavy-chain ferritin (HFn) nanocages and demonstrated selective accumulation in subcutaneous MDA-MB-231 breast tumor xenografts in mice via the ferritin nanocage interactions with transferrin receptors on cancer cells (Figure 2d) [39•]. Within the tumor, HFn-Gd yielded a significant increase in signal-to-noise ratio for up to 60 min post-injection, whereas Gd-DTPA was non-detectable over the same time range and demonstrated negligible cytotoxicity when incubated with MDA-MB-231 cells *in vitro*. Though they have a significantly smaller interior cavity compared to VLPs, HFn nanocages are a favorable alternative for delivery of small molecule cargos, such as Gd-DTPA, because they exhibit negligible immunogenicity as a result of their human origin [40].

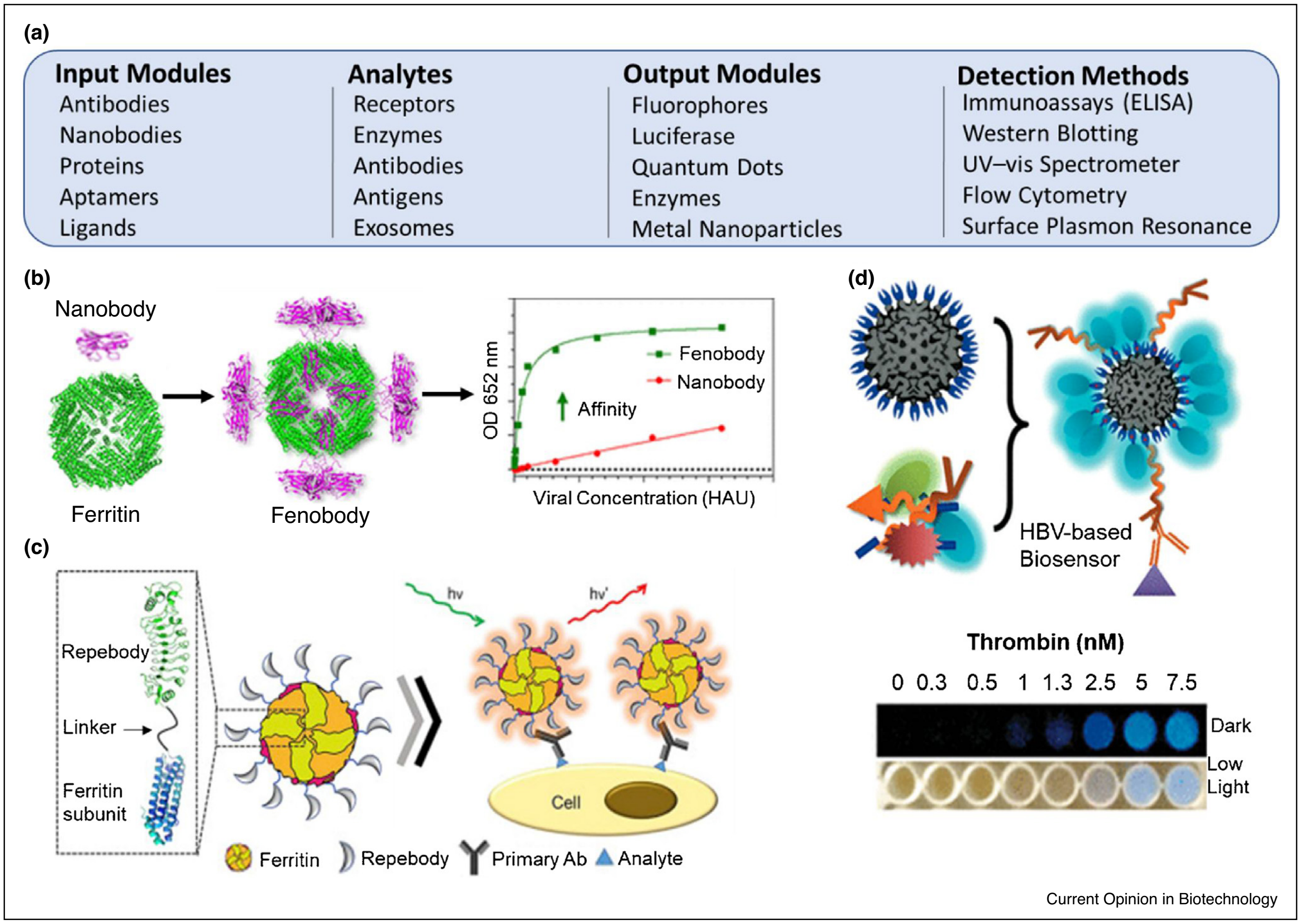
Besides protein nanocages, membrane vesicles (MV) are used as nanocarriers for bioimaging contrast agents because of their structural flexibility, in contrast to the structural rigidity of their protein nanoparticle counterparts. This structural flexibility increases the potential size of the surface modifications as well as their loading capacity, due to the ability for lipid-bound nanostructures to better tolerate steric stresses from surface modification or cargo encapsulation [24]. Mammalian-derived extracellular vesicles (EV) are extensively used for bioimaging due to their biocompatibility, limited immunogenicity, and potential for efficient, cell-selective uptake [41]. Lai *et al.* demonstrated the versatility of the EV platform as a multimodal imaging agent by fusing human Gaussia luciferase (hGluc) and biotin acceptor peptide (BAP) to a transmembrane domain, derived from platelet-derived growth factor receptor (PDGFR), which was expressed and anchored on the EV membrane (Figure 2e) [42]. Biotinylating BAP with a biotin ligase, the EVs were further modified with an Alexa Fluor 680-conjugated streptavidin for multimodal imaging, pairing the high sensitivity and high signal-to-noise ratio of bioluminescence with the high spatial and temporal resolution of fluorescence mediated tomography (FMT) to better pinpoint biodistribution. When administered intravenously in mice, both imaging modalities demonstrated EV localization in the liver and spleen and circulation half-lives of slightly less than 30 min. When human glioma cells (Gli36) were subcutaneously implanted into the left and right chest regions of mice, the EVs exhibited tumor accumulation via hGluc activity at comparable levels to that of both liver and spleen at 60 min post-injection. To achieve relatively high spatial and temporal resolution *in vivo*, another commonly employed technique is single-photon-emission computed tomography-computed tomography (SPECT-CT), which utilizes radioactive labels as contrast agents for *in vivo* bioimaging. To overcome poor circulation times of SPECT-CT agents, Faruqu *et al.* radiolabeled melanoma (B16F10) cell-derived exosomes with a bifunctional chelator, DTPA-anhydride, to bind indium-111 (^{111}In) onto surface-exposed amines on transmembrane proteins (Figure 2f) [43••]. The resulting [^{111}In]DTPA-Exo$_{B16}$ exosomes were radiochemically stable in 50% fetal bovine serum, retaining 80% of the initially chelated ^{111}In at 24 hour. When administered to melanoma-bearing C57Bl/6 mice, [^{111}In]DTPA-Exo$_{B16}$ demonstrated twice the radioactive signal at the tumor site in 24 hour compared to free [^{111}In]DTPA. Furthermore, owing to its larger size, [^{111}In] DTPA-Exo$_{B16}$ accumulated more in the liver and spleen and had reduced levels in excreted urine compared to its non-conjugated counterpart. Mammalian-derived EVs are attractive for bioimaging as they contain both surface-exposed targeting moieties for preferential uptake in specific cell types and the capacity for membrane fusion for enhanced cell internalization of their cargo, which can be selected for many applications of biomedical importance [17,41].

