Enhancing the Development of Therapeutics against SARS-CoV-2 by Exploring the Properties of Therapeutic Nano-structures

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Abstract

SARS-CoV-2 may be the most significant challenge that modern society (and modern medicine) has faced. The epidemiological characteristics of SARS-CoV-2 and its global impact will focus attention on all facets of medicine. Early evidence shows that SARS-CoV-2 initially infects alveolar epithelium and associated macrophages. Thus, a potential strategy to bring medicinals into close contact with infecting virus is to target nano-structures via inhalation; a factor in considering nano-therapeutic design being their localization to the tissues and cells of the alveoli. Strategies that target intracellular pathways utilized by ligand-directed nano-therapeutics appear to potentially have efficacy, focusing upon signalling mechanisms that activate endocytotic pathways that are also utilized by SARS-CoV when infecting cell targets. Foci of the present paper are the retrograde cellular transport pathways that direct proteins/peptides to the endoplasmic reticulum. Speculation in this perspective identifies multiple sub-cellular compartments at which nanostructure-delivered antivirals may intersect those utilized by SARS-CoV-2 (and other RNA viruses). These include endocytosis pathways, points of viral entry to cells, and sites of viral assembly. In the context of the development of antivirals against SARS-CoV-2, the discussion here provides the stimulus for scientific debate. In bringing the context of nano-therapeutic design together with the very preliminary knowledge of the pathophysiology of SARS-CoV-2, it is hoped that this perspective is useful.

Keywords:

SARS-CoV-2, anti-viral, nano-therapeutics, KDEL, retrograde transport, COVID-19

Purpose and Rationale:

COVID19, the disease caused by the virus recently named SARS-CoV-2, may be the most significant challenge that modern society (and modern medicine) has faced. Precision nanomedicine provides a suite of technologies that will contribute to the diagnosis, treatment through targeted delivery of antivirals, treatment of co-morbidities, and in exploring new pathways that diminish the pathologies and transmission of hideous, infectious threats to modern society. Knowledge of nano-structures and physiological mechanisms can aid in developing therapeutic strategies based upon physiological principles both for the design of nanomedicines, together with the principles that underpin the pathophysiology of SARS-

CoV-2. The intersection of these two areas may provide a useful platform in controlling the scourge of global SARS-CoV-2 infection.

(The author regularly uses the term "nano-structure" throughout this perspective, which is deliberate. The terminology used is intended to convey the following concepts: (a) Rigor in design and reproducibility of synthesis (manufacture) of nano-therapeutics (nano-structures) is paramount; (b) Highly efficacious nano-therapeutics will likely need to incorporate multi-functionality.

In the present discussion, the arguments for appropriate size, surface characteristics, and reversible (non-covalent binding) of an antiviral "payload" are presented. The term nano-structure is to emphasize that such

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sophisticated, multi-component tools will need to be precisely designed and manufactured.)

Introduction and Discussion:

Already, the fundamental structure of SARS-CoV-2 at both a superficial level (Figure 1) and detailed, structural, and genetic levels are well described (3).

The epidemiological characteristics of SARS-CoV-2 (4) and its global impact will focus attention on all facets of medicine (5) in the development of rapidly deployed, inexpensive and reliable diagnostics, vaccines, antivirals and other strategies to attenuate the severe pathologies that accompany infections, representing around 4 to 5% of infected patients. Fortunately, nanomedicine has the potential to develop technologies and therapeutics with efficacy at each of these diagnostic and therapeutic axes. Precision nanomedicine has recently provided novel approaches to addressing these facets to other emerging viral diseases, for example, the Nipah virus (6)

Figure 1. Electron micrograph of SARS-CoV-2 showing the corona features. (image provided by NIAID, RML, Montana).

In understanding the route of infection of SARS-CoV-2, early evidence shows that it initially infects alveolar epithelium and macrophages (3). Thus, a potential strategy to bring medicinals into close contact with the infecting virus is to target nano-structures via inhalation. Various inhalable nano-therapeutics under development include polymer-, liposome- and metal-based systems (7).

Nanostructure-based therapeutics hold exciting possibilities across many applications that can improve the treatment of viral aetiologies. It has been suggested that DNA-

conjugated gold NPs (AuNPs) may inhibit viral entry to cells (8) via mechanisms that facilitate cellular uptake of nano-structures including paracellular transport (9) and via endocytosis (10, 11). These shared pathways provide hints to develop therapeutic strategies that direct colocalization of virus and nano-therapeutic within specific, discrete subcellular compartments.

