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Commentary
Anthony Patch

In this month's edition of Entangled magazine, we present the cross-correlation of adverse neurological manifestations of the SARS-CoV-2 virus (Covid-19, the disease) to those of the vaccines specific to it.

Cross-correlation is defined as the comparison of two different time series to detect if there is a correlation between metrics with the same maximum and minimum values. Thus, herein, we compare the adverse effects found within the onset of SARS-CoV-2 (referred to here simply as "the virus") to those found within the follow-on vaccines specific to it.

As denoted by the published scientific literature, these cross-correlated adverse effects are common to those found in victims of envenomation by toxic animals such as serpents and scorpions.

By examining the adverse effects of the virus and its vaccines, we discover a direct connection in their aetiology (the study of the causes). The word "aetiology" is mainly used in medicine, where it is the science that deals with the causes or origin of disease, the factors which produce or predispose toward a certain disease or disorder.

Predominant to both the virus and its vaccines are neurological adverse effects/disorders/manifestations due to the presence of toxic-like peptides. The release of oligopeptides (ten to fifteen amino acids) are almost identical to toxic components of venoms from animals. Their involvement in a large set of heterogeneous extra-pulmonary COVID-19 clinical manifestations, like neurological ones, cannot be excluded. (Brogna, et al)**.**

While the focus of the presented literature is upon neurological manifestations specific to the virus, by cross-correlation of the same found within vaccinated persons a logical conclusion as to the contents of the vaccines is arrived at.

Akin to the process of reverse engineering, beginning with the so-called "adverse effects" of the vaccines in totality, their aetiology, peptides, is identical to the neurological manifestations exhibited by those persons diagnosed as having contracted the disease, COVID-19.

Peptides are short chains of between two and fifty amino acids, linked by peptide bonds. While polypeptides are a long, continuous, unbranched peptide chain of up to approximately fifty amino acids. Those consisting of fifty amino acids are known as a protein. For example, spike protein.

The July 2021 edition of Entangled magazine focused upon the expression of spike proteins by both the virus and each of the present vaccines addressing it. Peptides are the building blocks of proteins, including the spike versions of known and novel proteins.

Thus, we are noting the same neurological manifestations due to the presence of venomous neurotoxins within the virus as those exhibited post-vaccination. Neurotoxins are either synthetic or naturally occurring substances that damage, destroy, or impair the functioning of the central and/or peripheral nervous system. The January 2021 edition of Entangled magazine focused upon damage to the autonomic nervous system caused by the virus and its attendant spike proteins.

Logically, the adverse neurological manifestations are cross-correlated from the virus directly to the vaccines specific to it. As evidenced within several editions of Entangled magazine, additional technological components are contained within the vaccines, rendering these as more damaging than the disease, COVID-19, linked to the virus SARS-CoV-2.

Since January 2013, we have presented both the concept and process of *in silico* genetic design, and modelling within software, the artificial configuration of genes into a single (RNA) or double strand (DNA) of nucleic acid polymers (nucleotides). Likewise, both by selecting and ordering specific genes, while activating or silencing them, sequences of proteins are encoded in the genes. Proteins are made up of peptides.

By understanding the action of a specific peptide, the outcome of a *in silico* model can identified. When working backward from this outcome, the peptide is identified. Further, the protein from which the peptide originated is found. From this, the gene expressing a specific protein is located. The strand containing this gene is identified from a known sequence of proteins, thus the involved peptides.

When the action of a known or novel peptide results in a specific adverse neurological manifestation, identification of the causal peptide can be made. Discovery of such a peptide within a virus whose infection results in a specific manifestation provides a clue as to the ingredients of a vaccine formulated to terminate the virus.

If the ingredients of such a vaccine are unknown, the manifestation of so-called "adverse effects" are employed to identify them. Each effect can then be traced to a cause. In this case, adverse neurological manifestations can be traced back to one or more peptides. The peptides make up proteins linked to specific genes. Genes are arranged in a sequence of one (RNA) or two (DNA) strands.

In the recent past, a "traditional vaccine" produced from an attenuated (reduced effect) virus is itself a copy of the original. Following this example, today's mRNA-based (DNA-based results in mRNA) vaccines for SARS-CoV-2 (COVID-19 the disease) are a copy of this virus. Both the virus and the vaccines are man-made constructs as a single strand of mRNA.

The difference here is in the adverse neurological manifestations of the vaccines as compared to those of the virus alone. These are diagnosed in vaccinated populations more frequently and in greater severity, including deaths, as compared to data on the virus alone.

To further complicate comparison, four population groups need to be examined. First, asymptomatic survivors of exposure solely to the 'wild' virus. Second, the symptomatic survivors of exposure solely to the 'wild' virus. Third, the symptomatic convalescent having been vaccinated prior to exposure to the virus, either in the 'wild', or by infected transmission. Fourth, the symptomatic convalescent having been vaccinated post-recovery. Recently, the emergence of "variants" have added to these comparitive categories.

The focus here is upon those contracting and diagnosed with the disease COVID-19, whether vaccinated prior, or subsequent to infection by the virus SARS-CoV-2. The reason, the exacerbation of adverse neurological effects brought about by the actions of the vaccines themselves. Specifically, those of the peptides, and their increased quantities due to the concurrence of virus and vaccine.

As presented above, and as evidenced by public statements from pharmaceutical companies producing COVID-19 vaccines, one of their designed outcomes is the production of spike protein.

Subsequent published literature indicates successful production of spike protein by way of these vaccines. However, the quantities listed in these studies are 100-fold greater than therapeutic and on a continuous basis.

The result of such production is cellular entry by way of the receptor binding domain (RBC), most commonly the Angiotensin-converting enzyme 2 (ACE 2) attached to the membrane of cells located in the intestines, kidney, testis, gallbladder, and heart. Literature clearly points to the accumulation of spike protein within all the major organs, spleen, and by way of the vagus nerve, crossing of the blood brain barrier (BBB) into the brain itself.

Vaccine manufacturers attest to the production of spike protein. Their claim is to a level lower than that of the virus itself. Their expectation of a low-level response and production of antibodies by the human immune system has retrospectively and demonstrably been proven not to be case.

The increasing prominence in cases of adverse neurological manifestations are directly attributable to the actions of the peptides as ingredients of these vaccines. The very same peptides found in the venoms of serpents and scorpions.

In conclusion, the cross-correlation of adverse neurological manifestations of the SARS-CoV-2 virus (COVID-19, the disease) to the vaccines specific to it, is inescapable in its logic.

Luke 10:19 KJV

Behold, I give unto you power to tread on serpents and scorpions, and over all the power of the enemy: and nothing shall by any means hurt you.

Science with censorship,
is not science.

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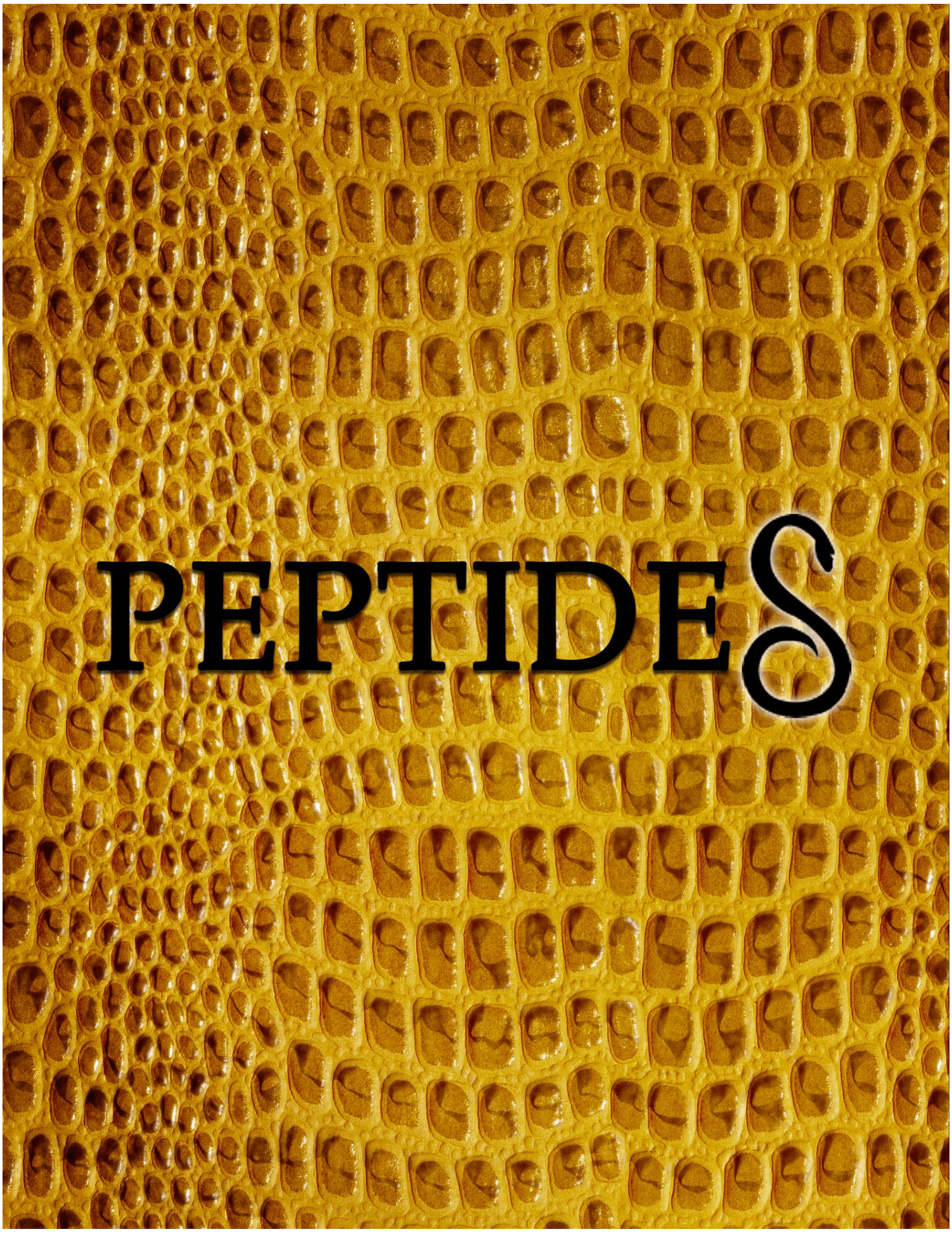
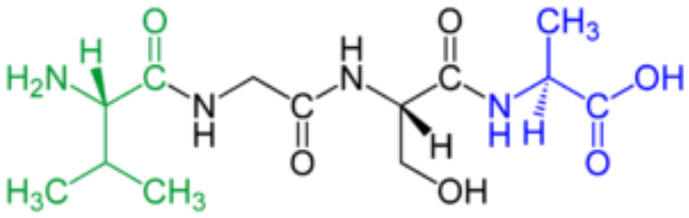

PEPTIDES

Peptides



Peptides (from Greek language πεπτός, *peptós* "digested"; derived from πέσσειν, *péssein* "to digest") are short chains of between two and fifty amino acids, linked by peptide bonds.[1][2] Chains of fewer than ten or fifteen amino acids are called oligopeptides, and include dipeptides, tripeptides, and tetrapeptides.

A polypeptide is a longer, continuous, unbranched peptide chain of up to approximately fifty amino acids.[3] Hence, peptides fall under the broad chemical classes of biological polymers and oligomers, alongside nucleic acids, oligosaccharides, polysaccharides, and others.

A polypeptide that contains more than approximately fifty amino acids is known as a protein.[3][4][5] Proteins consist of one or more polypeptides arranged in a biologically functional way, often bound to ligands such as coenzymes and cofactors, or to another protein or other macromolecule such as DNA or RNA, or to complex macromolecular assemblies.[6]

https://en.wikipedia.org/wiki/Peptide

RESEARCH ARTICLE

Toxin-like peptides in plasma, urine and faecal samples from COVID-19 patients [version 1; peer review: awaiting peer review]

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Abstract

Background: SARS-CoV-2 that causes COVID-19 disease and led to the pandemic currently affecting the world has been broadly investigated. Different studies have been performed to understand the infection mechanism, and the involved human genes, transcripts and proteins. In parallel, numerous clinical extra-pulmonary manifestations co-occurring with COVID-19 disease have been reported and evidence of their severity and persistence is increasing. Whether these manifestations are linked to other disorders co-occurring with SARS-CoV-2 infection, is under discussion. In this work, we report the identification of toxin-like peptides in COVID-19 patients by application of the Liquid Chromatography Surface-Activated Chemical Ionization – Cloud Ion Mobility Mass Spectrometry.
Methods: Plasma, urine and faecal samples from COVID-19 patients and control individuals were analysed to study peptidomic toxins' profiles. Protein precipitation preparation procedure was used for plasma, to remove high molecular weight proteins and efficiently solubilize the peptide fraction; in the case of faeces and urine, direct peptide solubilization was employed.
Results: Toxin-like peptides, almost identical to toxic components of venoms from animals, like conotoxins, phospholipases, phosphodiesterases, zinc metal proteinases, and bradykinins, were identified in samples from COVID-19 patients, but not in control samples.

Open Peer Review

Reviewer Status *AWAITING PEER REVIEW*

Any reports and responses or comments on the article can be found at the end of the article.

Conclusions: The presence of toxin-like peptides could potentially be connected to SARS-CoV-2 infection. Their presence suggests a possible association between COVID-19 disease and the release in the body of (oligo-)peptides almost identical to toxic components of venoms from animals. Their involvement in a large set of heterogeneous extra-pulmonary COVID-19 clinical manifestations, like neurological ones, cannot be excluded. Although the presence of each individual symptom is not selective of the disease, their combination might be related to COVID-19 by the coexistence of the panel of the here detected toxin-like peptides. The presence of these peptides opens new scenarios on the aetiology of the COVID-19 clinical symptoms observed up to now, including neurological manifestations.

Keywords
SARS-CoV-2, COVID-19, toxin-like peptides

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Introduction

Numerous clinical extra-pulmonary manifestations co-occurring with COVID-19 disease have been reported (e.g. neurological, haemorrhagic, and thrombotic) and evidence of their severity and persistence is increasing. Gupta *et al.* reviewed the extrapulmonary organ-specific pathophysiology of patients with COVID-19, '*to aid clinicians and scientists in recognizing and monitoring the spectrum of manifestations, and in developing research priorities and therapeutic strategies for all organ systems involved*'[1]. Liotta *et al.* characterized the incidence of neurological manifestations in a cohort of hospitalised patients with confirmed COVID-19: the most frequent were myalgia, headache, encephalopathy, dizziness, dysgeusia, and anosmia; encephalopathy was found to be '*associated with increased morbidity and mortality, independent of respiratory disease severity*'[2]. Whether these manifestations are linked to disorders co-occurring with SARS-CoV-2 infection is under discussion, including their concomitant occurrence, which could be strongly related COVID-19 disease. Frontera *et al.*, by conducting a prospective, multi-centre, observational study of hospitalised adults with laboratory-confirmed SARS-CoV-2 infection, concluded that *'neurologic disorders were detected in 13.5% of COVID-19 patients during the study timeframe. Many of these neurologic disorders occur commonly among patients with critical illness. Encephalitis, meningitis or myelitis referable to SARS-CoV-2 infection did not occur, though post-infectious Guillain-Barre syndrome was identified. Overall, neurologic disorders in the context of SARS-CoV-2 infection confer a higher risk of in-hospital mortality and reduced likelihood of discharge home*'[3].

Studies on the use of mass spectrometry in COVID-19 context focus on the search for augmented human inflammatory molecules to be used as biomarkers to assess the severity status of COVID-19 (see for example the work[4] of Messner and colleagues). Different studies report the use of proteomic approaches to characterise SARS-CoV-2 proteins[5–7]. Other studies highlight challenges in their use due to the need of enriching the protein fraction to be analysed for maximizing the technology sensitivity.[8]

Liquid Chromatography Surface-Activated Chemical Ionization – Cloud Ion Mobility Mass Spectrometry (LC-SACI-CIMS) is reported as a high sensitivity mass spectrometry technique able to maximize the peptide signal intensity[9–12]. We used LC-SACI-CIMS to reveal the presence of metabolites that could explain the clinical descriptions of neurological, coagulation and inflammatory symptoms, and here we present the results of our analyses. We found toxin-like peptides in plasma, urine, and faecal samples from COVID-19 patients, but not in control samples. As our findings do not correspond with current thinking of the aetiology related to the observed clinical manifestations in COVID-19 patients, we feel their immediate sharing with the scientific community is critical.

Methods

Rationale

Liquid Chromatography-Surface Activated Chemical Ionization – Cloud Ion Mobility Mass Spectrometry (LC-SACI-CIMS) exhibits a high selectivity in peptide detection thanks to its ability to selectively isolate peptide ions through an in-source ion mobility (IM) effect. In fact, it allows a selective regulation of the potential difference between the low voltage of the SACI surface (47 V) and the entrance lens (-50 / -600 V), and a selective focalization on solvent ion cloud containing species at low or high *m/z* ratio. By switching the entrance voltage lens between -50 and -600 V during the analysis, it is possible to separate the low *m/z* from the high *m/z* potential signal, to avoid ion trap saturation, and to maximize the number of detected compounds. The mass spectra chemical noise is also strongly reduced due to the lower amounts of solvent cluster ions that are produced in low voltage ionization conditions. Thus, the peptide detection efficiency is strongly increased by the IM selectivity and lower chemical noise with respect to the classical high voltage ionization approaches. Thanks to the specificity of the SACI-CIMS technology in focalizing the solvent ion clouds containing the high *m/z* (oligo-)peptide species, it was possible to increase the detection efficiency.

In the use of LC-SACI-CIMS, the following strategies have been adopted:

- To reduce the presence of contamination as much as possible and to avoid the formation of acetonitrile polymers occurring in acid conditions (as reported by Eizo *et al.*[13]), formic acid was not added to the CH_3CN chromatographic phase.
- To separate low from high *m/z* solvent ion clusters by reducing the ion trap saturation, the space/charge effect, and by increasing the detected compounds recovery, LC-SACI–CIMS entrance lens voltage was switched between -50 and -600 V every 10 ms during the analysis.
- To enhance the SACI ionization efficiency, NH_4HCO_3 was added to the samples. As reported in the literature[14,15], the peptide ionization efficiency (and consequently the sensitivity) is enhanced in SACI conditions when ionic salts are present in the sample, due to peptide ion specific coordination.
- To decrease the total run time, a shot gun chromatographic gradient was used to desalt the sample.
- To avoid sample molecular profile alteration, and to evaluate the potential biological activities of the circulating species, no enzymatic digestion was applied to samples.
- To normalize the *m/z* signal intensity, 5 µL of standard ESI tune mix (Agilent, USA) were added to each sample extract.

Chemicals

NH_4HCO_3, methanol, acetonitrile and formic acid were purchased from Sigma-Aldrich (Milan, Italy). Bi-distilled water was purchased from VWR (Milan, Italy).

Cohort

Samples used in the present study: plasma samples collected from 15 COVID-19 patients from different cities of Italy and from five control individuals (i.e. negative to SARS-CoV-2

tests and not affected by cancer or autoimmune diseases); urine samples collected from two additional COVID-19 patients and from two control individuals; stool samples from three COVID-19 patients and from three control individuals. The human biological samples used in the experimentation were collected and used with the expressed free and informed written consent, of the person from whom the material was taken, according to current legislation. The study received approval from "Comitato Etico Campania Sud" (n.36/2021, request submitted on 06-05-2020).

Sample preparation

Plasma. Each plasma sample was treated as follows: 5 µL of CH_3CN were added to 50 µL of plasma and vortexed for one minute. The procedure was repeated 10 times. Then the sample was centrifuged at 1,500 g for 10 minutes and two 100 µL aliquots of supernatant were dried and resuspended in 70 µL of NH_4HCO_3 50 mmol. The solution was analysed by LC-SACI-CIMS (see *Rationale*).

Urine. Each urine sample was treated as follows: an equivalent volume of bi-distilled water was added, followed by centrifugation at 1,500 g for 10 minutes. 100 µL were dried and resuspended in 70 µL of NH_4HCO_3 50 mmol. The sample was analysed by LC-SACI-CIMS (see *Rationale*).

Stool. Each stool sample was treated as described by Cristoni *et al.*[11] and analysed by LC-SACI-CIMS (see *Rationale*).