Biosensing

Biosensors can be used for a multitude of applications including viral, disease, and bacterial detection, glucose monitoring, and drug development [44]. A biosensor consists of an input modality, which is able to bind to the analyte, and an output modality for detection (Figure 3a). Bionanoparticles are ideal scaffolds for biosensing because of their potential to localize a high density of input and output modules on their surface for target binding and signal amplification, respectively [45]. The surface of protein nanoparticles and membrane vesicles are highly tunable and biocompatible making them particularly attractive for biosensing.

Protein cages, such as E2 and ferritin, have recently been utilized as biosensing scaffolds due to their heat resistance, high stability, and surface modularity. E2 - a 60-mer protein nanocage with a 24 nm diameter derived from *Bacillus stearothermophilus* [46,47] - was genetically modified to create a highly modular nanoprobe [48]. Sortase A mediated ligation [49] was used to attach a variety of sensing domains, such as antibodies and aptamers, and detection domains, such as nanoluciferase (NanoLuc) and fluorophore dyes, onto the surface of the E2 nanocage to detect thrombin and cancer cell markers. A 25-fold

Figure 3



Biosensing with bionanoparticles. **(a)** Examples of input (sensing) and output (detection) modules commonly scaffolded onto bionanoparticle sensing devices, along with common analytes and detection methods. **(b)** Fusion of nanobodies to the surface of ferritin cages to form fenobodies for enhanced detection of H5N1 viral titer. Adapted with permission from Ref. [53] Copyright 2018 American Chemical Society **(c)** Fusion of an IgG-specific repebody to the surface of ferritin cages for improved analyte specificity. Adapted with permission from Ref. [56] Copyright 2017 Elsevier **(d)** Site-specific ligation of Z-domain and NanoLuc to the surface of HBV though SpyCatcher/SpyTag binding for enhanced signal amplification in the detection of thrombin. Thrombin was visibly detectable in the dark at concentration as low as 1 nM. Adapted with permission from Ref. [14••] Copyright 2020 American Chemical Society.

increase in signal amplification was observed when 22 NanoLuc proteins were ligated to the surface of E2 as compared to just one, despite maintaining a constant number of sensing modules. E2 functionalized with immunoglobulin G (IgG)-binding Z-domains and elastin-like peptides has since been used to quantify antibody titer with UV–vis spectroscopy [50•].