The Nature of the Nano-structure Surfaces and Viral Capsids Presented to Cells

Many examples in the literature focus on the surface characteristics of nano-structures as the main driver of their cellular interactions (10, 12). These mechanisms are well understood, and strategies have been designed and broadly used to moderate cellular recognition of nano-structures. For example (PEG)ylation is reported to inhibit uptake via macrophages and phagocytes, the mechanism suggested is via preventing NPs from adsorbing proteins (the "stealth" effect), (9, 13). NP modifications that enhance cellular uptake are also shared with several classes of viruses.

Cell-Penetrating Peptides (CPPs).

These CPPs share short peptide domains that are also utilized by viruses in gaining cellular penetration. Examples include VP22, a herpes virus protein (14), and RNA-binding proteins such as HIV-1 Rev (15).

The Nature of the Optimal Nano-structure Sizes and Virus Size Range

Considering the size limitations of cellular uptake (via pinocytosis), mechanisms via caveolae and clathrin-coated pits (size range 80 to 200 nm, (11) together with rapid removal of NP via the kidneys, [< 3 nm, (11)] suggest that the optimal size range for nano-therapeutics will be between 3 nm and 200 nm. If systemic administration is the dosing route, most tissues are perfused by vasculature having a pore-size controlling endothelial layer that prevents the escape of nano-structures above the size range around 10 to 20 nm (16). Thus, intravascularly delivered therapeutic nano-structures may need to be in the size range of 3 to 20 nm for optimal delivery to the tissues.

In the case of anti-SARS-CoV-2 nano-therapeutics administered via inhalation, the vascular epithelial barrier becomes less

relevant. Nano-therapeutics administered via this route appear to have an effective size range between 6 and 600 nm (7). Thus, a factor in considering the nano-therapeutic design is their localization to the tissues and cells of the lower respiratory tract, particularly the alveoli. Prevention of passage into the circulation may well be facilitated through selecting nano-therapeutics larger than 20 nm (inhibiting escape via the vasculature), but less than 200 nm (size of clathrin-coated pits) to promote cellular uptake (11).

The parallels between what appear to be optimal nano-therapeutic sizes and the size of virus particles recapitulate the theme noted above focusing upon signalling mechanisms that activate endocytotic pathways. Again, these properties are shared by viruses and optimally designed nano-therapeutics. The example of SARS-CoV-2, which appears to utilize respiratory system facilitated entry to alveolar cells, is noted to be between 50 and 200 nm (5), further supporting the notion of size and expression or nano-design being in parallel.

Some key features that will likely impact the optimal design of anti-viral nano-structures

While my laboratory has extensively studied nano-gold (AuNP) to understand cellular physiology and as a basis of nano-therapeutics, it is essential to clarify to the reader that I

believe a broad range of nano-materials have characteristics that may provide advantages for specific designs. In the present discussion, we focus upon the potential for the development of potent nano-anti-virals. The examples provided here reflect my scientific experience (hopefully in the context of an unbiased thesis). We should remain entirely open in consideration of the optimal characteristics of the core of the design features discussed here.

How nano-structures are "seen" by cellular uptake mechanisms.

Many reports in the literature comment upon cellular uptake of nano-structures and the putative mechanisms involved. Here we will focus upon gold (AuNP) as an example. Whether we can generalize the following narrative to broader classes of nano-structures may give the reader pause to reflect.

It has been demonstrated that in vitro incubation of AuNP with minimal concentrations of the peptide (or protein) results in the rapid association between the two. The concentration range of 200 to 600 molar equivalents of N-terminal cysteine residue, 17-mer peptides was able to stabilize 20 nm AuNP, such that the nano-constructs readily migrated as discrete, single bands in electrophoresis (behaving similarly to a protein of a discrete size, (Figure 2) (1).