Liquid chromatography

The Ultimate 3000 LC (by ThermoFisher) was used to achieve separation of analytes for each sample prior to mass spectrometry (MS) analysis. A reversed phase Kinetex C-18 LC column (50 × 2.1 mm; particle size, 5 µm; pore size, 100 Å, by Phenomenex, USA) was used. The eluent flow was 0.25 mL/min and the injection volume was 15 µL. The mobile phases were:

A. 0.2% (v/v) formic acid (HCOOH)

B. acetonitrile (CH_3CN)

The elution gradient was: 2% (v/v) of B between 0 and 2 min; 2 to 30% between 2 and 7 min; 30 to 80% between 7 and 9 min; 80% between 9 and 12 min; 80-2% between 12 and 12.1 min. The column was rebalanced with 2% of B between 12.1 and 17 min.

Mass spectrometry

All samples were analysed for the presence of proteins with potential toxic effect by using the LC-SACI-CIMS as already described in the literature[9–12]. Samples were analysed with an ORBITRAP mass spectrometer (Breme, Germany) coupled to a surface-activated chemical ionization (SACI) source and operated in positive ion mode.

The surface voltage was 47 V and the entrance lens was switched between -50 and -600 V each 10 ms. Auxiliary gas: 2 L / min; Nebulizer gas: 80 psi; Temperature: 40 °C. Full scan spectra were acquired in the 40–3,500 *m/z* range for non-targeted metabolomics/proteomics analyses to detect analytes. The same *m/z* range was used for both discovery and selective biomarker identification, and to standardize (primarily in terms of scan rate) the instrument. The software used for data elaboration is SANIST, a modified version of the Global Proteome Machine (GPM, https://www.thegpm.org/GPM/), implanted as described in 9–12. SANIST output files are available as supplementary material[16] (see section *Data availability*).

SANIST software here used is freely available, upon email request to CranioMed group (dir.brogna@craniomed.it).

Mass spectrometry on samples was performed with collision-induced dissociation using data dependent scan and helium as the collision gas. The ion trap was applied to isolate and fragment the precursor ions (windows of isolation, ± 0.3 *m/z*; collision energy, 30% of its maximum value, which was 5V peak to peak), and the ORBITRAP mass analyser was used to obtain fragments with an extremely accurate *m/z* ratio (resolution 15,000; *m/z* error <10 ppm).

Data elaboration

Detected high *m/z* peptides were used to identify toxins thanks due to the selectivity given by their long chain.

The complete UniprotKB set of manually reviewed venom proteins and toxins (UniprotKB, Animal toxin annotation project. https://www.uniprot.org/program/Toxins, Accessed October 4, 2020), mixed with a subset of non-venom proteins and toxins from UniprotKB database[17] was used as reference protein dataset in order to give statistical significance to the results.

TBLASTN[18] was run at the National Center for Biotechnology Information (NCBI) website[19] with default options and parameters, with the exception of the following ones: max target sequences = 1,000; expect threshold = 100; word size = 3; gap cost existence = 9; gap cost extension = 1; filter of low complexity regions = No. Searches have been performed versus: Nucleotide collection (nr/nt); Reference RNA sequences (refseq_rna); RefSeq Genome Database (refseq_genomes); Whole-genome shotgun contigs (wgs) from metagenomic experiments; Sequence Read Archive (SRA) sequences from metagenomic experiments; Transcriptome Shotgun Assembly (TSA); Patent sequences (pat); Human RefSeqGene sequences (RefSeq_Gene); Betacoronavirus Genbank sequence dataset.

The information reported in Table 1 has been retrieved from the UniprotKB database and from the NCBI Taxonomy database[20], after confirmation by BLAST sequence comparison analysis[18].

SANIST was set to perform the database search considering all potential protein points and post-translational modifications, and to consider proton rearrangements. No enzyme cutting rules were specified, but all the protein subsequence combinations were considered. Database search calculation

Table 1. Overview of candidate proteins on which toxin-like peptides have been mapped. Thirty-six candidate protein sequences on which the identified toxin-like peptides have been mapped are here reported, together with information retrieved from UniprotKB and NCBI Taxonomy databases. The table is split in three sections according to the phylum of the reported species: *Chordata* (green), *Echinodermata* (pink), *Mollusca* (azure).

UNIPROTKB CANDIDATE'S INFORMATION							TAXONOMY CANDIDATE'S INFORMATION			
AC	***ID***	***Status***	***Protein name***	***ENZYME EC***	***Other name(s)***	***Length (aa)***	***ID***	***Species***	***Phylum - Family***	***Organism's common name(s)***
Q8AY46	VKTHB_BUNCA	reviewed	Kunitz-type serine protease inhibitor homolog beta-bungarotoxin B1 chain	NA	-	85	92438	*Bungarus Candidus*	***Chordata*** *- Elapidae*	. Malayan krait
A6MEY4	PA2B_BUNFA	reviewed	Basic phospholipase A2 BFPA	EC 3.1.1.4	. Antimicrobial phospholipase A2 . Phosphatidylcholine 2-acylhydrolase (svPLA2)	145	8613	*Bungarus fasciatus*	***Chordata*** *- Elapidae*	. Banded krait . Pseudoboa fasciata
F5CPF1	PA235_MICAT	reviewed	Phospholipase A2 MALT0035C	EC 3.1.1.4	. Phospholipase A2 MALT0035C (svPLA2)	142	129457	*Micrurus altirostris*	***Chordata*** *- Elapidae*	. Uruguayan coral snake . Elaps altirostris
A8QL59	VM3_NAJAT	reviewed	Zinc metalloproteinase-disintegrin-like NaMP	EC 3.4.24.-	. Snake venom metalloproteinase (SVMP)	621	8656	*Naja atra*	***Chordata*** *- Elapidae*	. Chinese cobra
Q9I900	PA2AD_NAJSP	reviewed	Acidic phospholipase A2 D	EC 3.1.1.4	. svPLA2 . APLA . Phosphatidylcholine 2-acylhydrolase	146	33626	*Naja sputatrix*	***Chordata*** *- Elapidae*	. Malayan spitting cobra . Naja naja sputatrix
Q58L90	FA5V_OXYMI	reviewed	Venom prothrombin activator omicarin-C non-catalytic subunit	NA	. vPA . Venom coagulation factor Va-like protein *Cleaved into 2 chains*	1460	111177	*Oxyuranus microlepidotus*	***Chordata*** *- Elapidae*	. Inland taipan . Diemenia microlepidota
Q58L91	FA5V_OXYSU	reviewed	Venom prothrombin activator oscutarin-C non-catalytic subunit	NA	. vPA . Venom coagulation factor Va-like protein *Cleaved into 2 chains*	1459	8668	*Oxyuranus scutellatus*	***Chordata*** *- Elapidae*	. Coastal taipan
Q9W7J9	3S34_PSETE	reviewed	Short neurotoxin 4	NA	. SNTX4 . Alpha-neurotoxin 4	79	8673	*Pseudonaja textilis*	***Chordata*** *- Elapidae*	. Eastern brown snake
P23028	PA2AD_PSETE	reviewed	Acidic phospholipase A2 homolog textilotoxin D chain	NA	. svPLA2 homolog	152	8673	*Pseudonaja textilis*	***Chordata*** *- Elapidae*	. Eastern brown snake
Q593B6	FA5_PSETE	reviewed	Coagulation factor V	NA	*Cleaved into 2 chains*	1459	8673	*Pseudonaja textilis*	***Chordata*** *- Elapidae*	. Eastern brown snake

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UNIPROTKB CANDIDATE'S INFORMATION							TAXONOMY CANDIDATE'S INFORMATION			
AC	***ID***	***Status***	***Protein name***	***ENZYME EC***	***Other name(s)***	***Length (aa)***	***ID***	***Species***	***Phylum - Family***	***Organism's common name(s)***
Q7SZN0	FA5V_PSETE	reviewed	Venom prothrombin activator pseutarin-C non-catalytic subunit	NA	. PCNS . vPA . Venom coagulation factor Va-like protein *Cleaved into 2 chains*	1460	8673	*Pseudonaja textilis*	***Chordata*** *- Elapidae*	. Eastern brown snake
Q2XXQ3	CRVP1_PSEPL	reviewed	Cysteine-rich venom protein ENH1	NA	. CRVP . Cysteine-rich secretory protein ENH1 (CRISP-ENH1)	239	338839	*Pseudoferania polylepis*	***Chordata*** *- Homalopsidae*	. Macleay's water snake . Enhydris polylepis
Q9PW56	BNP2_BOTJA	reviewed	Bradykinin-potentiating and C-type natriuretic peptides	NA	. Brain BPP-CNP . Evasin-CNP *Cleaved into the 12 chains*	265	8724	*Bothrops jararaca*	***Chordata*** *- Viperidae*	. Jararaca
A8YPR6	SVMI_ECHOC	reviewed	Snake venom metalloprotease inhibitor	NA	. 02D01 . 02E11 . 10F07 . Svmpi-Eoc7 *Cleaved into 15 chains*	308	99586	*Echis ocellatus*	***Chordata*** *- Viperidae*	. Ocellated saw-scaled viper
Q698K8	VM2L4_GLOBR	reviewed	Zinc metalloproteinase/ disintegrin [Fragment]	EC 3.4.24-	*Cleaved into 3 chains*	319	259325	*Gloydius brevicaudus*	***Chordata*** *- Viperidae*	. Korean slamosa snake . Agkistrodon halys brevicaudus
Q8AWI5	VM3HA_GLOHA	reviewed	Zinc metalloproteinase-disintegrin-like halysase	EC 3.4.24-	. Zinc metalloproteinase-disintegrin-like halysase . Snake venom metalloproteinase (SVMP) . Vascular apoptosis-inducing protein (VAP)	610	8714	*Gloydius halys*	***Chordata*** *- Viperidae*	. Chinese water mocassin . Agkistrodon halys
P82662	3L26_OPHHA	reviewed	Alpha-neurotoxin	NA	. Alpha-elapitoxin-Oh2b (Alpha-EPTX-Oh2b) . Alpha-elapitoxin-Oh2b . LNTX3 . Long neurotoxin OH-6A/OH-6B . OH-3	91	8665	*Ophiophagus hannah*	***Chordata*** *- Viperidae*	. King cobra . Naja hannah

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UNIPROTKB CANDIDATE'S INFORMATION							TAXONOMY CANDIDATE'S INFORMATION			
AC	**ID**	**Status**	**Protein name**	**ENZYME EC**	**Other name(s)**	**Length (aa)**	**ID**	**Species**	**Phylum - Family**	**Organism's common name(s)**
Q2PG83	PA2A_PROEL	reviewed	Acidic phospholipase A2 PePLA2	EC 3.1.1.4	. Phosphatidylcholine 2-acylhydrolase (svPLA2)	138	88086	*Protobothrops elegans*	***Chordata*** - *Viperidae*	. Elegant pitviper . Trimeresurus elegans
P06860	PA2BX_PROFL	reviewed	Basic phospholipase A2 PL-X	EC 3.1.1.4	. Phosphatidylcholine 2-acylhydrolase (svPLA2)	122	88087	*Protobothrops flavoviridis*	***Chordata*** - *Viperidae*	. Habu . Trimeresurus flavoviridis
P0C7P5	BNP_PROFL	reviewed	Bradykinin-potentiating and C-type natriuretic peptides	NA	. BPP-CNP *Cleaved into 6 chains*	193	88087	*Protobothrops flavoviridis*	***Chordata*** - *Viperidae*	. Habu . Trimeresurus flavoviridis
Q3C2C2	PA21_ACAPL	reviewed	Phospholipase A2 AP-PLA2-I	EC 3.1.1.4	. Phosphatidylcholine 2-acylhydrolase (svPLA2)	159	133434	*Acanthaster planci*	***Echinodermata*** - *Acanthasteridae*	. Crown-of-thorns starfish
D6C4M3	CU96_CONCL	reviewed	Conotoxin Cl9.6	NA	. Conotoxin Cl9.6	81	1736779	*Californiconus californicus*	***Mollusca*** - *Conidae*	. California cone - Conus californicus
D2Y488	VKT1A_CONCL	reviewed	Kunitz-type serine protease inhibitor conotoxin Cal9.1a	NA	-	78	1736779	*Californiconus californicus*	***Mollusca*** - *Conidae*	. California cone . Conus californicus
D6C4J8	CUE9_CONCL	reviewed	Conotoxin Cl14.9	NA	-	78	1736779	*Californiconus californicus*	***Mollusca*** - *Conidae*	. California cone . Conus californicus
P0DPT2	CA1B_CONCT	reviewed	Alpha-conotoxin CIB [Fragment]	NA	. C1.2	41	101291	*Conus catus*	***Mollusca*** - *Conidae*	. Cat cone
V5V893	CQG3_CONFL	reviewed	Conotoxin Fla16d	NA	. Conotoxin Fla16d *Cleaved into 2 chains*	76	101302	*Conus flavidus*	***Mollusca*** - *Conidae*	. Yellow Pacific cone
P58924	CS8A_CONGE	reviewed	Sigma-conotoxin GVIIIA	NA	. Sigma-conotoxin GVIIIA	88	6491	*Conus geographus*	***Mollusca*** - *Conidae*	. Geography cone . Nubecula geographus
P0DM19	NF2_CONMR	reviewed	Conotoxin Mr15.2	NA	. Conotoxin Mr15.2 (Mr094)	92	42752	*Conus marmoreus*	***Mollusca*** - *Conidae*	. Marble cone
P0C1N5	M3G_CONMR	reviewed	Conotoxin mr3g	NA	. Conotoxin mr3g (Mr3.6)	68	42752	*Conus marmoreus*	***Mollusca*** - *Conidae*	. Marble cone

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UNIPROTKB CANDIDATE'S INFORMATION							TAXONOMY CANDIDATE'S INFORMATION		
AC	*ID*	*Status*	*Protein name*	*ENZYME EC*	*Other name(s)*	*Length (aa)*	*ID*	*Species*	*Phylum - Family*	*Organism's common name(s)*
D2DGD8	I361_CONPL	reviewed	Conotoxin Pu6.1	NA	-	83	93154	*Conus pulicarius*	***Mollusca*** *- Conidae*	. Flea-bite cone
P0C8U9	CA15_CONPL	reviewed	Alpha-conotoxin-like Pu1.5	NA	-	81	93154	*Conus pulicarius*	***Mollusca*** *- Conidae*	. Flea-bite cone
A1X8B8	CAI_CONQU	reviewed	Putative alpha-conotoxin Qc alphaL-1	NA	. QcaL-1	68	101313	*Conus quercinus*	***Mollusca*** *- Conidae*	. Oak cone
P58786	COW_CONRA	reviewed	Contryphan-R	NA	. Bromocontryphan *Cleaved into 2chains*	63	61198	*Conus radiatus*	***Mollusca*** *- Conidae*	. Rayed cone
P58811	CA1A_CONTU	reviewed	Rho-conotoxin TIA	NA	. Rho-TIA	58	6495	*Conus tulipa*	***Mollusca*** *- Conidae*	. Fish-hunting cone snail . Tulip cone
Q5K0C5	016A_CONVR	reviewed	Conotoxin 10	NA	-	79	89427	*Conus virgo*	***Mollusca*** *- Conidae*	. Virgin cone
B3FIA5	CVFA_CONVR	reviewed	Conotoxin Vi15a	NA	. Conotoxin Vi15.I	74	8765	*Conus virgo*	***Mollusca*** *- Conidae*	. Virgin cone

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was performed by means of General Processing Graphic Processing Units (GPGPU).

The MS data are available on the ZENODO platform[16] (see section *Data availability*).

Results and discussion

The presence of (oligo-)peptides characterised as toxic components of animal venoms was observed in plasma and urine samples from SARS-CoV-2 infected patients and never in plasma, urine and faecal samples from control individuals. Examples of SACI-CIMS chromatograms are reported in Figure 1 and Figure 2 (panels a and b), showing the spectra acquired by means of the LC-SACI-CIMS technology. Figure 2c and d show the spectra obtained using ESI extracted at the same retention time. SACI-CIMS give rise to higher signal intensities probably due to the low ion trap saturation.

Several (oligo-)peptides (between 70 and 115, depending on the analysed sample) matched to different animal venom proteins and toxins like conotoxins, phospholipases A2, metalloproteinases (86% of assignments have a *-log(e)* higher than 25). An overview of 36 proteins covered by the toxin-like peptides found is reported in Table 1; details of -log(e) and false discovery rates are reported in Table 2. Examples of mass spectra peptide characterization together with the peptide ion fragmentation pathways are shown in Figure 3a. All the MS/MS signal were assigned to the different N-terminal y,z (blue and purple colour) and c-terminal b,c (red and yellow colour) fragmentation series (see Figure 3b for fragmentation series details). In the defined SACI-CIMS conditions, doubly charged *m/z* ion of medium-high molecular weight peptide species are produced, allowing high identification accuracy, in line with what is already described in the literature that high identification statistical rates are achieved

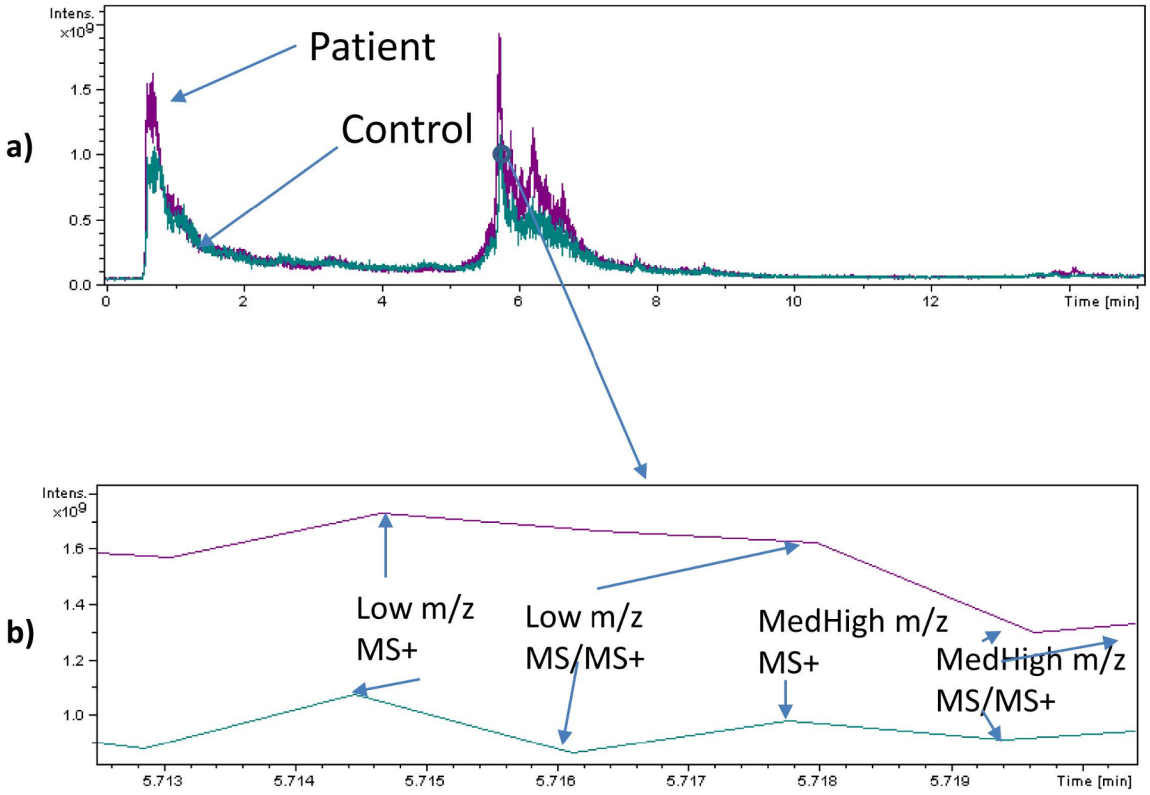


Figure 1. (**a**) Base peak LC Full Scan (MS), tandem mass (MS/MS) chromatogram of an extracted plasma sample of a patient and a control subject and (**b**) a blow-up of a specific chromatogram region (5.713–5.719 min). The blow-up shows the four regions of data acquisition: 1) Full scan mass spectrum originated by the cloud containing low *m/z* ratio molecular species; 2) Tandem mass spectra (MS/MS) mass spectrum originated by the cloud containing low *m/z* ratio molecular species; 3) Full scan mass spectrum originated by the cloud containing medium-high (MedHigh) *m/z* ratio molecular species; 4) Tandem mass spectra (MS/MS) mass spectrum originated by the cloud containing medium-high (MedHigh) *m/z* ratio molecular species.