Ferritin - a 24-subunit protein nanocage 12 nm in diameter - has also been genetically modified to incorporate output moieties ranging from gold nanoparticles to quantum dots, and input moieties capable of detecting cancer markers and viral proteins [51,52]. Recently, ferritin was genetically modified to display 24 nanobodies capable of binding to the H5N1 virus, to create a particle termed fenobody (Figure 3b) [53]. A 360-fold increase in the apparent binding affinity and a 100-fold increase in immunoassay sensitivity were observed for the fenobody compared to a single nanobody when IgG capture antibodies were used for detection. The same approach has since been used to detect Newcastle disease virus (NDV) using fenobodies and a nanobody-fused reporter, demonstrating the versatility of this approach for detecting a variety of antigens through nanobody selection [54••]. In another example, fluorescently labeled ferritin was fused with 24 IgG-specific repebodies, leucine-rich repeat modules derived from antibodies [55], resulting in three-order of magnitude higher binding affinity compared to the free repebody for IgG detection via immunoassays (Figure 3c) [56].

VLPs have also shown promise for biosensing applications [15,57,58]. VLPs are often larger than other protein nanocages, making the number of potential surface modifications higher. For example, cowpea mosaic virus (CPMV) has been utilized as a nanoscaffold for biosensing applications due to the 300 reactive lysine residues on its surface amenable to modification [59,60]. Other commonly used VLPs, HBV and bacteriophage P22, self-assemble from 240 and 420 copies, respectively, of core-forming proteins [61,62], and these VLPs have been functionalized with up to 235 motifs for HBV [14••] and 150 motifs for P22 [63], higher decoration of P22 led to aggregation. In recent work, 240 SpyCatcher binding sites were inserted onto the surface loops of HBV. SpyCatcher/SpyTag [64] conjugation was used to attach 12 Z-domains to recruit antibodies of interest, and the remaining SpyCatcher sites were used to recruit an increasing number of NanoLuc moieties, ranging from 42 to 211 copies, for detection [14••]. The biosensor was used to detect thrombin, a biomarker for diagnosis of pulmonary metastases and coagulation abnormalities, using a thrombin-specific antibody in an ELISA format. Compared to a single Z-domain NanoLuc fusion, the HBV-based biosensor showed up to 1521-fold amplification in signal at 120 copies of NanoLuc. Visible detection of thrombin at concentrations as low as 1 nM was observed using HBV modified with 211 copies of NanoLuc, making this a highly attractive platform for low-cost detection of waterborne pathogens (Figure 3d).

Tobacco Mosaic Virus (TMV), which forms filamentous nanorods comprises 2130 coat proteins [65], has been functionalized with up to 2000 modifications enabling the scaffolding of enzymes for the detection of penicillin [66,67], glucose [68], and antibodies [69,70]. Another filamentous bionanoparticle, M13 bacteriophage, exhibits angle independent color change properties, where exposure to external chemicals can cause a color change due to bacteriophage bundle swelling [71,72]. M13 can be genetically engineered through phage display, allowing the bionanoparticle to be used for recognition of a variety of analytes [72]. These properties make M13 an ideal candidate for biosensor platforms that utilize color sensors [73], fluorescence [74], and surface plasmon resonance (SPR) [75] for detection.

Conclusion and future outlook

Now more than ever, there is a pressing need to improve diagnostics for detecting viruses, bacteria, and diseases. The COVID-19 pandemic has made it clear that our current diagnostic tools need to be faster and more affordable to reach populations around the world to prevent mass infection. Bionanoparticles are intrinsically biocompatible, stable, uniform, and tolerant of genetic modifications, making high levels of surface functionalization with sensing and detection modules possible. Additionally, adding small ligation tags provides the opportunity to tune the ratio of these modules for signal enhancement and analyte specificity with high modularity so diagnostics can be created rapidly when new contagions emerge. Finally, bionanoparticles are easy and affordable to manufacture and often do not require severe storage conditions making rapid global distribution possible.

While the benefits of bionanoparticles are clear, the next generation of biosensor and bioimaging systems must focus on making diagnostic tools that are more broadly applicable with high specificity and sensitivity for rapid detection. Engineering biosensing platforms capable of rapid visual detection through signal amplification would remove the need for expensive equipment, increasing the scope of usage beyond biomedical applications toward low-cost environmental detection. Additionally, bioimaging platforms often suffer from clearance and immune cell phagocytosis, greatly reducing their signal. Immune shielding and strategies to increase circulation half-life could greatly improve their sensitivity and ease of use. While most bioimaging applications have focused on protein-based bionanoparticles and mammalian EVs, bacterial outer membrane vesicles (OMVs) have recently shown promise as biosensor scaffolds because they can tolerate sterically demanding modifications and demonstrate high cargo loading and will likely play a role in future biosensor design [18,76,77••]. Engineering strategies to further improve the modularity and toxicity of bionanoparticles continue to be realized today strengthening their capabilities to act as drug delivery vehicles, vaccine platforms, and biosensing and bioimaging scaffolds and will likely play a major role in diagnosis, treatment, and even prevention of future contagions.

Conflict of interest statement

Nothing declared.

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