Figure 2. AuNP were incubated with N-terminal cysteine-containing peptides. Peptide A contained the C-terminal sequence: lysine, aspartate, glutamate, leucine (KDEL), and the C-terminal moiety of Peptide B was designed with random amino acid residues. Agarose gel electrophoresis profiles are shown: (A) Incubation of Au NP-peptide A nanoconjugates in DMEM + 10% FBS (DMEM + S) for 0, 15 min, 1, 6 and 24 h. (B) Incubation of Au NP-peptide A nanoconjugates or (C) Au NP-peptide B nanoconjugates in DMEM, DMEM + FBS (DMEM + S), PBS, PBS + 10% Rat Serum (PBS + S) or PB, or (B + C) Incubation of Au NPs alone in DMEM + S or PBS + S. After incubation, in 5% CO₂ at 37°C for the indicated times (A) or 24 h (B) and (C), the nanoconjugates or Au NPs were recovered and resolved in electrophoresis. After Wang et al. (1).

At lower peptide concentrations, the AuNP did not migrate and appeared to become oxidized, forming black deposits in the

application wells. Similarly, a classical pharmacological assay protocol, Scatchard Analysis, was borrowed to more completely

characterize the binding between the 17-mer (TYR-radio-iodinated) peptides and the 20 nm AuNP. The analogy here is that AuNP were proposed to behave similarly to classical cell-bound receptors, being uniform in binding characteristics. The model also assumes reversible binding, dissociation rendering both ligand (peptide), and receptor (AuNP) in a form that is unmodified from that before binding, and that the interactions are non-covalent and reversible. The majority of the underlying assumptions likely hold true; however, the study used both association kinetics and dissociation kinetics designs; the dissociation studies suggesting that around 20% of the maximum binding was non-dissociative (likely covalent linkage). This may be a source of some error in the estimates of the maximum number of binding sites (4182 molar equivalents) and the dissociation constant (K_D), which is also the peptide concentration at which half of the maximum available binding sites are occupied (30 nM peptide). Furthermore, incubation of the stable nano-conjugates with or without serum proteins for 15 minutes resulted in protein-peptide exchange, with serum proteins becoming bound into the structure which retained similar, stable electrophoretic profiles

up to 24 hours incubation. Thus, serum protein incubation maintained the stability of the nano-conjugates, and they migrated more slowly as discrete, single bands in electrophoresis suggesting that the serum proteins had displaced some of the 17-mer peptides and that their association with the nano-constructs was quite stable and of a predictable total molecular mass (Figure 2).

Interpretation of these data suggests the following: AuNP, when incubated with peptide or serum protein solutions, very rapidly adsorb (and likely form Au-S covalent linkage, where sulphur moieties on peptides and proteins are available). Under physiological conditions (in the presence of cell culture medium, DMEM, or the presence of PBS) levels of protein binding result in discrete and predictable association that is highly stable at equilibrium. The majority of the binding is non-covalent and reversible; thus, the binding is dynamic, and protein or peptide exchange is likely driven through predictable association and dissociation kinetics. The final hydrodynamic diameters of these forms are shown in Table 1.

Table 1. Dynamic Light Scattering Data of Gold-peptide Nanoconjugates and Gold Nanoparticles After Wang et al. (1)

Sample Name	Hydrodynamic
	Diameter (nm)
AuNP-peptide A	26 ± 0.5
AuNP-peptide B	24 ± 0.1
AuNP	22 ± 0.0
AuNP-peptide A with Serum	45 ± 0.9
AuNP-peptide B with Serum	46 ± 0.9
AuNP with Serum	83 ± 6.0

Data are presented as Mean ± SE (Standard Error) from 3 independent measurements.

What does this mean for studies that incubate nanoparticle or nano-constructs in biological systems in vitro or in vivo?

The rapid and stable association of these peptides or proteins (even at relatively low molar equivalents, ~ 200) with AuNP (< 15 min) suggests that "naked" nanoparticles, when incubated (delivered) into a biological matrix, will rapidly adsorb protein. This is also true in cases where nano-structures are pre-complexed

with other drugs and macromolecules (ligands). However, the specifics of the association and dissociation kinetics will be different in each case. Thus, cells or tissues "see" nano-constructs as peptide/protein/macromolecular complexes. The core nanoparticle is "invisible" to the cells and tissues. This includes all cell types, whether normal somatic cells in the tissues or cancer cells or migrating cell types of the immune system, present in tissues, lymph, or blood.

Furthermore, if administered as a therapeutic, the non-covalently linked fraction of the ligands bound to the nano-therapeutic once administered will begin to dissociate at a predictable rate constant. If the nano-therapeutic is designed (or the surface moieties happen) to be recognized by cellular uptake mechanisms, including specific ligand-receptor interactions or non-specific interactions, micropinocytosis, leads to rapid cellular internalization (maximum nano-structure size limit around 150 to 200 nm).