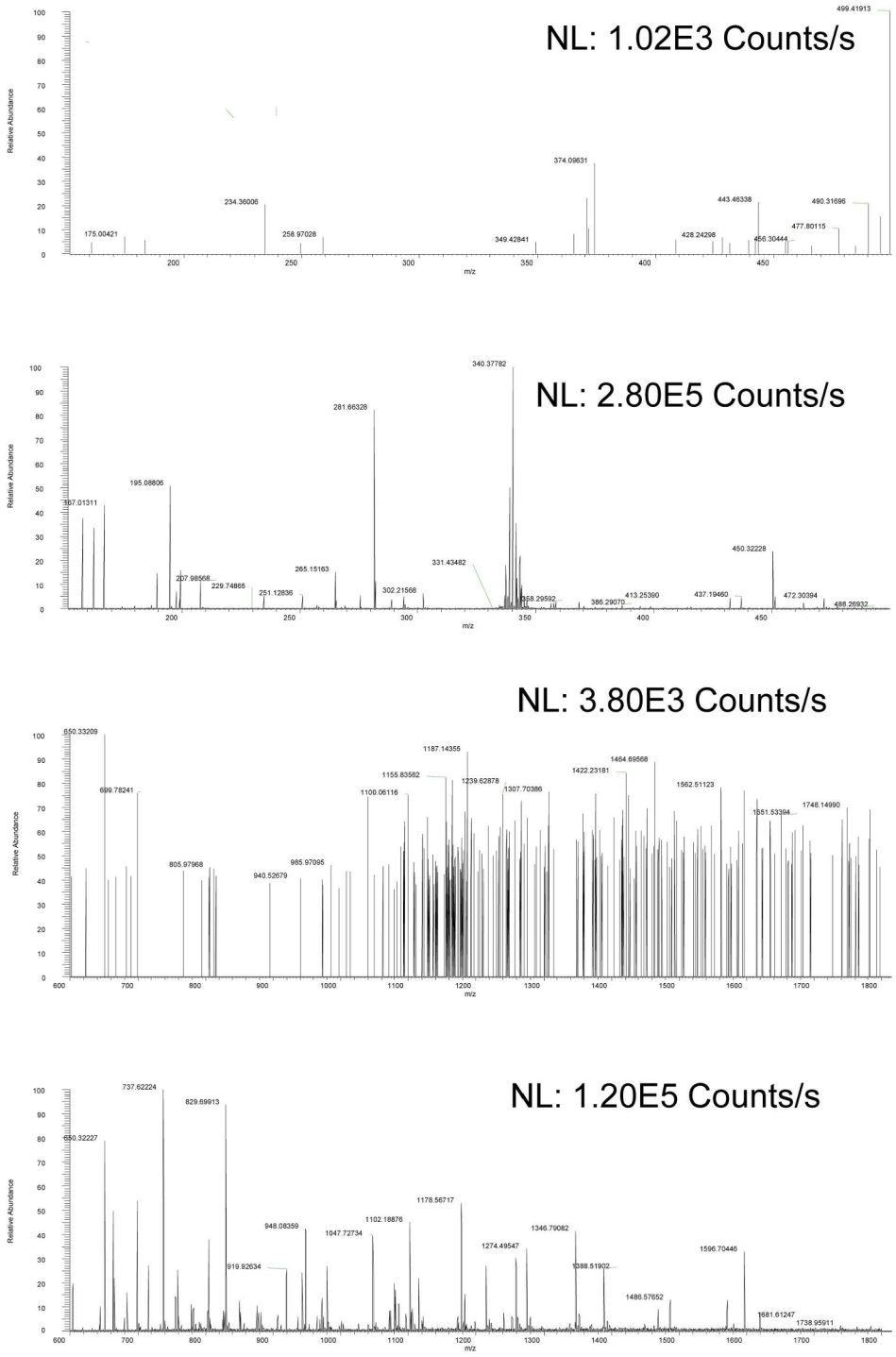
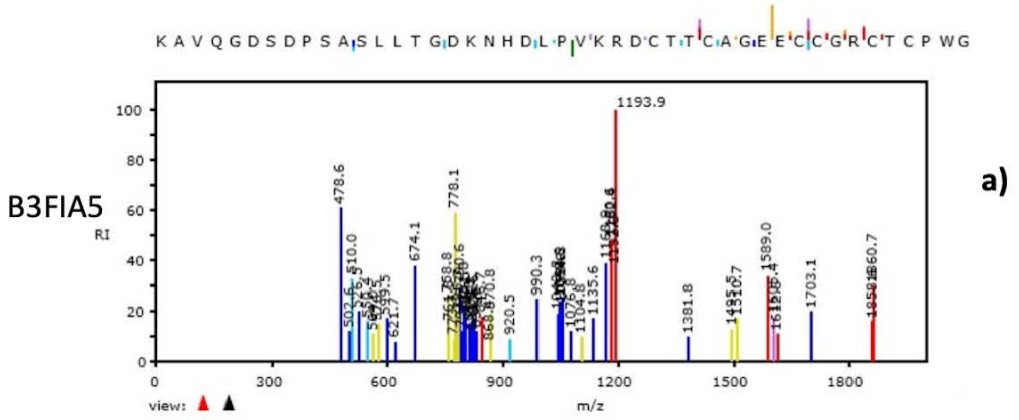


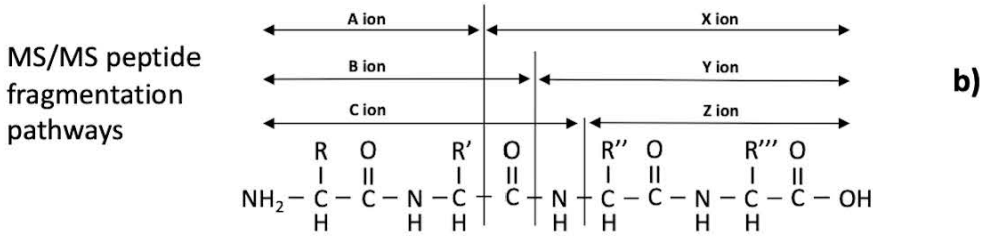
Figure 2. Examples of full scan mass spectra, obtained by analysing a COVID-19 positive urine sample and acquired focalizing solvent ion cloud species containing **a**) low, **b**) high *m/z* species extracted in the 5.713–5.719 min chromatographic region and ESI full scan mass spectrum obtained analysing the same sample and extracting the signal at the same retention time extracting **c**) low and **d**) high m/z ratio.

Table 2. List of proteins and the related -log(e) and false discovery ratio (FDR) expressed as p value.

Protein	ID	Database	-log(e)	FDR p value
Conotoxin Pu6.1	D2DGD8	Uniprot	75	0.001
Conotoxin Vi15a	B3FIA5	Uniprot	89	0.005
Putative alpha-conotoxin Qc alphaL-1	A1X8B8	Uniprot	76	0.005
Conotoxin 10	Q5K0C5	Uniprot	76	0.001
Rho-conotoxin TIA	P58811	Uniprot	54	0.001
Kunitz-type serine protease inhibitor conotoxin Cal9.1a	D2Y488	Uniprot	67	0.001
Alpha-conotoxin Pu1.5	P0C8U9	Uniprot	57	0.002
Conotoxin Fla16d	V5V893	Uniprot	67	0.003
Phospholipase A2 MALT0035C	F5CPF1	Uniprot	87	0.003
Phospholipase A2 AP-PLA2-I	Q3C2C2	Uniprot	81	0.004
Acidic phospholipase A2 PePLA2	Q2PG83	Uniprot	66	0.001
Basic phospholipase A2 BFPA	A6MEY4	Uniprot	69	0.001
Basic phospholipase A2 PL-X	P06860	Uniprot	70	0.001
Complement factor B Ba fragment	Q91900	Uniprot	74	0.001
Acidic phospholipase A2 homolog textilotoxin D chain	P23028-1	Uniprot	73	0.002
Acidic phospholipase A2 homolog textilotoxin D chain	P23028-2	Uniprot	65	0.002
Venom prothrombin activator pseutarin-C non-catalytic subunit	Q7SZN0	Uniprot	60	0.002
Coagulation factor V	Q593B6	Uniprot	61	
Venom prothrombin activator oscutarin-C non-catalytic subunit	Q58L91	Uniprot	87	0.001
Short neurotoxin 4	Q9W7J9	Uniprot	69	0.001
Conotoxin Cl9.6	D6C4M3	Uniprot	58	0.002
Zinc metalloproteinase-disintegrin-like halysase	Q8AWI5	Uniprot	57	0.003
Alpha-elapitoxin-Oh2b	P82662	Uniprot	96	0.003
Sigma-conotoxin GVIIIA	P58924	Uniprot	43	0.002
Conotoxin Mr15.2	P0DM19	Uniprot	47	0.001
Conotoxin mr3g	P0C1N5	Uniprot	74	0.001
Contryphan-R	P58786	Uniprot	58	0.002
Snake venom metalloprotease inhibitor 02D01	A8YPR6	Uniprot	43	0.002
Bradykinin-potentiating and C-type natriuretic peptides	P0C7P5	Uniprot	51	0.003
Bradykinin-potentiating and C-type natriuretic peptides	Q9PW56	Uniprot	51	0.003
Zinc metalloproteinase/ disintegrin	Q698K8	Uniprot	49	0.004

analysing peptide doubly charged species with medium high molecular weight. Different fragmentation anomalies with proton rearrangements have also been detected and considered in phase of data elaboration. Only mass spectra exhibiting a statistical *-log(e)* score higher that 10 and a false discovery rate lower than 0.05 were considered for the identification (see Figure 3c). False discovery rate and statistical score were estimated by means of reverse sequence approach.





Protein	ID	Ion Type	m/z	Database	-log(e)	FDR pvalue
Conotoxin Vi15a	B3FIA5	$[M+2H]^{2+}$	2362.53991	Uniprot	89	0.005

c)

Figure 3. Examples of mass spectra peptide characterization together with the peptide ion fragmentation pathways. Example of how MS/MS signal were assigned to the different N-terminal y,z (blue and purple colour in panel ***a***) and c-terminal b,c (red and yellow colour) fragmentation series (detailed in panel ***b***). Only mass spectra exhibiting a statistical -log(e) score higher that 10 and a false discovery rate lower than 0.05 were considered for the identification (reported in panel ***c***). False discovery rate and statistical score were estimated by means of reverse sequence approach.

Some of the toxin-like peptides found mapped on the same reference protein (UniprotKB: D2DGD8), are reported in Figure 4: these peptides were found in the five plasma samples and in the three faecal samples.

The types of toxic-like peptides found resemble known conotoxins, phospholipases A2, metalloproteinases, prothrombin activators, coagulation factors, usually present in animal venoms, which are known to have high specificity and affinity towards human ion channels, receptors, and transporters of the nervous system, like the nicotinic acetylcholine receptor.

The same results have been observed in an additional set of 10 plasma samples from 10 different patients.

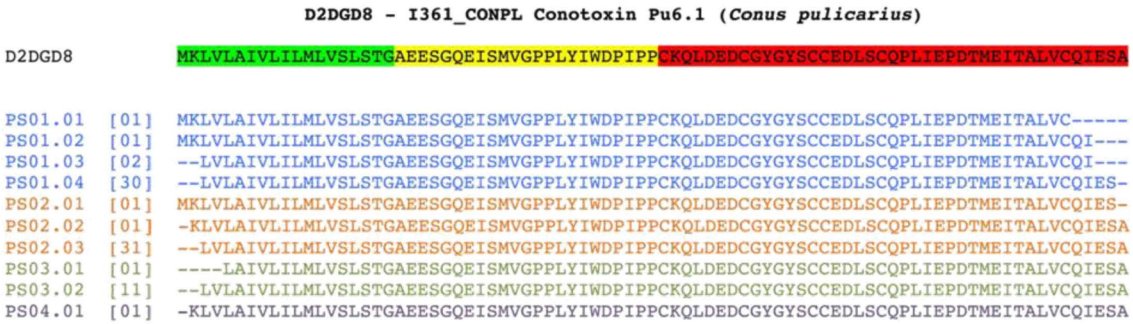


Figure 4. Alignment of toxin-like peptides to Conotoxin Pu6.1. Conotoxin Pu6.1 from *Conus pulicarius* (UniprotKB:D2DGD8) is aligned with the toxin-like peptides identified in four out of five plasma samples. Being the protein secreted and cleaved, leader-region pro-peptide and mature cysteine rich domains are highlighted in green, yellow and red, respectively. Each identified toxin-like peptide is named according to the sample of origin and its uniqueness. For each of them, the number reported in square brackets indicates the number of identical toxin-like peptides identified in the same sample.

What follows is our attempt to elaborate a potential relation between their presence and extra-pulmonary COVID-19 symptomatology.

Conotoxins

Conotoxins are neurotoxic peptides isolated from the venom of marine (genus *Conus*) cone snails. In their mature form, they consist of 10 to 30 amino acid residues, with often one or more disulphide bonds, which are used to classify them in structural classes (μ-conotoxins, ω-conotoxins, and α-conotoxins are the major classes). The mechanism of action of conotoxins is not yet fully understood[21]. Studies have found that they are able to modulate the activity of several receptors, including ion channels, nicotinic acetylcholine receptors (nAChRs) and acetylcholine-degrading enzymes (acetylcholinesterases), thus resulting in the alteration of acetylcholine levels and of cholinergic transmission[22–24]. Regarding cholinesterases, a potential association between cholinesterase levels and severity of pneumonia in COVID-19 patients has been proposed[25].

The presence of conotoxin peptides might explain the occurrence of many symptoms (like hyposmia, hypogeusia and the signs typical of Guillain-Barre syndrome) observed in some COVID-19 patients. Their presence can alter normal functioning of ion channels, nicotinic acetylcholine receptors and of acetylcholine levels.

Phospholipases A2

Phospholipases A2 (PLA_2, E.C. 3.1.1.4) hydrolyse phospholipids and lead to release of lysophosphatidic acid and arachidonic acid[26]. Arachidonic acid is a major precursor of many pro-inflammatory mediators like leukotriene, thromboxane and prostaglandin; as a consequence, abnormal presence of active PLA_2 can induce severe inflammation[27]. In animal venoms, PLA_2 act as neurotoxic proteins: they hydrolyse membrane phospholipids of the motor nerve terminal, and the plasma membrane of skeletal muscle, thus triggering a severe inflammatory degenerative response, which in turn leads to degeneration of the nerve terminal and skeletal muscle[26]. The drug dexamethasone can inhibit prostaglandins synthesis and leukotriene formation[28]. As dexamethasone is still the only therapeutic shown to be effective against the novel coronavirus in patients[29] with severe symptoms, it can be that the positive effect of this drug on COVID-19 patients is also due to the reduction of the here identified PLA_2-like peptides.

Metalloproteinases

The last example of identified toxin-like peptides is those recognised as metalloproteinases present in animal venoms, zinc-dependent enzymes of varying molecular weight having multidomain organization. These toxic enzymes cause haemorrhage, local myonecrosis, skin damage, and inflammatory reaction[30]. It has been reported that symptomatic COVID-19 patients have significantly lower zinc levels in comparison to controls and that zinc deficient patients develop more complications[31]. The presence of this specific group of toxin-like peptides, which capture zinc, can be one of the reasons for such significantly low zinc levels in symptomatic COVID-19 patients.

Similarity searches by TBLASTN[14] with relaxed parameters at the National Center for Biotechnology Information (NCBI) website (see Methods) revealed (in addition to mRNA sequences from the animal species reported in Table 1) almost identical short stretches (up to 10 amino acids) of these peptides in potential coding regions of many bacterial and viral sequences, but no long potential coding frame entirely covering any of them was found. Consequently, at the time of writing we have not yet identified the "genetic source" of these peptides, which could be:

- The SARS-CoV-2 RNA genome with its protein reading set, as proposed by Brogna[32], who reported the identification in SARS-CoV-2 RNA of many regions

encoding for oligopeptides (four–five amino acids long) identical to neurotoxin peptides typical of animal venoms.

- The SARS-CoV-2 genome directly read by bacteria, assuming that the SARS-CoV-2 genome, or parts thereof, is capable of replicating with a possible 'bacteriophage-like' mode of action, as previously described[33].

- Genomes of bacteria, which, as a reaction to the presence of the virus, secrete these peptides. This could happen by using still not well known and debated mechanisms, like alternative reading due to rRNA sequence heterogeneity (as described in 34,35), or the involvement of small bacterial ncRNA (sRNAs), known to be key players of gene regulation under conditions like stress response, quorum sensing, or virulence (in this context, in 1984 Coleman *et al.* reported the *micF* non-coding RNA as a functional bacterial sRNA[36]).

- A combination of the above e.g. the 'toxin' genetic code is present in the bacteria and expression may be triggered by SARS-CoV-2, acting like temperate bacteriophages, which are known to interact with bacteria so that they express (or not) certain genes, as described by Carey *et al.*[37].

A detailed 3D structural similarity analysis between the toxin-like peptides found and reference proteins has not yet been conducted. Accordingly, at the time of writing, we can only speculate that these toxin-like peptides are involved in the clinical extra-pulmonary manifestations in symptomatic COVID-19 patients. According to our knowledge, these toxin-like peptides have never been searched in animals considered reservoirs of SARS-CoVs.

Conclusions

The presence of (oligo-)peptides almost identical to toxic components of venoms from animals has been observed. Data and results reported here suggest an association between COVID-19 disease and the release in the body of these, and raise a series of questions:

- Are these findings in line with what was proposed by Tizabi *et al.*[38], i.e. a potential therapeutic role for nicotine, nicotinic agonists, or positive allosteric modulators of nicotinic cholinergic receptors in COVID-19?

- If induced by SARS-CoV-2, can the production of toxin-like peptides be involved in the neurological disorders and injuries observed in hospitalized COVID-19 patients?

- If induced by SARS-CoV-2, can the production of toxin-like peptides influence complex diseases apparently triggered or enhanced by COVID-19, like e.g. Guillain-Barré Syndrome[39] or Parkinson's disease[40]?

- Are toxin-like peptides associated with SARS-CoV-2 infection or to other viral infections or, more in general, is their presence related to sickness condition?

- Are our findings supporting the suggestion made by the iVAMP Consortium[41] on the relationships between animal venom glands and microorganisms' microenvironments?

We consider that the immediate sharing of these results can contribute to the untangling of the multifaceted set of clinical manifestations in symptomatic COVID-19 patients, and to the further understanding of the mechanisms involved.