Depending upon the nature of the molecules that are receptor-bound in these internalized vesicles, signalling between ligands and receptors may have a direct effect on retrograde cellular transport pathways. Thus, some vesicles may be directed to specific organelles. If there is "therapeutic intent" in the design of nano-construct-bound ligands, it is a stronger strategy to direct vesicles away from pathways that result in fusion of the vesicle with lysosomes (containing a multitude of degradative enzymes in a low pH matrix).

The rationale presented here does not discount alternative strategies. For example, Fuller and Köper (17) have recently reviewed the flexibility of ligand binding to AuNP evoked via polyelectrolyte (PE) coating. However, given the diversity of proteins present in biological fluids and the dynamics of non-covalent interactions of proteins with nano-structures, strategies such as PE coating likely alter the specifics of the interactions. Nonetheless, overlaying of endogenous proteins must be included in our understanding of the dynamic state of these interesting alternative approaches to nano-therapeutics.

What features of nano-structures or viruses are recognized by cells?

Working further into the theme of shared characteristics of developing nano-therapeutics and viruses, the example of the cellular retrograde transport signal is explored further here.

It has been hypothesized that the C-terminal sequence KDEL (standard amino acid residue single letter codes), utilizes retrograde cellular transport pathways that direct proteins/peptides bearing this sequence to the endoplasmic reticulum (ER) and away from the degradative lysosomes.

Figure 3. Confocal image of cells with over-expressed KDEL receptor-GFP fusion protein (green). AuNP-delivered KDEL-peptide (red). Peptide-bound receptor (yellow). The overexpressed KDEL-receptor locates to the ER where nano-structure-delivered KDEL peptides co-localize. After Wang et al. (2).

This retrograde transport pathway is also hijacked by some bacteria (e.g., *Vibrio cholera*) as part of their host attack machinery (18). The pathway has evolved in eukaryotic cells to recover and recycle valuable proteins and peptides. The signalling peptide C-terminal sequence KDEL (lysine, aspartate, glutamate, leucine), binds receptors in a Coat Protein I (CoPI) mediated pathway that directs vesicles through the Golgi and into the ER. Utilization of this pathway has been shown to accumulate nano-structures in the ER (Figure 3)

The cellular uptake and migration of AuNP-bound KDEL to the ER happens quickly *in vitro* (< 15 min). Escape from the ER to the cytoplasm is hypothesized to occur through the protein translation machinery, specifically via translocons (2). Assuming that this model holds true for a range of AuNP-delivered drugs/macromolecules and interpreting the discussion above around association and dissociation of the nano-complexes, the dynamics of these interactions support the idea that cargo drugs can be readily delivered into the ER and cytoplasm, as the non-covalently linked fraction of ligand can be delivered to target cellular organelles and will slowly, but readily dissociate from the carrier AuNP. Viruses utilize similar pathways, their targets being ER/cytoplasm and the nucleus, where the cellular transcription and translation machinery are hijacked in the replication of viral

elements and their assembly into viral particles. Thus, the theory of co-localization of nano-therapeutics to sub-cellular sites of viral accumulation is supported.

A further point on association and dissociation dynamics: Suitable cell culture models for in vivo systems.

As it is well known that there is a wide range of metabolic / protein and other macromolecular dynamics across different cell types, we should consider the question of nanostructure-based drug delivery in this context. In vitro, cancer cell types typically exhibit the most rapid turnover, with features such as short cell number doubling time (typical range 14 to 20 hours), while normal cell types tend to have longer turnover dynamics (doubling times 18 to 30 hours). Within normal cells, examples such as stem cells tend to have higher metabolic turnover rates, while there are also terminally differentiated cell types that exhibit much slower metabolic rates. Examples of cell culture models from the literature include pre-adipocytes (stem cells) and differentiated adipocytes (19, 20) and cells of muscle lineage, myocytes (stem cells) – myoblasts – and terminally differentiated myotubes (21). The dynamics of these cell types are recapitulated in vivo. Thus, in considering drug delivery to terminally differentiated cells/tissues, in the case argued in this perspective, let's explore how ligand-directed nano-structures compare in "cargo" delivery dynamics against other systems. We keep in mind that fast and efficient drug delivery to peripheral tissues (alveolae in the case argued here) is a core requirement.

In this example, the model "drug" delivered by AuNP was a siRNA, a relevant model as a potential antiviral. This study (21) used the peptide noted above to direct the AuNP-based nano-structures, carrying siRNA into two cell types, myogenic stem cells and the same cell line that had been differentiated to form myotubes (precursors and sub-structures that integrate to form mature muscle). Notably, the stem cell myoblasts have relatively high metabolic rates, while the differentiated myotubes have much lower metabolic rates. In comparing the efficacy of siRNA delivery to myoblast (stem cells) versus differentiated myotubes, it was found that the classically used experimental transfection reagent,

Lipofectamine was more effective at delivering siRNA to the stem cells (high metabolic rate), while the AuNP-based transfection resulted in greater efficacy of siRNA delivery into terminally differentiated myotubes. Thus, AuNP as a carrier/vector for drug delivery appears to have some advantages that potentially translate as favourable features for in vivo drug delivery. Because differentiated cells such as alveolar epithelial cells and bronchial epithelial cells expressing the ACE2 receptor (required for binding of the SARS-CoV-2 virus) exhibit slower metabolic turnover dynamics, and at least in vitro appear to be infected by SARS-CoV by either plasma membrane fusion or endosomal pathways (22) this suggests that nano-therapeutics designed to interact at the endosomal pathway will encounter the virus, if it is present. Furthermore, the more specific cellular retrograde transport pathways utilized by KDEL-directed AuNP appear to have greater efficacy in terminally differentiated (mature tissue type) cells compared to the generic, non-specific pathways (direct plasma membrane interactions) utilized by Lipofectamine.

The confluence of proposed nano-therapeutics and SARS-CoV in sub-cellular pathways

As discussed above, KDEL-bearing nano-structures are directed through cellular retrograde transport pathways, shuttling via the Golgi to the ER. Interestingly, the initial intracellular target of SARS-CoV is the ER, where the spike, envelope, membrane, neocapsid and accessory proteins are translated and shuttled through anterograde pathways via the ER-Golgi intermediate compartment, the site of SARS-CoV assembly (22). Thus, speculation in this perspective identifies multiple sub-cellular compartments at which nanostructure-delivered antivirals may intersect those utilized by SARS-CoV-2. These include endocytosis pathways, points of viral entry to cells, and sites of viral assembly.

Consideration for candidate antivirals: The greatest need appears to be combating the devastating symptoms that arise in a low percentage of patients who seem to generally fall into older age groups. Early evidence suggests that non-symptomatic patients and those with severe symptoms have moderate to

high levels of viral RNA in the throat and nasal samples (23), thus, the strategy of focusing upon therapeutics directed to the respiratory system appears to have potential in treating respiratory symptoms at the source of the most severe pathologies. There are many sophisticated approaches to identifying drugs of choice as SARS-CoV-2 antivirals (24). While there are many non-expert opinions on trialling candidate antivirals, the present commentary recognizes the need for thorough and

systematic studies. As noted above, strategies that target viral transcription and/or inhibit translation of viral proteins offer great specificity but are also subject to similar shortcomings should the target be prone to mutation. A considered recommendation is that such strategies have many advantages over some other recently proposed therapeutics that are contra-indicated due to their off-target effects.

Conclusions:

This perspective has intended to stimulate discussion around the underlying mechanisms of ligand-directed, nano-therapeutic delivery to virally infected cells in vivo and to generate further ideas that may evolve for the development of antivirals for the treatment of insidious SARS-CoV-2 infections. We have explored some of the mechanisms that regulate the in vivo distribution and cellular trafficking of potential nano-therapeutics and some of these features that are also exploited by viruses. Although speculative in the context of the development of antivirals against SARS-CoV-2, the discussion here provides the stimulus for scientific debate. So, little of SARS-CoV-2 biology is understood in this period of its early and devastating global impact. However, elements of its biology inferred from knowledge of SARS-CoV and other related viruses provide a theoretic basis in which to control the impact of SARS-CoV-2. In bringing the context of nano-therapeutic design together with the very preliminary knowledge of SARS-CoV-2, it is hoped that this perspective is useful.

Conflict of Interests

The author declares no conflicts of interest. For a signed statement, please contact the journal office: editor@precisionnanomedicine.com

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