Data availability

Underlying data

Uniprot: Kunitz-type serine protease inhibitor homolog beta-bungarotoxin B1 chain [*Bungarus candidus* (Malayan krait)]. Accession number Q8AY46, https://identifiers.org/uniprot:Q8AY46

Uniprot: Basic phospholipase A2 BFPA, svPLA2, EC 3.1.1.4 (Antimicrobial phospholipase A2) (Phosphatidylcholine 2-acylhydrolase) [*Bungarus fasciatus* (Banded krait) (Pseudoboa fasciata)]. Accession number A6MEY4, https://identifiers.org/Uniprot:A6MEY4

Uniprot: Phospholipase A2 MALT0035C, svPLA2, EC 3.1.1.4 [*Micrurus altirostris* (Uruguayan coral snake) (Elaps altirostris)]. Accession number F5CPF1, https://identifiers.org/Uniprot:F5CPF1

Uniprot: Zinc metalloproteinase-disintegrin-like NaMP, EC 3.4.24.- (Snake venom metalloproteinase, SVMP) [*Naja atra* (Chinese cobra)]. Accession number A8QL59, https://identifiers.org/Uniprot:A8QL59

Uniprot: Acidic phospholipase A2 D, svPLA2, EC 3.1.1.4 (APLA) (Phosphatidylcholine 2-acylhydrolase) [*Naja sputatrix* (Malayan spitting cobra) (Naja naja sputatrix)]. Accession number Q9I900, https://identifiers.org/Uniprot:A9I900

Uniprot: Venom prothrombin activator omicarin-C non-catalytic subunit, vPA (Venom coagulation factor Va-like protein) [Cleaved into: Omicarin-C non-catalytic subunit heavy chain; Omicarin-C non-catalytic subunit light chain] [*Oxyuranus microlepidotus* (Inland taipan) (Diemenia microlepidota)]. Accession number A58L90, https://identifiers.org/Uniprot:Q58L90

Uniprot: Venom prothrombin activator oscutarin-C non-catalytic subunit, vPA (Venom coagulation factor Va-like protein) [Cleaved into: Oscutarin-C non-catalytic subunit heavy chain; Oscutarin-C non-catalytic subunit light chain] [*Oxyuranus scutellatus* (Coastal taipan)]. Accession number Q58L91, https://identifiers.org/Uniprot:Q58L91

Uniprot: Short neurotoxin 4, SNTX4 (Alpha-neurotoxin 4) [*Pseudonaja textilis* (Eastern brown snake)]. Accession number Q9W7J9, https://identifiers.org/Uniprot:Q9W7J9

Uniprot: Acidic phospholipase A2 homolog textilotoxin D chain, svPLA2 homolog [*Pseudonaja textilis* (Eastern brown

snake)]. Accession number P23028, https://identifiers.org/Uniprot:P23028

Uniprot: Coagulation factor V [Cleaved into: Coagulation factor V heavy chain; Coagulation factor V light chain] [*Pseudonaja textilis* (Eastern brown snake)]. Accession number Q593B6, https://identifiers.org/Uniprot:Q593B6

Uniprot: Venom prothrombin activator pseutarin-C non-catalytic subunit, PCNS, vPA (Venom coagulation factor Va-like protein) [Cleaved into: Pseutarin-C non-catalytic subunit heavy chain; Pseutarin-C non-catalytic subunit light chain] [*Pseudonaja textilis* (Eastern brown snake)]. Accession number Q7SZN0, https://identifiers.org/Uniprot:Q7SZN0

Uniprot: Cysteine-rich venom protein ENH1, CRVP (Cysteine-rich secretory protein ENH1, CRISP-ENH1) [*Pseudoferania polylepis* (Macleay's water snake) (Enhydris polylepis)]. Accession number Q2XXQ3, https://identifiers.org/Uniprot:Q2XXQ3

Uniprot: Bradykinin-potentiating and C-type natriuretic peptides (Brain BPP-CNP, bBPP-CNP) (Evasin-CNP) [Cleaved into 12 chains] [*Bothrops jararaca* (Jararaca)]. Accession number Q9PW56, https://identifiers.org/Uniprot:Q9PW56

Uniprot: Snake venom metalloprotease inhibitor 02D01 (02E11) (10F07) (Svmpi-Eoc7) [Cleaved into 15 chains] [*Echis ocellatus* (Ocellated saw-scaled viper)]. Accession number A8YPR6, https://identifiers.org/Uniprot:A8YPR6

Uniprot: Zinc metalloproteinase/disintegrin [Cleaved into: Snake venom metalloproteinase brevilysin L4, SVMP (Snake venom metalloproteinase hxl-1, EC 3.4.24.-) ; Disintegrin brevicaudin-1a; Disintegrin brevicaudin-1b (Disintegrin adinbitor) (Disintegrin halystatin)] [Gloydius brevicaudus (Korean slamosa snake) (Agkistrodon halys brevicaudus)]. Accession number Q698K8, https://identifiers.org/Uniprot:Q698K8

Uniprot: Zinc metalloproteinase-disintegrin-like halysase, EC 3.4.24.- (Snake venom metalloproteinase, SVMP) (Vascular apoptosis-inducing protein, VAP) [*Gloydius halys* (Chinese water mocassin) (Agkistrodon halys)]. Accession number Q8AWI5, https://identifiers.org/Uniprot:Q8AWI5

Uniprot: Alpha-elapitoxin-Oh2b, Alpha-EPTX-Oh2b (Alpha-neurotoxin) (LNTX3) (Long neurotoxin OH-6A/OH-6B) (OH-3) [*Ophiophagus hannah* (King cobra) (Naja hannah)]. Accession number P82662, https://identifiers.org/Uniprot:P82662

Uniprot: Acidic phospholipase A2 PePLA2, svPLA2, EC 3.1.1.4 (Phosphatidylcholine 2-acylhydrolase) [*Protobothrops elegans* (Elegant pitviper) (Trimeresurus elegans)]. Accession number Q2PG83, https://identifiers.org/Uniprot:Q2PG83

Uniprot: Basic phospholipase A2 PL-X, svPLA2, EC 3.1.1.4 (Phosphatidylcholine 2-acylhydrolase) [*Protobothrops elegans* (Elegant pitviper) (Trimeresurus elegans)]. Accession number P06860, https://identifiers.org/Uniprot:P06860

Uniprot: Bradykinin-potentiating and C-type natriuretic peptides (BPP-CNP) [Cleaved into six chains] [*Protobothrops flavoviridis* (Habu) (Trimeresurus flavoviridis)]. Accession number P0C7P5, https://identifiers.org/Uniprot:P0C7P5

Uniprot: Phospholipase A2 AP-PLA2-I, PLA2, EC 3.1.1.4 (Phosphatidylcholine 2-acylhydrolase 2) [*Acanthaster planci* (Crown-of-thorns starfish)]. Accession number Q2C2C2, https://identifiers.org/Uniprot:Q3C2C2

Uniprot: Conotoxin Cl9.6 [*Californiconus californicus* (California cone) (Conus californicus)]. Accession number D6C4M3, https://identifiers.org/Uniprot:D6C4M3

Uniprot: Kunitz-type serine protease inhibitor conotoxin Cal9.1a [*Californiconus californicus* (California cone) (Conus californicus)]. Accession number D2Y488, https://identifiers.org/Uniprot:D2Y488

Uniprot: Conotoxin Cl14.9 [*Californiconus californicus* (California cone) (Conus californicus)]. Accession number D6C4J8, https://identifiers.org/Uniprot:D6C4J8

Uniprot: Alpha-conotoxin CIB (C1.2) [*Conus catus* (Cat cone)]. Accession number P0DPT2, https://identifiers.org/Uniprot:P0DPT2

Uniprot: Conotoxin Fla16d (Conotoxin Fla16.1) [Cleaved into: Conotoxin fla16a; Conotoxin fla16b; Conotoxin fla16c] [*Conus flavidus* (Yellow Pacific cone)], Accession number V5V893, https://identifiers.org/Uniprot:V5V893

Uniprot: Sigma-conotoxin GVIIIA [*Conus geographus* (Geography cone) (Nubecula geographus)]. Accession number P58924, https://identifiers.org/Uniprot:P58924

Uniprot: Conotoxin Mr15.2 (Mr094) [*Conus marmoreus* (Marble cone)]. Accession number P0DM19, https://identifiers.org/Uniprot:P0DM19

Uniprot: Conotoxin mr3g (Mr3.6) [*Conus marmoreus* (Marble cone)]. Accession number P0C1N5, https://identifiers.org/Uniprot: P0C1N5

Uniprot: Conotoxin Pu6.1 [*Conus pulicarius* (Flea-bitten cone)]. Accession number D2DGD8, https://identifiers.org/Uniprot:D2DGD8

Uniprot: Alpha-conotoxin-like Pu1.5 [*Conus pulicarius* (Flea-bitten cone)]. Accession number P0C8U9, https://identifiers.org/Uniprot:P0C8U9

Uniprot: Putative alpha-conotoxin Qc alphaL-1, QcaL-1 [*Conus quercinus* (Oak cone)]. Accession number A1X8B8, https://identifiers.org/Uniprot:A1X8B8

Uniprot: Contryphan-R (Bromocontryphan) [Cleaved into: [Des-Gly1]-contryphan-R] [*Conus radiatus* (Rayed cone)]. Accession number P58786, https://identifiers.org/Uniprot:P58786

Uniprot: Rho-conotoxin TIA, Rho-TIA [*Conus tulipa* (Fish-hunting cone snail) (Tulip cone)]. Accession number P58811, https://identifiers.org/Uniprot:P58811

Uniprot: Conotoxin 10 [*Conus virgo* (Virgin cone)]. Accession number Q5K0C5, https://identifiers.org/Uniprot:Q5K0C5

Uniprot: Conotoxin Vi15a (Vi15.1) [*Conus virgo* (Virgin cone)]. Accession number B3FIA5, https://identifiers.org/Uniprot:B3FIA5

Zenodo: Underlying data for 'Toxin-like peptides in plasma, urine and faecal samples from COVID-19 patients', https://doi.org/10.5281/zenodo.4903154[16]

This project contains the following underlying data:

- Data file 1: Toxins.fasta
- Data file 2: Toxins.mgf

Consent

The human biological samples used in the experimentation were collected and used with the expressed free and informed written consent of the person from whom the material was taken, according to current legislation.

Acknowledgements

The authors thank Martina Larini and Simone Madama for paper revision.

Declarations

The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication.

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Comments on this article

Version 1

Author Response 22 Jul 2021

Mauro Petrillo, European Commission, Joint Research Centre (JRC), Ispra, Italy

Dear Dr. de Bernardis,

Thanks a lot for your valuable comment.

You are perfectly right: *it can't be excluded that the findings aren't specific to COVID and that might be common to other conditions*.

And in fact, one of the questions of the Conclusions section of the manuscript is "*Are toxin-like peptides associated with SARS-CoV-2 infection or to other viral infections or, more in general, is their presence related to sickness condition?*"

The aim of our manuscript is to immediately share these observations with the scientific community as they are (together with a series of other observations which we have recently reported in https://doi.org/10.12688/f1000research.52540.3) quite unexpected, at least to us.

Thanks again for your time and interest. I am happy to further discuss, also privately.

Best regards,

Mauro Petrillo

Competing Interests: None

Reader Comment 19 Jul 2021

Ernesto de Bernardis, ASP SR, Italy

I don't understand why the Authors' hypotheses about the origin of these peptides don't include the host response during severe inflammation or ARDS, or during a pharmacological therapy similar to those given to COVID patients.

The paper compares peptides from COVID patients with those from healthy controls, but doesn't include controls with other diseases, e.g. inflammatory diseases, other viral diseases, or people treated with the same medications that were administered to their sample of COVID patients.

So, I guess it can't be excluded that the findings aren't specific to COVID and that might be common to other conditions.

Competing Interests: No competing interests were disclosed.

RESEARCH ARTICLE

REVISED Increase of SARS-CoV-2 RNA load in faecal samples prompts for rethinking of SARS-CoV-2 biology and COVID-19 epidemiology [version 3; peer review: 2 approved]

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Abstract

Background

Scientific evidence for the involvement of human microbiota in the development of COVID-19 disease has been reported recently. SARS-CoV-2 RNA presence in human faecal samples and SARS-CoV-2 activity in faeces from COVID-19 patients have been observed.

Methods

Starting from these observations, an experimental design was developed to cultivate *in vitro* faecal microbiota from infected individuals, to monitor the presence of SARS-CoV-2, and to collect data on the relationship between faecal bacteria and the virus.

Results

Our results indicate that SARS-CoV-2 replicates *in vitro* in bacterial growth medium, that the viral replication follows bacterial growth and it is influenced by the administration of specific antibiotics. SARS-CoV-2-related peptides have been detected in 30-day bacterial cultures and characterised.

Discussion

Our observations are compatible with a 'bacteriophage-like' behaviour of SARS-CoV-2, which, to our knowledge has not been observed or described before. These results are unexpected and hint towards a novel hypothesis on the biology of SARS-CoV-2 and on the COVID-19 epidemiology. The discovery of possible new modes of action of SARS-CoV-2 has far-reaching implications for the prevention and the treatment of the disease.

Open Peer Review

Reviewer Status ✓ ✓

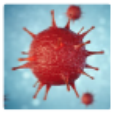
	Invited Reviewers	
	1	**2**
version 3 (revision) 01 Jul 2021	✓ report	
version 2 (revision) 23 Jun 2021	? report	✓ report
version 1 11 May 2021	? report	✓ report

1. **Kourosh Honarmand Ebrahimi**, University of Oxford, Oxford, UK

2. **Margarita Aguilera**, University of Granada, Granada, Spain

Any reports and responses or comments on the article can be found at the end of the article.

Keywords
SARS-CoV-2, COVID-19, gut microbiota



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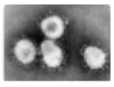

This article is included in the Coronavirus collection.

Corresponding author: Mauro Petrillo (mauro.petrillo@ec.europa.eu)

Author roles: Petrillo M: Conceptualization, Data Curation, Formal Analysis, Methodology, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Brogna C**: Conceptualization, Formal Analysis, Methodology, Resources, Supervision, Writing – Review & Editing; **Cristoni S**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Resources, Validation, Writing – Review & Editing; **Querci M**: Conceptualization, Data Curation, Methodology, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Piazza O**: Conceptualization, Writing – Review & Editing; **Van den Eede G**: Conceptualization, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED **Amendments from Version 2**

This version contains text additions within the Discussion following the suggestion and comment from Dr. Ebrahimi in his referee report on version 2.

Any further responses from the reviewers can be found at the end of the article

Introduction

Recent scientific articles and reviews[1–3] discuss the relationship between gastrointestinal microbiota and COVID-19 disease. In particular, the prolonged presence of SARS-CoV-2 RNA in human faecal samples from COVID-19 patients has been reported[4] and the potential role of orofecal transmission of SARS-CoV-2 has been examined in systematic reviews[5,6] and open evidence briefs[7,8]. Cases of SARS-CoV-2 detection in faecal samples from patient with typical symptoms of COVID-19 but negative to multiple SARS-CoV-2 real-time reverse transcription polymerase chain reaction (rRT-PCR) tests on oropharyngeal and nasopharyngeal swabs have been reported[9]. SARS-CoV-2 faecal viral activity was depicted in association with gut microbiota composition in patients with COVID-19[10], and the replicating virus was detected in faeces[11]. At the same time, Wölfel *et al.*[12] observed high viral RNA concentration in stool samples, but reported isolation of infectious virus only from throat- and lung-derived samples, while Yao *et al.*[13] had indication of viable SARS-CoV-2 particles in stool samples, denoting that the detailed biology of SARS-CoV-2 is not yet fully elucidated. Moreover, the interaction between SARS-CoV-2 and individual variable microbiota composition could drive the differential pathophysiological effects and severity of symptoms (Yeoh YH *et al.* 2021, Zuo T *et al.* 2021, Zuo T *et al.* 2020).

Our experiments further explored the relationship between COVID-19 disease and SARS-CoV-2 infected faeces to provide data relevant for pandemic understanding and disease management. The results however did not correspond with current thinking of the epidemiology of SARS-CoV-2 and, therefore, we believe a quick sharing with the scientific community of our findings is imperative.

Methods

The experimental design included:

1) the inoculation of NutriSelect™ Plus nutrient broth at 37°C, fit for the growth of more fastidious bacteria, with a faecal sample (stool) from one subject positive to SARS-CoV-2 and from a healthy individual (here called sample A and sample B, respectively) following written informed consent.

2) The assessment of the presence of SARS-CoV-2 RNA in both samples, after seven days of culture, using the Luminex technology; with confirmation of the presence of SARS-CoV-2 RNA in sample A, and of its absence in sample B.

3) Inoculation of sample B with the supernatant of sample A, obtained after centrifugation (hereafter called sample $B_{(A+)}$) and resuspension of the formed pellet (sample C).

4) Incubation of all samples (A, B, $B_{(A+)}$ and C) for 30 days under the same conditions in NutriSelect™ Plus nutrient broth at 37°C with measurement of the viral RNA load in each sample at days 1, 2, 3, 7, 14, 21, and 30, following the date of inoculation (day 0).

5) Antibiotic treatment on 18 aliquots derived from sample $B_{(A+)}$ at day 21, consisting in the addition of a specific antibiotic (each of the following: metronidazole, clindamycin, lincomycin, piperacillin+tazobactam, vancomycin, amoxicillin, ampicillin, cefixime, ceftriaxone, meropenem, rifaximin, azithromycin, erythromycin, gentamicin, ciprofloxacin, colistin, levofloxacin, and teicoplanin) to each aliquot. SARS-CoV-2 RNA load was measured by Luminex technology in each aliquot before and 3 days after antibiotic administration.

6) In parallel, additional analyses were performed to evaluate and monitor over time the bacterial growth and metabolic activity of all samples and all aliquots of sample $B_{(A+)}$, using SANIST Biotyper according to the method described by Cristoni *et al.*[14]

7) Purification and analysis of the peptides present in sample $B_{(A+)}$ at day 30.

The details of the procedures and protocols used are presented in the extended data, together with a schematic representation of the experimental design (Graphical abstract)[15].

Results

The experimental design included a series of analyses (performed on all samples A, B, $B_{(A+)}$ and C) aimed at verifying: 1) the permanence/survival over time and the eventual multiplication of SARS-CoV-2 RNA *in vitro*; 2) the presence/synthesis of SARS-CoV-2 peptides in the cultures having confirmed SARS-CoV-2 RNA presence; 3) the effect of antibiotics administration in sample $B_{(A+)}$; 4) the concomitant presence of other metabolites; and 5) the characterisation of the bacterial samples, including the verification of the presence of eukaryotic cells.

Presence of SARS-CoV-2 RNA

The results presented and discussed here, carried out over a period of 30 days, confirmed the extra-corporal multiplication of SARS-CoV-2 RNA: viral load highly increased over time in sample $B_{(A+)}$, slightly increased in sample A, decreased in sample C while, as expected, sample B was found constantly negative (Figure 1).

In order to verify the reproducibility of our results, the whole experiment was repeated independently three times using the same infected and healthy samples (with the exception that the repetition experiments were stopped at day 14 instead of day 30). The results of the SARS-CoV-2 RNA load measurements in the repetitions are reported in Figure 2, where results are depicted as the average of the measurements of the three

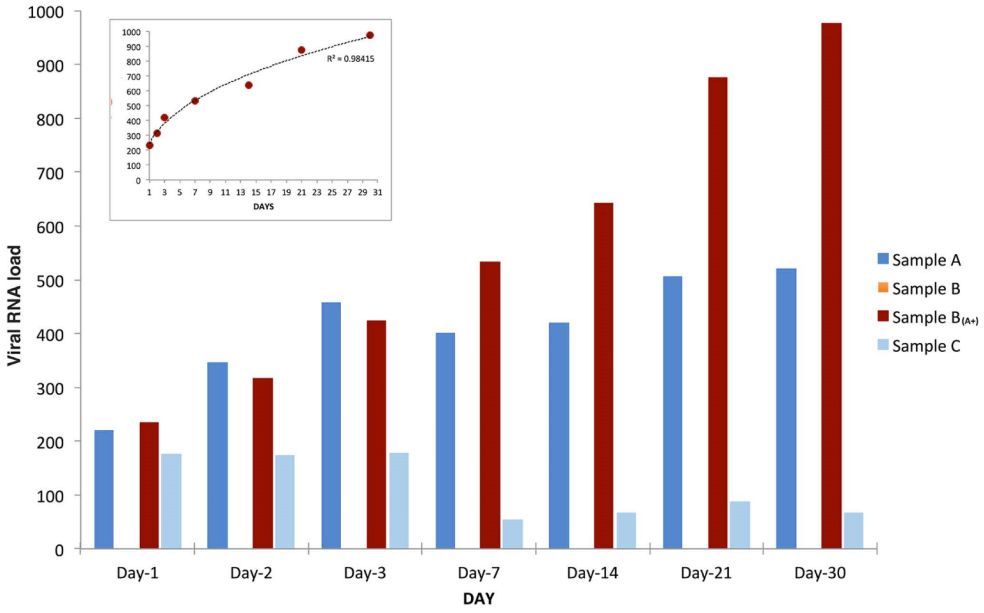

Figure 1. SARS-CoV-2 RNA load variation over time. SARS-CoV-2 RNA load measurements (reported as AU, see extended data) of samples A (blue bars), B (orange bars), $B_{(A+)}$ (red bars), and C (azure bars) grown, all under the same conditions for thirty days from inoculation (day 0). SARS-CoV-2 RNA load in sample $B_{(A+)}$ had a power increase trend over time (as shown in the small frame on top-left), slightly increased in sample A, and decreased in sample C. As expected, sample B was found constantly negative.

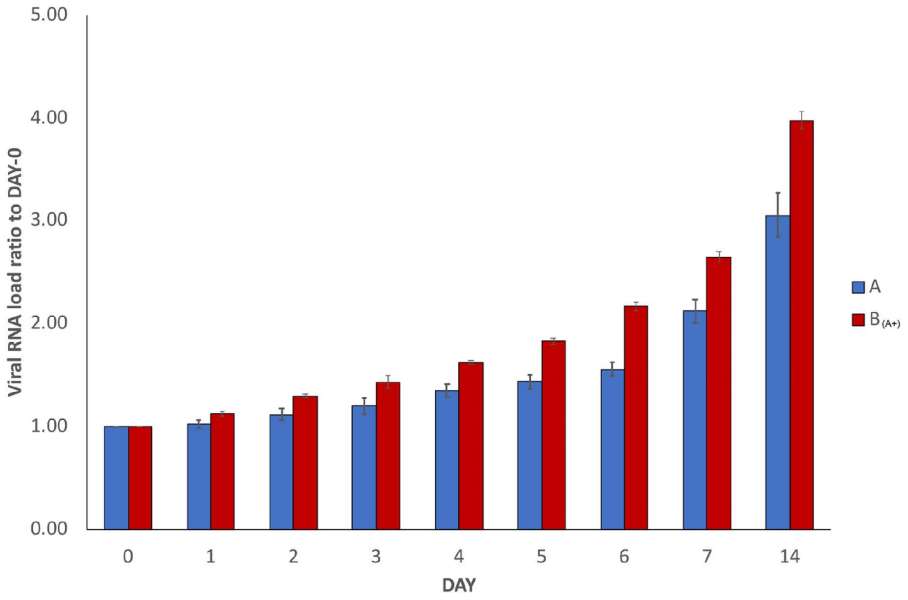

Figure 2. Average SARS-CoV-2 RNA load variation in repetitions. The graph reports average results of three repetitions of the experiment conducted using the same starting material as described in Figure 1 (with the exception that the repetitions were stopped at day 14 instead of day 30). To normalise the measurements, all values at day 0 were used as denominator (at day 0 all values = 1), *i.e.* for each sample, at day X, the ratio between LuminexCountAtDayX/LuminexCountAtDay0 was calculated. Each bar represents the average of the SARS-CoV-2 RNA load ratio of samples A (blue bars) and $B_{(A+)}$ (red bars), together with the calculated standard deviations.

repetitions, together with the calculated standard deviations. The trend was confirmed, with the increase over time of measured viral RNA load in sample A and sample $B_{(A+)}$. Decrease in sample C and no detection in sample B were also confirmed, but they are not reported in Figure 2.

In addition, three new couples of faecal samples from different "infected donors" (*i.e.* sources of A) and "healthy recipients" (i.e. sources of B) have been recruited, and subject to the same experimental procedure. Samples were collected from anonymous donors, and no information (*i.e.* age, sex, blood serotype, severity of the disease, time of the collection, fatality, *etc.*) is available. All combinations of "infected donors" sources (A1, A2 and A3) and "healthy recipients" donors (B1, B2 and B3) were subject to the same experimental procedure. Although with certain differences, the observed trends are similar (Figure 3A), confirming the increase over time of SARS-CoV-2 RNA load in samples of type A and in samples of type $B_{(A+)}$. Lastly, for each "recipient", we measured SARS-CoV-2 RNA load (Figure 3B).

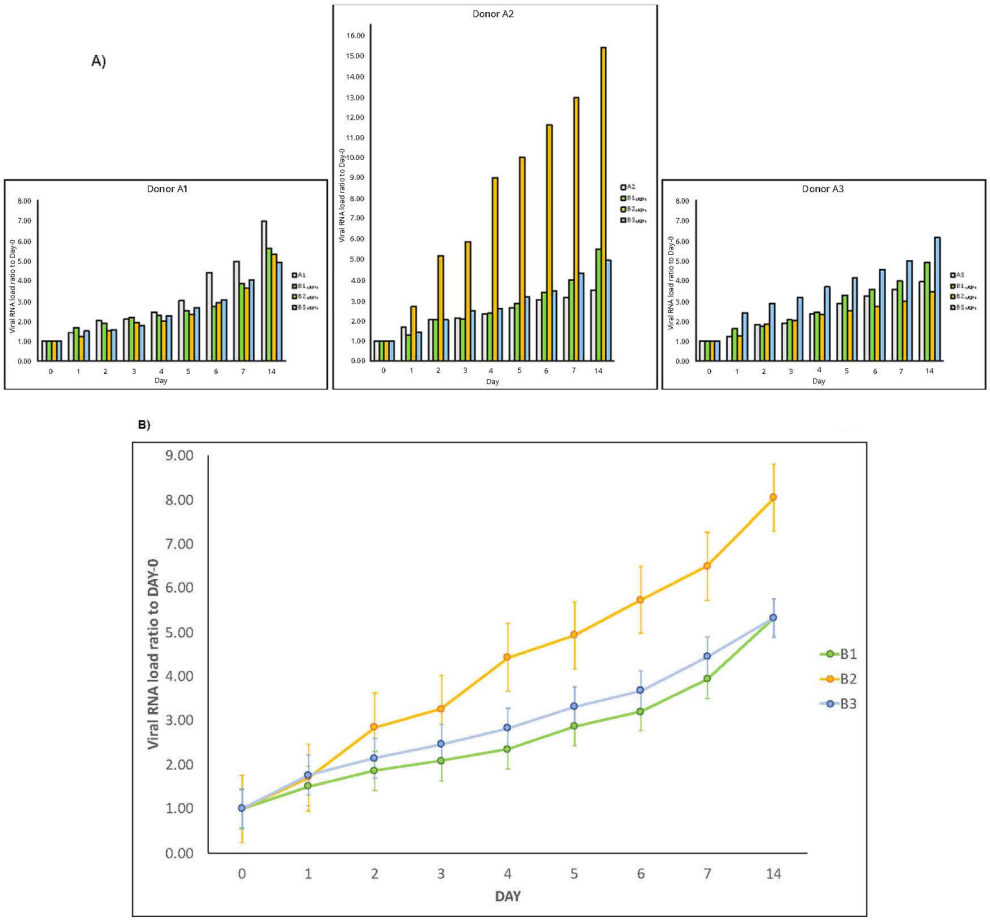

Figure 3. SARS-CoV-2 RNA load increase in different donor and recipient samples. Results of experiments combining samples from three "infected donor" sources (A1, A2 and A3) and from three "healthy recipient" sources (B1, B2 and B3). **A**) The graphs report results of nine combinations. To normalise the measurements, all values at day 0 were used as denominator (at day 0 all values = 1), i.e. for each sample, at day X, the ratio between LuminexCountAtDayX/LuminexCountAtDay0 was calculated. Each bar represents the SARS-CoV-2 RNA load ratio. Although with certain differences, the observed trends are similar, confirming the increase over time of SARS-CoV-2 RNA load in samples of type A and samples of type $B_{(A+)}$, independently from the sources of A and B. **B**) Each line represents the average of the SARS-CoV-2 RNA load ratio of samples B1 (green line), B2 (yellow line), and B3 (azure line) infected each one with three different A donor sources. To normalise the measurements, all values at day 0 were normalised as described in panel A).

Effect of antibiotics administration

Aliquots of sample $B_{(A+)}$ tested after three days of culture in the presence of the single different antibiotics belonging to different classes were analysed and the SARS-CoV-2 RNA load measured in each of them. SARS-CoV-2 RNA load was found to be influenced by the presence of antibiotics in different ways (Figure 4):

- SARS-CoV-2 RNA load was reduced to undetectable levels in the four aliquots treated with metronidazole, vancomycin, amoxicillin and azithromycin, respectively.
- SARS-CoV-2 RNA load decreased by 20% to 85% in the aliquots treated with piperallicin+tazobactam, ampicillin, cefixime, ceftriaxone, meropenem, gentamicin, ciprofloxacin and teicoplanin. For example, cefixime induced a decrease of viral RNA load of 85%, ciprofloxacin of 61% and teicoplanin of 56%.
- SARS-CoV-2 RNA load was not substantially affected by the presence of clindamycin, lincomycin, rifaximin, erythromycin, colistin and levofloxacin.

Presence of SARS-CoV-2 peptides

After 30 days of bacterial growth in culture, aliquots of samples $B_{(A+)}$ (from couple 0) were collected and tested for the presence of SARS-CoV-2-related peptides using mass spectrometry (details are described in extended data[15]). Several peptides found in the aliquot from sample $B_{(A+)}$ were assigned to SARS-CoV-2 proteins. As shown in Table 1, the sequence of some of the peptides (pep51 and pep121, matching on NSP3; pep199, matching on the spike protein; pep25 and pep68, matching on NS3 and N, respectively) have one or more amino acid (AA) changes (highlighted in red) with respect to the translations of CDS regions reported in the reference 'Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome sequence' (GenBank LOCUS: NC_045512.2). No SARS-CoV-2-related peptide was identified in the aliquot of sample B.

The identified AA changes have been checked for their existence among the observed variations in SARS-CoV-2 sequenced isolates available in GISAID[16] at time of writing. As shown in Table 2, all of them except NSP3:A274K in pep51 have

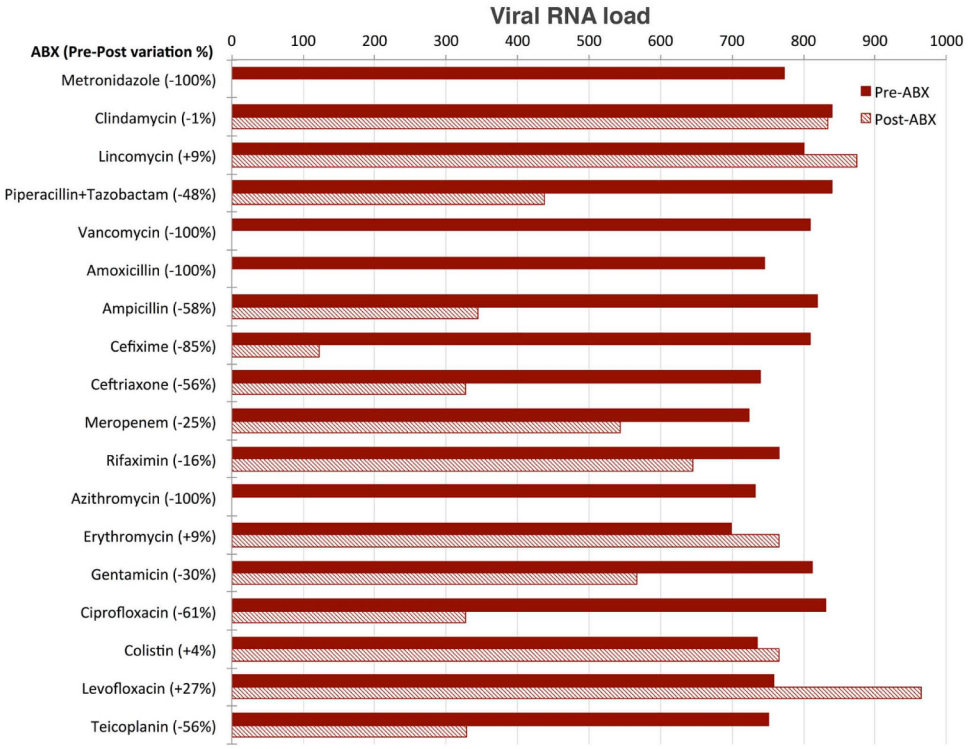

Figure 4. Effect of antibiotics on viral load. SARS-CoV-2 RNA load measurements (reported as AU, see Supplementary Material) of eighteen aliquots pre- (red) and post- (three days, dashed) treatment with the following selection of antibiotics (ABX): Metronidazole (class: Azoles); Clindamycin, Lincomycin, Piperacillin+Tazobactam, Vancomycin (class: Carboxylic acids and derivatives); Amoxicillin, Ampicillin, Cefixime, Ceftriaxone, Meropenem (class: Lactams); Rifaximin (class: Macrolactams); Azithromycin, Erythromycin, Gentamicin (class: Organooxygen compounds); Ciprofloxacin, Colistin, Levofloxacin (class: Quinolines and derivatives); Teicoplanin (semisynthetic glycopeptide antibiotic). SARS-CoV-2 RNA load is reported as preABX-postABX variation in percentage.

Table 1. Examples of 12 peptides (named as in the first column) mapping on different SARS-CoV-2 proteins (column "Match") are here reported. Amino acids highlighted in red represent changes with respect to the translations of CDS regions reported in the reference severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome sequence (GenBank LOCUS: NC_045512.2). Pep51, pep121, and pep230 were found with a different mass spectrometry approach using the Q Exactive HF Hybrid Quadrupole-Orbitrap with an ultra-high-field analyser (Brogna, personal communication).

Peptide ID	Length (AA)	Fragment	Match	From	To
pep51	27	ESDDYIKLNGPLTVGGSCLLSGHNLAK	NSP3	268	294
pep121	82	LILSVCLGSLIYSTAALGVLMSNLGMPSYCTGYREGYLNSTNVTIATYCTGSIPCSVCLSGLDSLDTYPSLETIQITISSFK		1416	1497
pep20	77	CLGSLIYSTAALGVLMSNLGMPSYCTGYREGYLNSTNVTIATYCTGSIPCSVCLSGLDSLDTYPSLETIQITISSFK		1421	1497
pep230	62	WVLNNDYYRSLPGVFCGVDAVNLLTNMFTPLIQPIGALDISASIVAGGIVAIVVTCLAYYFM	NSP4	241	302
pep199	62	TDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQGVNCTEVPVAIHADQLTPTWR	S	573	634
pep33	69	DPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQDVNCTEVPVAIHADQLTPTWSVYSTGSNVFQTR		578	646
pep190	74	SVASQSIIAYTMLLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSF		686	759
pep181	75	SIIAYTMSLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNR		691	765
pep103	31	IQDSLSSTASALGKLQDVVNQNAQALNTLVK		934	964
pep25	77	DCVVLHSYFTSDYYQLYSTQLSTDTGVEHVTFFIYNKIVDEPEEHVQIHTIDGSSGVVNPVMEPICDEPTTTTSVPL	NS3	199	275
pep68	13	GISPGRMAGNGGDAALALLLLDR	N	204	216
pep38	31	EAVGTNLPLQLGFSTGVNLVAVPTGYVDTPN	NSP14	99	129

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been already reported in humans; the majority of them have been never reported in the country of origin of the samples (Italy), the remaining ones have been observed in samples sequenced in Italy, but after the time of collection of the infected sample A (February 2020). Some of the found peptides mapping on the SARS-CoV-2 spike protein are shown in Figure 5.

Presence of other metabolites

We have already described the detection of toxin-like peptides in plasma, urine and faecal samples from COVID-19 affected individuals (Cristoni *et al.*[17], *under review*). The evaluation on the potential release of toxic-like peptides in aliquots from sample $B_{(A+)}$ has been assessed by performing the same analyses. Toxin-like peptides have been observed, but their presence was completely reduced to negligible levels in the aliquots treated with metronidazole and vancomycin administration (data not shown). These results need to be carefully interpreted, taking into account the different antimicrobials kinetics.

Presence of eukaryotic cells and virus-like particles

Samples A and $B_{(A+)}$ were found to contain some bacterial genera particularly abundant and metabolically active during the whole experiment, as shown in Figure 6.

Table 2. Amino acid changes reported in Table 1 have been checked for their existence among the observed variations in SARS-CoV-2 sequenced isolates available GISAID at time of writing. All of them, except one, have been already reported in humans, and only two in Italy. For each amino acid change, the number of occurrences in GISAID isolates is reported, together with details of the first human isolate recorded in GISAID with reported collection date. AA change NSP3:A274K of pep51 has never been reported in human SARS-CoV-2 sequences, but it has been found in beta-CoV genome sequences from bats (isolate hCoV-19/bat/Yunnan/RmYN01/2019, collection date 25-06-2019).

Peptide ID	AA change	Observed in human?	#Occurrence in human	Observed in Italy?	Observed in other than human?	First human isolate recorded in GISAID with reported collection date	Collection date
pep51	NSP3:A274K	No	0	No	Yes	-	-
pep51	NSP3:K280T	Yes	2	No	No	hCoV-19/Finland/HEL-18-471/2021	23/01/2021
pep51	NSP3:V286L	Yes	1	No	Yes	hCoV-19/USA/TX-HMH-MCoV-25096/2021	20/01/2021
pep121	NSP3:L1417I	Yes	4	No	No	hCoV-19/USA/WA-UW163/2020	13/03/2020
pep199	S:D614G	Yes	728,982	Yes	Yes	hCoV-19/Australia/NSW2153/2020	25/01/2020
pep33	S:R634S	Yes	2	No	Yes	hCoV-19/India/MH-NIV-815-3/2020	07/04/2020
pep190	S:S698L	Yes	398	Yes	No	hCoV-19/USA/AZ-TG666166/2020	25/03/2020
pep25	NS3:Y264C	Yes	106	No	No	hCoV-19/Canada/ON-S738/2020	09/04/2020
pep68	N:T205I	Yes	25,665	Yes	No	hCoV-19/Beijing/Wuhan_IME-BJ07/2020	29/01/2020
pep68	N:A208G	Yes	514	No	No	hCoV-19/USA/MD-HP00076/2020	11/03/2020

Figure 5. Peptides identified in sample $B_{(A+)}$ mapped on SARS-CoV-2 spike protein. Local alignments of peptides identified in sample $B_{(A+)}$ mapping on three different regions of SARS-CoV-2 reference spike protein (NCBI protein LOCUS YP_009724390.1). Amino acids highlighted in red correspond to changes described in Table 1 and Table 2.

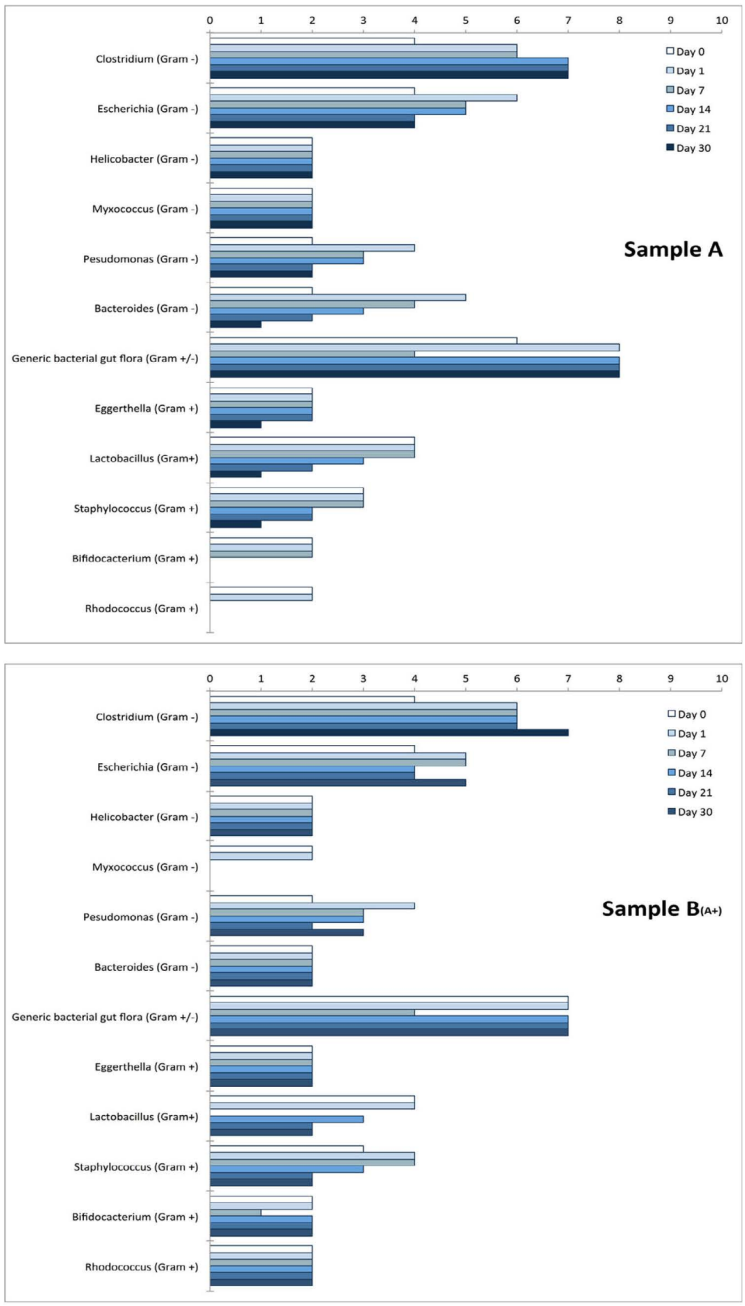

Figure 6. Bacteria genera. The presence of bacteria genera was monitored over time by looking at their metabolic activity as described by Cristoni *et al.*[14] Measures on Y-axis are reported as "detection frequency" (range 0–10). The two charts report the most metabolically active genera identified together with the "generic bacterial gut microbiota " (representing other bacterial genera not classified by the instrument) at day 0, 1, 7, 14, 21, and 30 for samples A, and $B_{(A+)}$. Other microbial organisms were observed at low levels (2 or less, at day 7) and not reported in the figure: *Mycobacterium, Actinobacteria, Bacteroidetes, Blautia, Brevibacterium, Brevundimonas, Candida (C. albicans), Collinsella, Enterococcus, Eubacterium, Klebsiella, Lactonifactor, Microbacterium, Porphyromonas, Propionibacterium, Sphingomonas, Stenotrophomonas, Streptococcus gordonii, Xanthomonas.*

Aliquots of samples A, B, and $B_{(A+)}$, collected at different times, were analysed with both transmission electron microscope (TEM) and scanning electron microscope (SEM) to verify the presence of eukaryotic cells. More than 30 different preparations (including at 30 days of culture) have been observed: none was found to contain any structure resembling cells with nuclei, but only bacterial cells. Analyses of images on samples A and $B_{(A+)}$ revealed the presence of virus-like particles interacting with bacterial cells. Immune electron microscopy is ongoing for confirming that these particles are of SARS-CoV-2 origin (*in preparation*).

Discussion

Our results indicate that the SARS-CoV-2 genome, in addition to its known interactions with eukaryotic cells, is additionally capable of replicating outside the human body, suggesting a possible 'bacteriophage-like' mode of action. It is not clear yet whether the SARS-CoV-2 genome could just be replicated by its RNA polymerase (which would correspond to a bacteriophage pseudo-lysogenic mechanism), or if the production of full-blown SARS-CoV-2 replicating particles within the bacteria occur (which would correspond to the typical lytic cycle of bacteriophages). In either case, according to our knowledge, this is a novel observation and has never described before for SARS-CoV-2.

The experiment here described (Figure 1) was repeated three additional times using the same samples (Figure 2). In addition, independent replications were performed in a 3×3 design using different starting material (Figure 3). In all of them, very similar trends were observed and the increase of SARS-CoV-2 RNA load in sample A and sample $B_{(A+)}$ was confirmed in all experiments.

In the cases of replications with different starting material (*i.e.* faecal samples from different "infected donors" as sources of A, and "healthy recipients" as sources of B), the trends are similar and confirm that the experiment is reproducible. In terms of relevance, we noticed that SARS-CoV-2 RNA load was particularly high in one combination (A2×B2). It is thus plausible that viral RNA load depends on the gut eubiotic/dysbiotic condition met by the virus. This is also a plausible explanation of why in the initial couple of A-B sources (Figure 1) the difference between viral RNA load measurements in A and B(A+) is notable. SARS-CoV-2 is considered as a respiratory virus, and many bacteria reside in the upper respiratory tract (URT) interacting with different viruses like influenza (see Schenk *et al.*, 2016 for an overview). With this respect, our observations are in line with the hypothesis formulated by Shah, who has recently proposed the existence of a gut-lung equilibrium mediated by multiple mechanisms of action, including the abundance of certain microorganisms in the gut microbiota as responsible for determining the sensitivity and severity of SARS-CoV-2 infections.

Moreover, recent reports suggest an interaction of URT microbiota with SARS-CoV-2 (Ebrahimi, 2020, Budding *et al.*, 2020). In particular, Ebrahimi identified *in silico* a series of serine protease TMPRSS2 and peptidyl peptidases with high similarity to the angiotensin-converting enzyme 2 (ACE2) receptor peptidase domain (ACE2-PD) in members of *Proteobacteria* phylum. It can't be excluded that these or other similar proteins act as the cellular receptors for SARS-CoV-2 in bacteria. At the same time, ACE2 receptor gene, whose protein is known to be critical in SARS-CoV-2 transmission[18], is expressed in various tissues and organs of the human body, including the small intestine[19]. It is thus very likely that SARS-CoV-2 found in faecal samples of infected individuals is from infections occurring in human body cells. Our observations are not in contrast, and they suggest that, in the gastrointestinal tract, human cells, like those of small intestine, are not the sole SARS-CoV-2 target. Looking for taxa, species or consortia that can be prone to act as receptor of virus is imperative, and, with this respect, a whole-genome metagenomic sequencing on the samples is ongoing, aimed at characterising further which bacterial species are candidate target(s) of the observed behaviour of SARS-CoV-2.

Whereas our experimental design was intended to grow bacterial cells, the possibility that SARS-CoV-2 RNA increase could be due to replication in human cells present in the original faecal samples, was considered. The human cells most abundantly present in faecal samples are colonic epithelial cells (colonocytes). Loktionov[17], reported that cell exfoliation events from colonic epithelium are rare under normal conditions, while they dramatically increase in cases of uncontrolled growth of cells not under physiologic control (like in neoplasia), when cell removal by apoptosis does not function properly. In addition, Iyengar *et al.*[20] reported that colonic epithelial cells terminally differentiated are devoid of proliferative activity. More recently, Nair *et al.*[21] and Chandel *et al.*[22] developed specific methodologies to recover viable colonocytes from stool. In our case, both sample A and B originated from adult individuals with no diagnosis of cancer. In addition, it is unlikely that human cells potentially present in samples A and B are able to:

- grow in a culture medium typically formulated for bacteria and not containing growth factors, serum, nor other important components for eukaryotic cell sustainment;
- survive in such a medium for 30 days, and in co-occurrence with an event of SARS-CoV-2 infection;
- multiply in the absence of specific CO_2 concentration conditions.

Also, the possibility of interaction between SARS-CoV-2 and other eukaryotic organisms potentially present in the cultures, as *e.g.* parasitic nematodes and fungal cells, has been considered.

During the whole experiment, parasitic nematodes were not noted at visual inspections by eye. In addition, stool of sample B was independently analysed and certified to be free of known

parasites and microbial pathogens (certification provided by the Italian diagnostic laboratory Biomolecular Diagnostic Srl). Parasitic nematodes are usually not able to survive outside the host and many intestinal roundworms (like those of genus *Ascaris*) release antimicrobial factors that interfere with bacterial growth[23], in contrast with the found high increase of metabolic activities of some bacterial genera. In the used medium, chemical elements relevant for (parasitic and not) nematodes (*e.g.* cholesterol and traces of metals) are missing.

The possibility of involvement of the mycobiome fraction present in the stool was considered. As highlighted by Chin *et al.*[24], multifaceted and multidisciplinary approaches are necessary to identify uncultivatable, low-abundance, permanent and transient fungal species residing in the gut, confirming that the human mycobiome is not yet fully characterised. Accordingly, while the ability of unknown fungi to grow in the used culture medium cannot be excluded, no significant metabolic activity of *Candida albicans,* most commonly found in the microbiota, was observed.

Finally, inspections of images from TEM and SEM on more than 30 different preparations did not reveal presence of eukaryotic cells (*in preparation*). If on the one hand, the possibility that a nematode or another unknown eukaryotic cell is able grow in the medium cannot be excluded, the used conditions make this possibility very unlikely. Anyhow, the ability of SARS-CoV-2 to interact either with nematodes or with fungal cells has never been observed before and would be a novel and surprising observation as well.

As indicated above, several peptides matching to SARS-CoV-2 proteins were found in the aliquot from sample $B_{(A+)}$. The identification of peptides with amino acid changes, compared to the translations of CDS regions of the reference SARS-CoV-2 genome, is intriguing but is compatible with the mechanism of viral replication in bacteria. RNA viruses such as SARS-CoV-2 inhabit the host as a population of variants called *quasispecies*, *i.e.* a group made of different variants that are genetically linked through mutation events, and contribute collectively to the characteristics of the whole (viral) population in the host[25]. Recent studies highlighted the significant amount of intra-host genomic diversity in SARS-CoV-2 samples[26,27]. In a 'bacteriophage-like' mode of action, as bacteria were grown for 30 days, it can't be excluded that the observed amino acid changes represent viral *quasispecies* emerged through replication events in bacterial hosts. In relation to this, recent studies[28–30] evidenced hypermutations occurrences in SARS-CoV-2 genomes and suggested APOBEC and ADAR deaminases as the possible responsible of these phenomena. The APOBEC family is related to bacterial, yeast, and plant deaminases all possessing highly conserved amino acid motifs responsible for coordination of zinc in the active site[31]. As no sequencing was performed on the original infected stool sample, the presence of SARS-CoV-2 haplotypes in the initial SARS-CoV-2 population used to perform the experiments, therefore justifying the amino acid changes observed, cannot be excluded. However, all the amino acid changes found have been reported in sequences of SARS-CoV-2 found for the first time after the date of collection of the infected sample A (February 2020), and some of them have never been reported in Italy, the country of origin of the samples.

On the other hand, other mechanisms like those at the basis of diversity-generating retroelements (DGR) systems[32] have that could contribute to SARS-CoV-2 hypermutation phenomena have recently been described in bacteria, and could therefore be responsible of the AA changes found. Additional experiments aimed to verify increase of viral peptides similar to viral RNAover time are planned.

These results can potentially provide new insights in the epidemiology of SARS-CoV-2. Considering the possible impact and implications that such relationship has on the manifestation, therapy and control of COVID-19 disease, some questions immediately arise like *e.g.*:

- Can this 'bacteriophage-like' behaviour of SARS-CoV-2 explain the long-term presence of SARS-CoV-2 observed in some recovered patients?[33]
- Can antibiotics and/or bacteriophage-based therapies play a role in the treatment of COVID-19 affected patients?[34]
- How would the (antecedent) administration of antibiotics to patients, influencing the microbiota population, impact the clinical course of the disease?[35]
- Can the involvement of bacteria in COVID-19 epidemiology help to explain clinical observations, like the elevated serum C-reactive protein, procalcitonin, D-dimer, and ferritin associated with poor outcomes in COVID-19?[36]

These questions are only examples of the plethora of questions to be addressed. Our results support the way to tackle COVID-19 pandemic proposed by Mushi[37], *i.e.* by using the One Health holistic approach. If individuals are considered not only human bodies, but as 'holobionts', *i.e.* discrete ecological units that need to be studied and treated as such, a deeper understanding of the role of the microbial community living in the human body is fundamental to tackle COVID-19 disease.

Consent

Faecal samples were collected and handled by CranioMed S.R.L. from anonymous donors who agreed to participate in this study by signing informed consent, as foreseen by Italian legislation. No personal information (*i.e.* age, sex, blood serotype, severity of the disease, time of the collection, fatality, *etc.*) were collected.

The study is compliant with the JRC Scientific Integrity and Research Ethics guidance.

Declarations

The scientific output expressed does not imply a policy position of the European Commission. Neither the European

Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication.

Data availability

Underlying data

Zenodo: Underlying data for 'Increase of SARS-CoV-2 RNA load in faecal samples prompts for rethinking of SARS-CoV-2 biology and COVID-19 epidemiology'. https://doi.org/10.5281/zenodo.4723549[15].

The project contains the following underlying data:

Mass spectrometry raw data of the peptides.

NCBI Genome: Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome. Accession number: NC_045512.2; https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2.

NCBI Protein: Surface glycoprotein [Severe acute respiratory syndrome coronavirus 2]. Accession number: YP_009724390.1; https://www.ncbi.nlm.nih.gov/protein/1796318598.

Extended data

Zenodo: Extended data for 'Increase of SARS-CoV-2 RNA load in faecal samples prompts for rethinking of SARS-CoV-2 biology and COVID-19 epidemiology'. https://doi.org/10.5281/zenodo.4723549[15].

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Authors' notes

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PubMed Abstract | Publisher Full Text | Free Full Text

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PubMed Abstract | Publisher Full Text | Free Full Text

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PubMed Abstract | Publisher Full Text | Free Full Text

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 3

Reviewer Report 01 July 2021

https://doi.org/10.5256/f1000research.58157.r88861

✔ **Kourosh Honarmand Ebrahimi**
Department of Chemistry, University of Oxford, Oxford, UK

I thank the authors for all their efforts. They have fully answered my concerns. I recommend the indexing of this work.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virology and immunology and bioinorganic chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 25 June 2021

https://doi.org/10.5256/f1000research.57680.r88114

? **Kourosh Honarmand Ebrahimi**
Department of Chemistry, University of Oxford, Oxford, UK

The authors have fully addressed my original concerns. I found their hypothesis very interesting, however, they should note and somewhere in the paper they mention that ACE2 receptor is expressed in other tissues like the small intestine, and what they have observed could be due to

the replication of the virus in the gastrointestinal tract tissue (Han *et al.* (2020[1])).

References

1. Han T, Kang J, Li G, Ge J, et al.: Analysis of 2019-nCoV receptor ACE2 expression in different tissues and its significance study.*Ann Transl Med*. 2020; **8** (17): 1077 PubMed Abstract | Publisher Full Text

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virology and immunology and bioinorganic chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 25 Jun 2021

Mauro Petrillo, European Commission, Joint Research Centre (JRC), Ispra, Italy

Dear Dr Kourosh Honarmand Ebrahimi,

Thanks a lot for your comment.

Your point is relevant and we modified the Discussion section accordingly:

Moreover, recent reports suggest an interaction of URT microbiota with SARS-CoV-2 (Ebrahimi, 2020, Budding et al., 2020). In particular, Ebrahimi identified in silico a series of serine protease TMPRSS2 and peptidyl peptidases with high similarity to the angiotensin-converting enzyme 2 (ACE2) receptor peptidase domain (ACE2-PD) in members of Proteobacteria phylum. It can't be excluded that these or other similar proteins act as the cellular receptors for SARS-CoV-2 in bacteria. ***At the same time, ACE2 receptor gene, whose protein is known to be critical in SARS-CoV-2 transmission [Yan 2020], is expressed in various tissues and organs of the human body, including the small intestine [Han 2020]. It is thus very likely that SARS-CoV-2 found in faecal samples of infected individuals is from infections occurring in human body cells. Our observations are not in contrast, and they suggest that, in the gastrointestinal tract, human cells, like those of small intestine, are not the sole SARS-CoV-2 target.***

We hope that the quality of the manuscript, thanks to your comment, has been improved and you consider it suitable for publication.

Best regards,

Mauro Petrillo, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Reviewer Report 25 June 2021

https://doi.org/10.5256/f1000research.57680.r88113

Margarita Aguilera
Department of Microbiology, Faculty of Pharmacy, Campus of Cartuja, University of Granada, Granada, Spain

Authors have fully improved the manuscript according to all the suggestions proposed. No further actions are needed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiota, Probiotics, taxonomy, Microbiology, Molecular biology, Biotechnology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 25 Jun 2021

Mauro Petrillo, European Commission, Joint Research Centre (JRC), Ispra, Italy

Dear Prof. Aguilera,

Thanks a lot for your valuable comments and suggestions that you provided. They surely improved the quality of the manuscript.

Best regards,

Mauro Petrillo, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 07 June 2021

https://doi.org/10.5256/f1000research.55836.r85802

Margarita Aguilera
Department of Microbiology, Faculty of Pharmacy, Campus of Cartuja, University of Granada, Granada, Spain

The work titled *"Increase of SARS-CoV-2 RNA load in faecal samples prompts for rethinking of SARS-CoV-2 biology and COVID-19 epidemiology"* has been well designed and soundly performed.

After the urgent needs to invest efforts on clinical research for handling the COVID-19, new questions should be posed and the present work has done innovatively. The hypothesis is relevant due to the high differential effects within SARS-CoV-2 patients, based on the individual health statuses that determine variable host responses. The Authors have shown experimental data to demonstrate that SARS-CoV-2 could have a bacteriophage activity through evaluating SARS-CoV-2 long-term survival, proliferation and the potential interaction between the virus and gut microbiota taxa. Multiple methodologies and experimental data support appropriately the results and conclusions: viral RNA by Luminex technology; impact of antibiotic treatments; Biotyper for cultured microbiota predominant taxa over the time, eukaryotic cells, peptides, other metabolites molecular alignments, etc.

Therefore, I consider that Petrillo *et al.* have carried out an excellent and holistic approach; **I would suggest its publication after minor corrections/explanations:**

- Please use the term gut microbiota instead gut flora along the document.
- Please add the underlined phrase at the end of this paragraph: *At the same time, Wölfel et al.12 observed high viral RNA concentration in stool samples, but reported isolation of infectious virus only from throat- and lung-derived samples, while Yao et al. had indication of viable SARS-CoV-2 particles in stool samples, denoting that the detailed biology of SARS-CoV-2 is not yet fully elucidated.* Moreover, the interaction between SARS-CoV-2 and individual variable microbiota composition could drive the differential pathophysiological effects and severity of symptoms.
- Please explain if the medium NutriSelect™Plus nutrient used for the inoculation and cultivation of microorganisms could have an impact on the specific taxa cultured as well as metabolites measured. Have you tried other media?
- Please explain if you have been used only aerobic conditions for all experimental culturing data.
- Please specify the concentration of antibiotics added to the experiment.
- Figure 1: Please give a plausible explanation for the data shown in this figure - Could it be that microbiota of healthy individuals contain eubiotic taxa that allow a better proliferation of SARS-CoV-2?
- Figure 3: Please explain the differences observed between the three graphics. The

microbiota composition of the sample receptors seems to have an impact. Please give a plausible explanation.
- Future research is directly derived from these results: to look for the taxa, species or consortia that can be prone to act as receptor of virus. Please comment on that.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiota, Probiotics, taxonomy, Microbiology, Molecular biology, Biotechnology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 07 Jun 2021

Mauro Petrillo, European Commission, Joint Research Centre (JRC), Ispra, Italy

Dear Prof. Aguilera,

Thanks a lot for your valuable comments and suggestions that you have provided in the report.

We will address all of them, and will provide a revised version of the manuscript.

Best regards,

Mauro Petrillo, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Author Response 09 Jun 2021

Mauro Petrillo, European Commission, Joint Research Centre (JRC), Ispra, Italy

Dear Dr Aguilera,

Thanks a lot for your valuable comments and suggestions that you have provided in the report.

As anticipated, we have addressed all your points, and provided a new version of the manuscript:

- *Please use the term gut microbiota instead gut flora along the document.*
- **Response:** Done, thanks.

- *Please add the underlined phrase at the end of this paragraph: 'At the same time, Wölfel et al.12 observed high viral RNA concentration in stool samples, but reported isolation of infectious virus only from throat- and lung-derived samples, while Yao et al. had indication of viable SARS-CoV-2 particles in stool samples, denoting that the detailed biology of SARS-CoV-2 is not yet fully elucidated. Moreover, the interaction between SARS-CoV-2 and individual variable microbiota composition could drive the differential pathophysiological effects and severity of symptoms.'*
- **Response:** Very relevant point. We added it, together with references.

- *Please explain if the medium NutriSelect™Plus nutrient used for the inoculation and cultivation of microorganisms could have an impact on the specific taxa cultured as well as metabolites measured. Have you tried other media?*
- *Please explain if you have been used only aerobic conditions for all experimental culturing data.*
- *Please specify the concentration of antibiotics added to the experiment.*
- **Response:** With respect to these points, the information has been added in the supplementary material, points 1, 3, 8). The new link for get the supplementary material is https://doi.org/10.5281/zenodo.4723549
- *Figure 1: Please give a plausible explanation for the data shown in this figure - Could it be that microbiota of healthy individuals contain eubiotic taxa that allow a better proliferation of SARS-CoV-2?*
- *Figure 3: Please explain the differences observed between the three graphics. The microbiota composition of the sample receptors seems to have an impact. Please give a plausible explanation.*
- *Future research is directly derived from these results: to look for the taxa, species or consortia that can be prone to act as receptor of virus. Please comment on that.*
- **Response:** Your points are relevant and we modified the Discussion section accordingly: 'In terms of relevance, we noticed that SARS-CoV-2 RNA load was particularly high in one combination (A2×B2). It is thus plausible that viral RNA load depends on the gut eubiotic/dysbiotic condition met by the virus. This is also a plausible explanation of why in the initial couple of A-B sources (Figure 1) the difference between viral RNA load measurements in A and B(A+) is notable.SARS-CoV-2 is considered as a respiratory virus, and many bacteria reside in the upper

respiratory tract (URT) interacting with different viruses like influenza (see Schenk et al., 2016 for an overview). With this respect, our observations are in line with the hypothesis formulated by Shah, who has recently proposed the existence of a gut-lung equilibrium mediated by multiple mechanisms of action, including the abundance of certain microorganisms in the gut microbiota as responsible for determining the sensitivity and severity of SARS-CoV-2 infections. Moreover, recent reports suggest an interaction of URT microbiota with SARS-CoV-2 (Ebrahimi, 2020, Budding et al., 2020). In particular, Ebrahimi identified in silico a series of serine protease TMPRSS2 and peptidyl peptidases with high similarity to the ACE2 peptidase domain (ACE2-PD) in members of Proteobacteria phylum. It can't be excluded that these or other similar proteins act as the cellular receptors for SARS-CoV-2 in bacteria. Looking for taxa, species or consortia that can be prone to act as receptor of virus is imperative, and, with this respect, a whole-genome metagenomic sequencing on the samples is ongoing, aimed at characterising further which bacterial species are candidate target(s) of the observed behaviour of SARS-CoV-2.'

We believe that, thanks to your comments, the quality of the manuscript has improved a lot.

Best regards,

Mauro Petrillo, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Reviewer Report 17 May 2021

https://doi.org/10.5256/f1000research.55836.r85393

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Kourosh Honarmand Ebrahimi
Department of Chemistry, University of Oxford, Oxford, UK

Petrillo and colleagues report that SARS-CoV-2 could have a bacteriophage activity in faecal samples. They measure the presence of viral RNA using Luminex technology and viral proteins using mass spectrometry. The experimental design is sound and their findings are exciting, which support the publication of this work. However, I have few concerns that must be addressed before publication. Therefore, I recommend publication after minor revisions.

- SARS-CoV-2 is a respiratory virus. Many bacteria reside in the upper respiratory tract (URT) and interact with different viruses like influenza (Schenk *et al.*, 2016[1]). Moreover, recent reports suggest an interaction of URT microbiota with SARS-CoV-2 (e.g. (Ebrahimi, 2020[2]); (Budding *et al.*, 2020[3])). The authors should cite these literatures and discuss their findings with respect to a similar bacteriophage behaviour of SARS-CoV-2 in URT.

- In Figure 3, the graph for donor A2: Why in B2(A2+) sample the amount of RNA is hugely different than the other samples? The authors need to explain.

- The authors suggest that the presence of SARS-CoV-2 peptides is compatible with the mechanism of viral replication in bacteria. If this is true, shouldn't the authors observe an increase in viral peptides similar to viral RNA?

References

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3. Budding A, Sieswerda E, Wintermans B, Bos M: An Age Dependent Pharyngeal Microbiota Signature Associated with SARS-CoV-2 Infection. *SSRN Electronic Journal*. 2020. Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virology and immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 May 2021

Mauro Petrillo, European Commission, Joint Research Centre (JRC), Ispra, Italy

Dear Dr Kourosh Honarmand Ebrahimi,

Thanks a lot for your valuable comments and suggestions that you have provided in the report.

We will address all of them, and wait for those of other reviewers, in order to provide a fully revised version of the manuscript.

Best regards,

Mauro Petrillo, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Author Response 09 Jun 2021

Mauro Petrillo, European Commission, Joint Research Centre (JRC), Ispra, Italy

Dear Dr Kourosh Honarmand Ebrahimi,

Thanks a lot for your valuable comments and suggestions that you have provided in the report.

As anticipated, we have addressed all your points, and provided a new version of the manuscript:

- *SARS-CoV-2 is a respiratory virus. Many bacteria reside in the upper respiratory tract (URT) and interact with different viruses like influenza (Schenk et al., 2016). Moreover, recent reports suggest an interaction of URT microbiota with SARS-CoV-2 (e.g. (Ebrahimi, 2020); (Budding et al., 2020). The authors should cite these literatures and discuss their findings with respect to a similar bacteriophage behaviour of SARS-CoV-2 in URT.*
- **Response:** Your point is relevant and we modified the Discussion section accordingly: 'SARS-CoV-2 is considered as a respiratory virus, and many bacteria reside in the upper respiratory tract (URT) interacting with different viruses like influenza (see Schenk *et al.*, 2016 for an overview). With this respect, our observations are in line with the hypothesis formulated by Shah, who has recently proposed the existence of a gut-lung equilibrium mediated by multiple mechanisms of action, including the abundance of certain microorganisms in the gut microbiota as responsible for determining the sensitivity and severity of SARS-CoV-2 infections. Moreover, recent reports suggest an interaction of URT microbiota with SARS-CoV-2 (Ebrahimi, 2020, Budding *et al.*, 2020). In particular, Ebrahimi identified in silico a series of serine protease TMPRSS2 and peptidyl peptidases with high similarity to the ACE2 peptidase domain (ACE2-PD) in members of Proteobacteria phylum. It can't be excluded that these or other similar proteins act as the cellular receptors for SARS-CoV-2 in bacteria. Looking for taxa, species or consortia that can be prone to act as receptor of virus is imperative, and, with this respect, a whole-genome metagenomic sequencing on the samples is ongoing, aimed at characterising further which bacterial species are candidate target(s) of the observed behaviour of SARS-CoV-2.'

- *In Figure 3, the graph for donor A2: Why in B2(A2+) sample the amount of RNA is hugely different than the other samples? The authors need to explain.*
- **Response:** We have modified Figure 3, by adding an additional panel. In addition, we modified the Discussion section accordingly: 'In terms of relevance, we noticed that SARS-CoV-2 RNA load was particularly high in one combination (A2×B2). It is thus plausible that viral RNA load depends on the gut eubiotic/dysbiotic condition met by the virus. This is also a plausible explanation of why in the initial couple of A-B sources (Figure 1) the difference between viral RNA load measurements in A and B(A+) is notable.'

- *The authors suggest that the presence of SARS-CoV-2 peptides is compatible with the mechanism of viral replication in bacteria. If this is true, shouldn't the authors observe an increase in viral peptides similar to viral RNA?*
- **Response:** Your point is relevant and we modified the Discussion section accordingly: 'Additional experiments aimed to verify increase of viral peptides similar to viral RNA over time are planned.'

We hope that the quality of the manuscript, thanks to your comments, has been improved and you consider it suitable for publication.

Best regards,

Mauro Petrillo, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Modern trends in animal venom research - omics and nanomaterials

Yuri N Utkin, Laboratory of Molecular Toxinology, Shemyakin Ovchinnikov Institute of Bioorganic Chemistry, 117997 Moscow, Russia

World J Biol Chem 2017 February 26; 8(1): 4-12 ISSN 1949-8454
DOI: 10.4331/wjbc.v8.i1.4

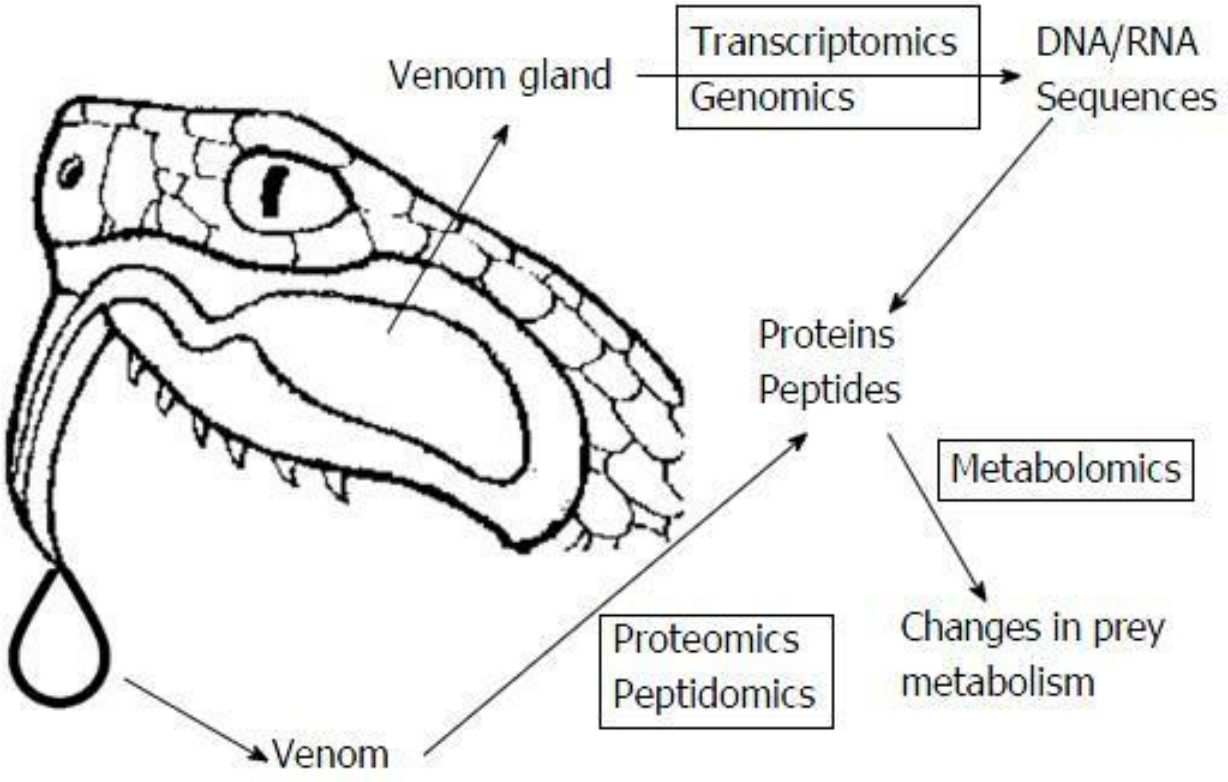
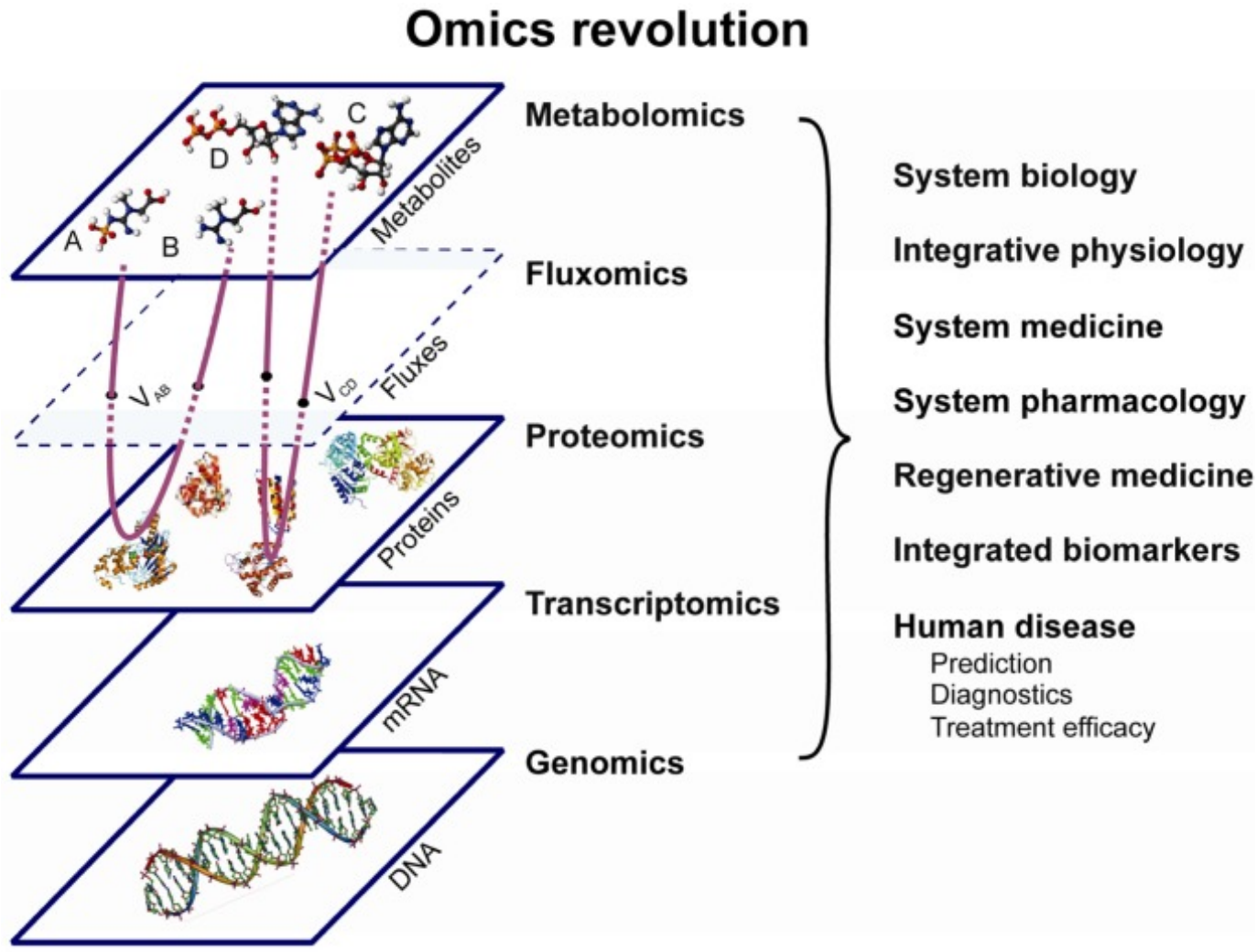


Figure 1 The relationship between "omics" technologies.

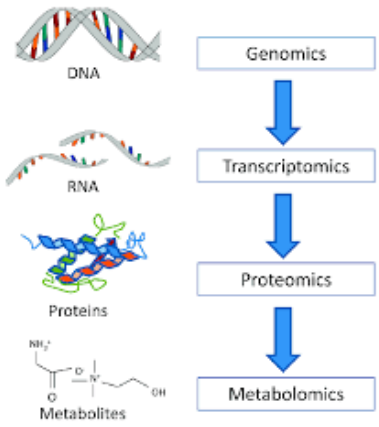
Various omics technologies: Proteomics, peptidomics, transcriptomics, genomics and metabolomics. As in other research fields, these omics technologies ushered in a revolution for venom studies, which is now entering the era of big data.



Excerpts Pages -

omics

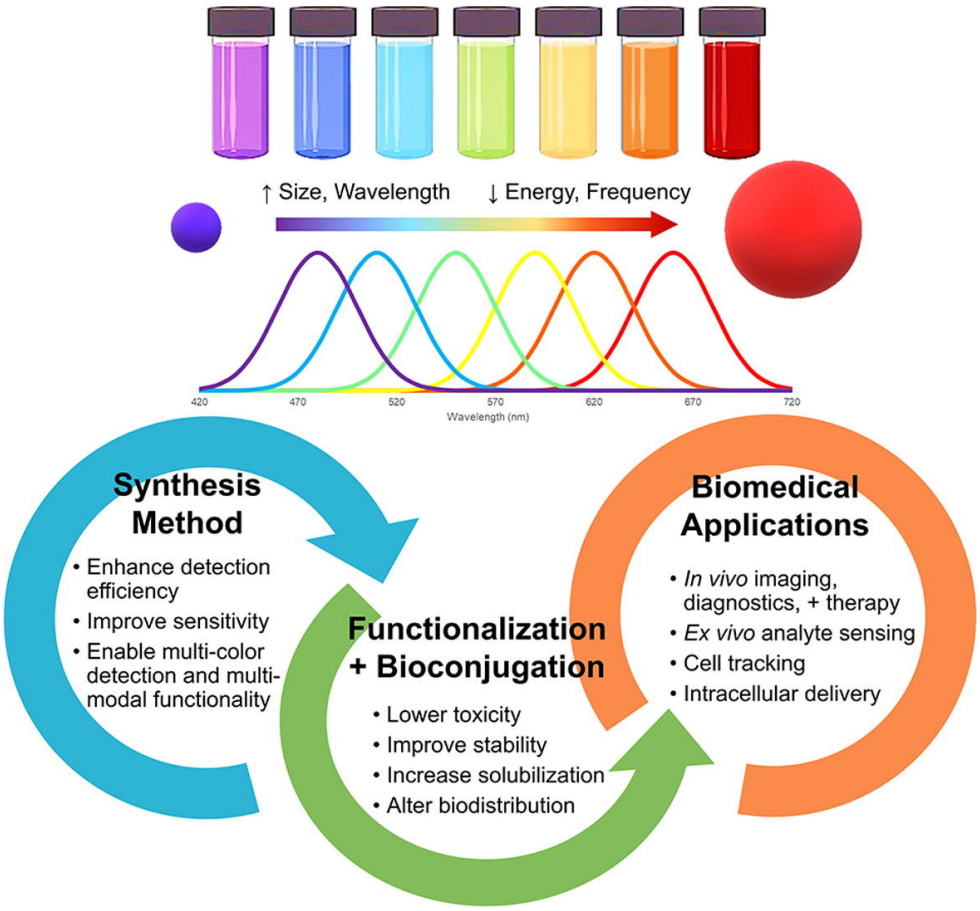
Three main categories within omics technologies are genomics, proteomics, and metabolomics/metabonomics. Genomics techniques are used to analyze the structure and function of genomes, while proteomic techniques deal with cellular and tissue-wide protein expression and metabolomics techniques are concerned with the identification and quantification of all the metabolites in a biological system. Within or in addition to these main techniques, some other omics techniques exist, such as transcriptomics, peptidomics, etc., Several omics techniques have already been applied to venom studies, resulting in more comprehensive characterization of venoms and their effects on organisms.



Most recently, venom research was revolutionized by the introduction of new mass spectrometry (MS) methods and the development of "omics" technologies, including but not limited to genomics, proteomics and metabolomics. These English-language neologisms specify fields of study in biology that deal with very large-scale data collection and analysis, in particular characterization and quantification of pools of biological molecules[1]. In molecular biology, the suffix - ome refers to a "totality of some sort" and is used to address the objects of omics studies, such as the genome, proteome or metabolome.

Nanotechnology

Nanotechnology is a very new branch of technology and developing at an extremely rapid pace. It has found application in many spheres and has not bypassed the venom studies. Nanomaterials are quite promising in medicine, and most studies combining venoms and nanomaterials are dedicated to medical applications. Conjugates of nanoparticles with venom components have been proposed for use as drugs or diagnostics. For example, nanoparticles conjugated with chlorotoxin - a toxin in scorpion venom, which has been shown to bind specifically to glioma cells - are considered as potential glioma-targeted drugs, and conjugates of neurotoxins with fluorescent semiconductor nanoparticles or quantum dots may be used to detect endogenous targets expressed in live cells. The data on application of omics and nanotechnologies in venom research are systematized concisely in this paper.



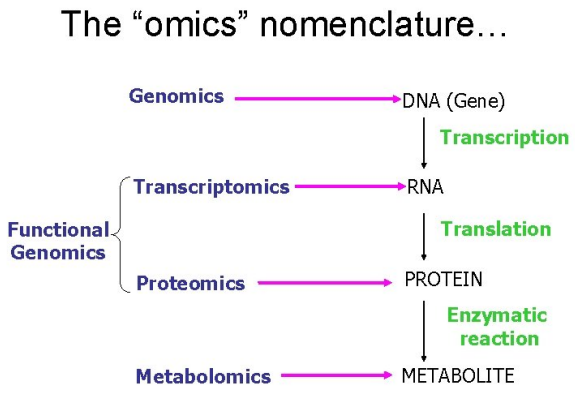
Conjugates of neurotoxins with fluorescent semiconductor nanoparticles or quantum dots

Proteomics

As was mentioned earlier in this Editorial, animal venoms are complex mixtures of different substances, but mostly proteins and peptides. In some venoms, the content of peptides is quite high; this is especially true for spider venoms[9]. The proteomic approach is also used to study peptide components of the venom. This peptide-aimed type of study was given the name "peptidomics". Thus far, peptidomics has been used mostly for peptide profiling of invertebrate venoms and its application has resulted in the discovery of several new toxins[10,11]

Genomics

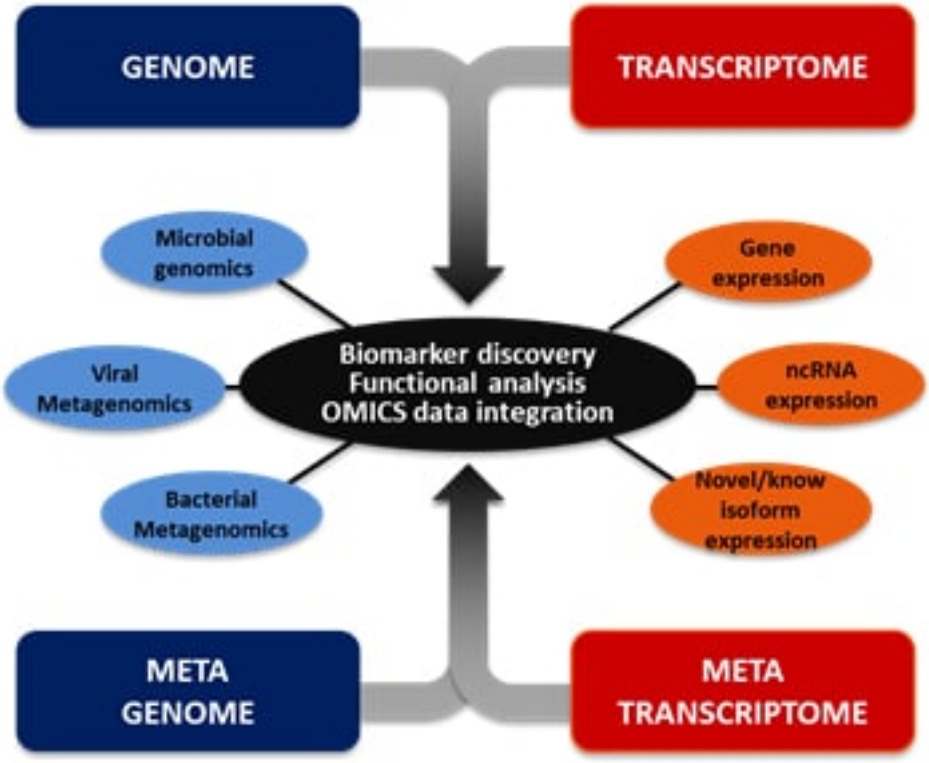
Genomics represents another versatile omics technology that is used widely in the life sciences. By definition "genomics is a discipline in genetics that applies recombinant DNA, DNA sequencing methods, and bioinformatics to sequence, assemble, and analyze the function and structure of genomes"[15]



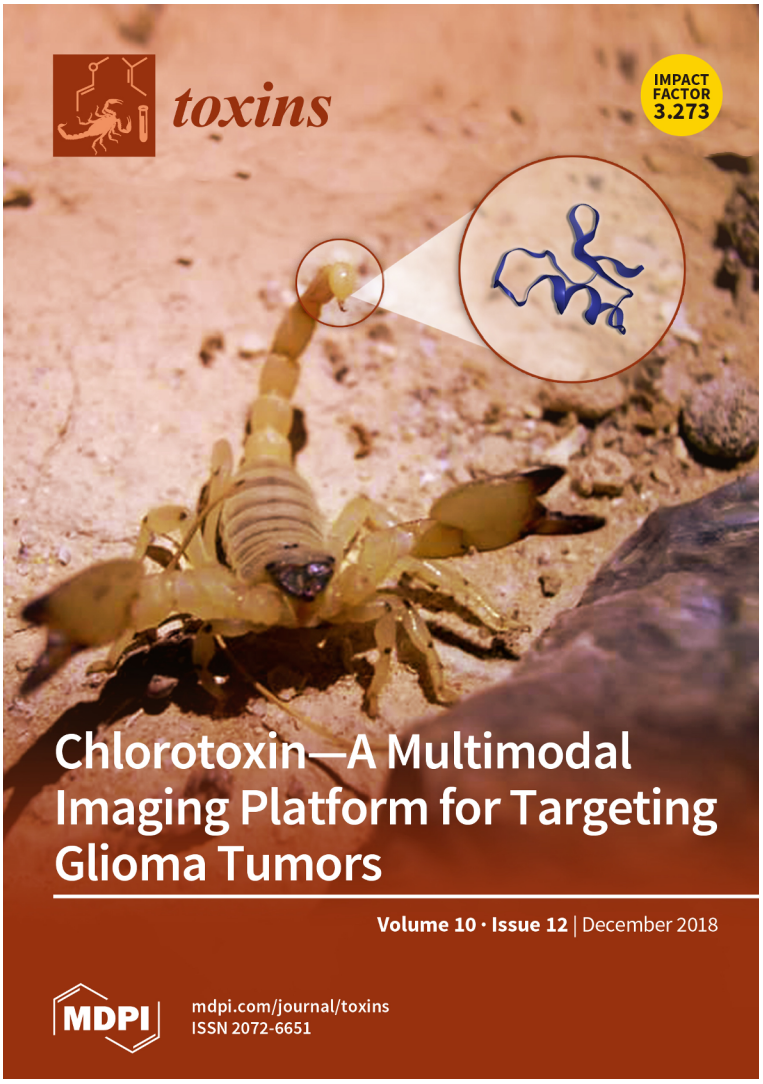
The rapid progress in sequencing methods has resulted in cost effective highthroughput sequencing (or next-generation sequencing) technologies that allow for the obtainment of millions of sequences at once. This makes the use of genomics more attractive for scientists involved in venom studies. And, according to a search of the up-to-date publicly available literature, among the snake species, the genomes of more than 10 are currently being studied[20].

Transcriptomics

Venom proteins are produced by the venom gland, wherein translation of the information encoded by messenger (m)RNA takes place. This means that the data about venom protein composition can be obtained from an array of mRNA present in the venom gland. This approach is the basis of transcriptomic studies.

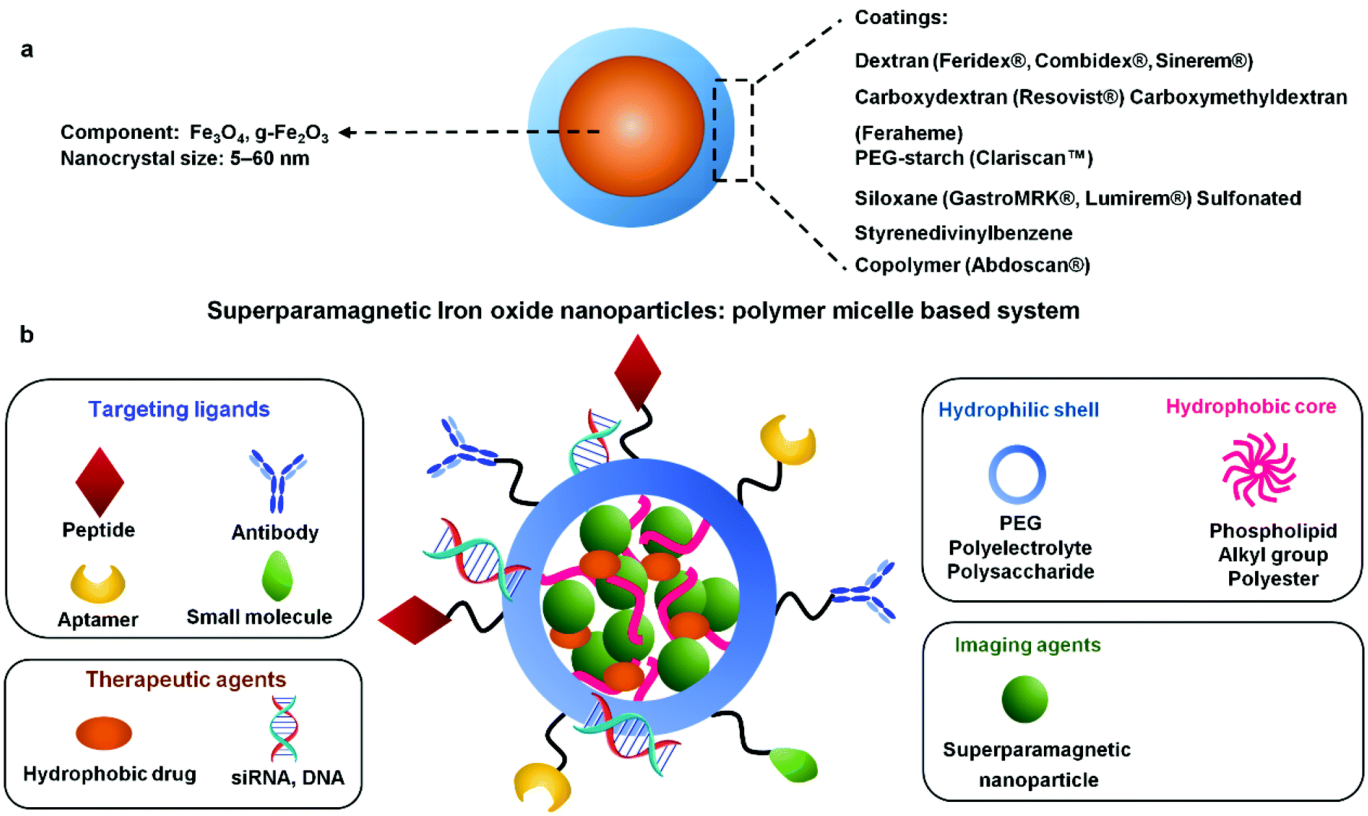


Regarded sometimes as a part of genomics, transcriptomics can be defined as the study of the expression level of mRNAs in a given cell population, often by using high-throughput techniques. Transcriptomics has been embraced by venom researchers and its application has provided valuable information on the anticipated protein array in a given venom gland under a given biological condition.



Chlorotoxin nanoconjugates - the best example of nanomaterials in venom studies

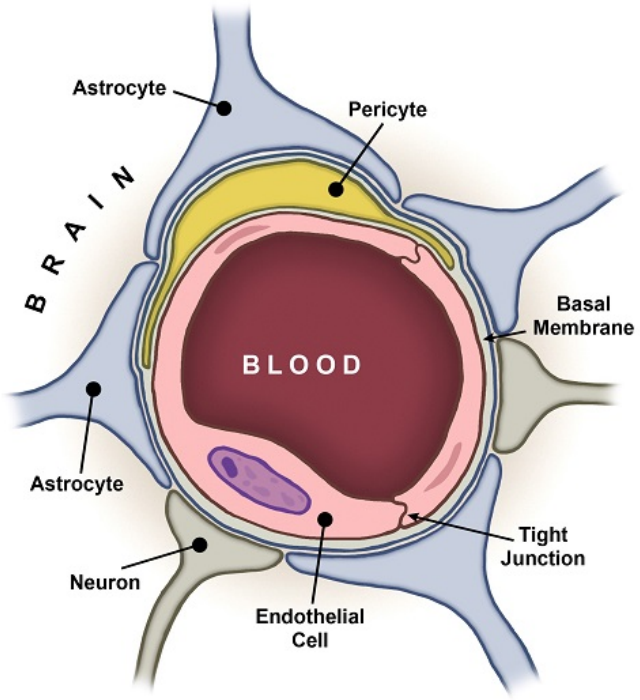
Chlorotoxin (CTX) found in the Israeli Leiurus quinquestriatus scorpion's venom binds preferentially to glioma cells, as compared with non-neoplastic cells or normal brain cells. This finding has allowed for the development of new methods for the treatment and diagnosis of several types of cancer with CTX-targeted NPs now being used fairly often for imaging and therapy of gliomas[36].



Using supermagnetic iron oxide as a nano-vector, a CTX conjugate with methotrexate was obtained[37]. This conjugate demonstrated preferential accumulation in and high cytotoxicity against glioma cells in vitro. Moreover, the prolonged retention of this NP conjugate was observed in tumor cells in vivo.

In another study, CTX conjugated with an aminefunctionalized polysilane and supermagnetic iron oxide NPs was developed[38]. As a result, there was an increased uptake of the toxin conjugate into cancer cells and the tumor invasiveness was retarded compared to cells treated with the unconjugated CTX (98% vs 45% respectively). Moreover, the CTX-conjugates deactivated membrane-bound MMP2 and caused an increase in the internalization of lipid rafts. Because of its therapeutic action, this type of conjugate is considered a possible candidate for use both in non-invasive diagnosis and in treatment of various tumors.

A CTX-conjugate with near-infrared fluorophore and iron oxide particles coated with a biocompatible polyethylene glycol-modified chitosan was obtained[39]. This conjugate was able to cross the blood-brain barrier, capable of mainly targeting brain tumor cells. The obtained compound showed no toxic properties and remained for a long time in the tumor cells.



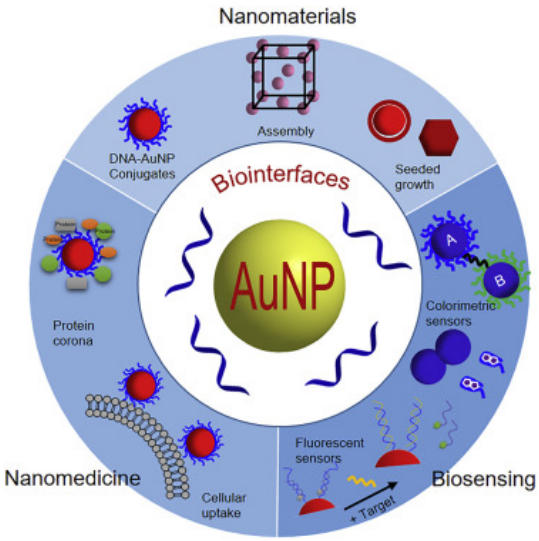
Blood-brain Barrier (BBB)

Polyethyleneglycol-mediated synthesis was used to create highly stable iron oxide NPs. These NPs were conjugated to CTX and Cy5.5 fluorescent dye of nearinfrared range. Near-infrared fluorescence imaging showed the specific accumulation of this conjugate in mice glioblastoma cells[40].

Thus, using different conjugation methods, CTX can be tethered to iron oxide NPs, QDs, and upconversion NPs for targeted imaging of gliomas. In addition, CTX nanoconjugates can also be used as carriers to deliver anticancer drugs to gliomas.

Potential adverse effects of nanomaterials

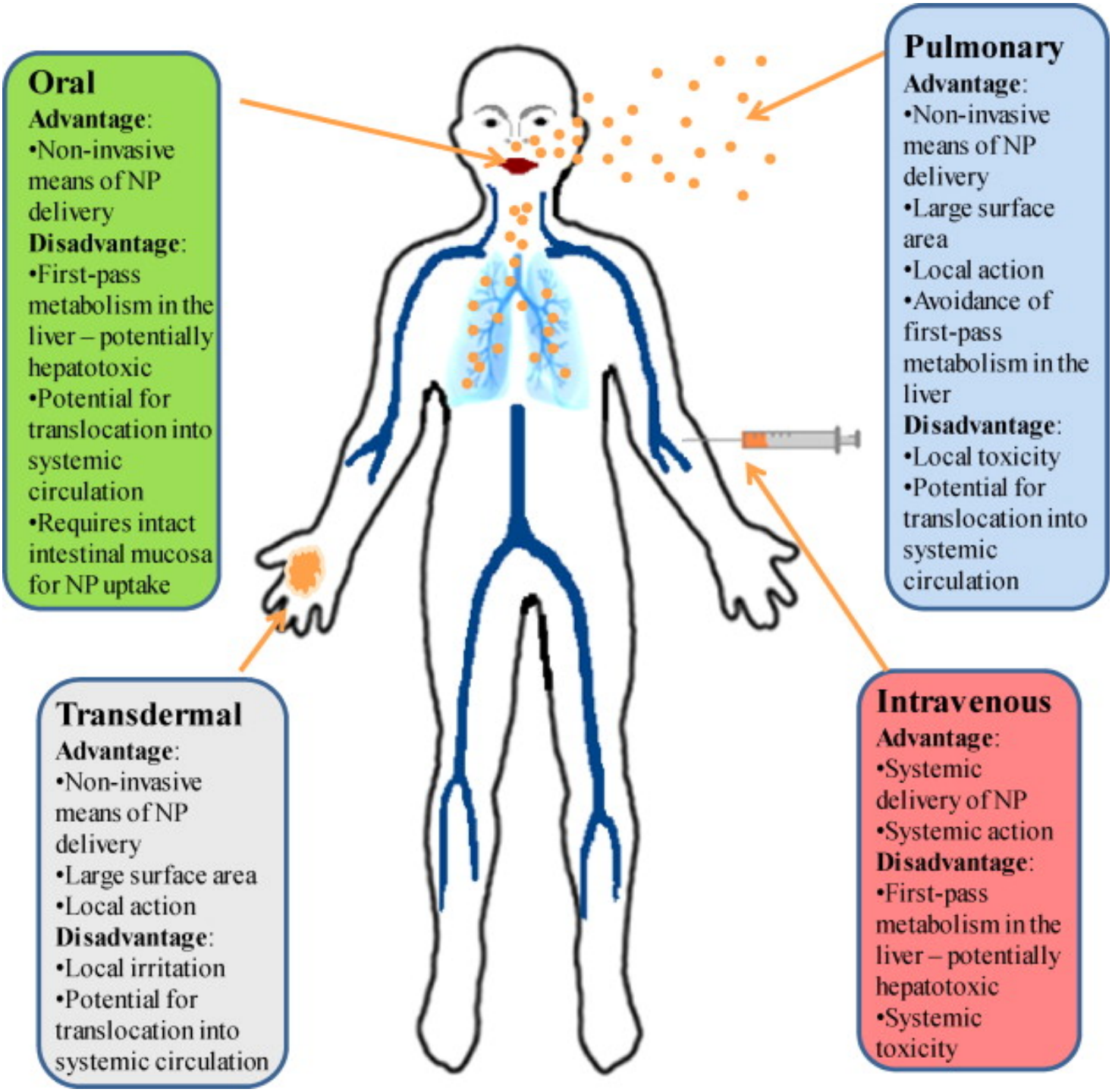
Although nanomaterials have numerous applications and possess great advantages over the traditional materials, they can have dangerous properties, which have not yet been completely studied and can cause adverse effects in humans. NPs can enter the human body through several routes, including inhalation, ingestion, skin penetration or injection. [42,43]. After entering the body, NPs can interact with different components and localize in various organs, wherein they may remain intact or be subjected to modifiction or metabolism. NPs can cross cell boundaries, accumulating within the cells.



Gold Nanoparticles (AuNP)

Once in the cell, they may bind to DNA or proteins and interfere with normal cell functions or trigger an inflammatory response. The production of excess ROS[44], including free radicals, which can cause oxidative stress, inflammation and other cellular damage, is one of the main known toxicity mechanisms of NPs. Similar to the toxicity of the NPs' parent bulk materials, the NPs themselves have toxicity that is determined by their chemical composition; however, size, surface chemistry, shape, and/or surface smoothness or roughness may enhance the toxicity profile of an NP, and all of these features can be altered substantially.

While some negative NP effects have become understandable through detailed research, considerable efforts are still needed to study the physiological effects of acute and chronic exposure to NPs. Concerning the safety of NP conjugates with venoms or toxins, their application in vivo should be carried out with great care, keeping in mind the toxicity of starting materials.



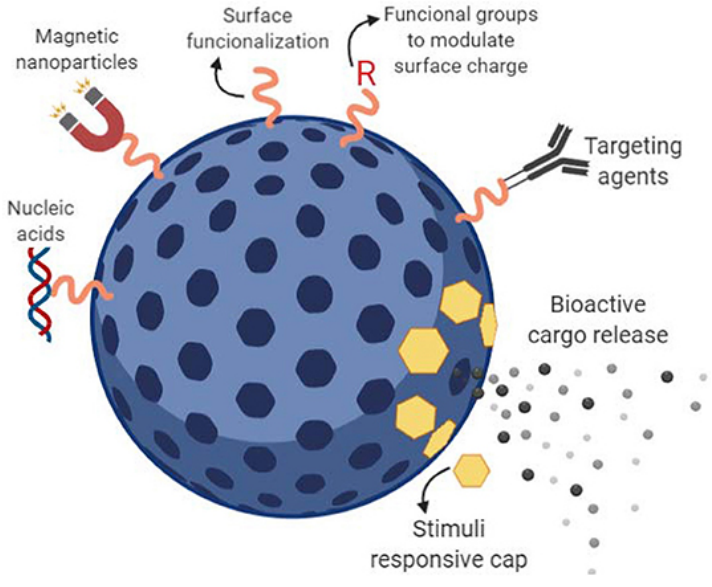
Peptides for specific intracellular delivery and targeting of nanoparticles: implications for developing nanoparticle-mediated drug delivery

James B Delehanty, Kelly Boeneman, Christopher E Bradburne, Kelly Robertson, Jason E Bongard & Igor L Medintz

15 Sep 2010https://doi.org/10.4155/tde.10.27

Introduction

The use of peptides to mediate the delivery and uptake of nanoparticle (NP) materials by mammalian cells has grown significantly over the past 10 years. This area of research has important implications for the development of new therapeutic materials and for the emerging field of NP-mediated drug delivery. In this review, we highlight recent advances in the delivery of various NPs by some of the more commonly employed cellular delivery peptides and discuss important related factors such as NP–peptide bioconjugation, uptake efficiency, intracellular fate and toxicity. We also highlight various demonstrations of therapeutic applications of NP–peptide conjugates where appropriate. The paper concludes with a brief forward-looking perspective discussing what can be expected as this field develops in the coming years.



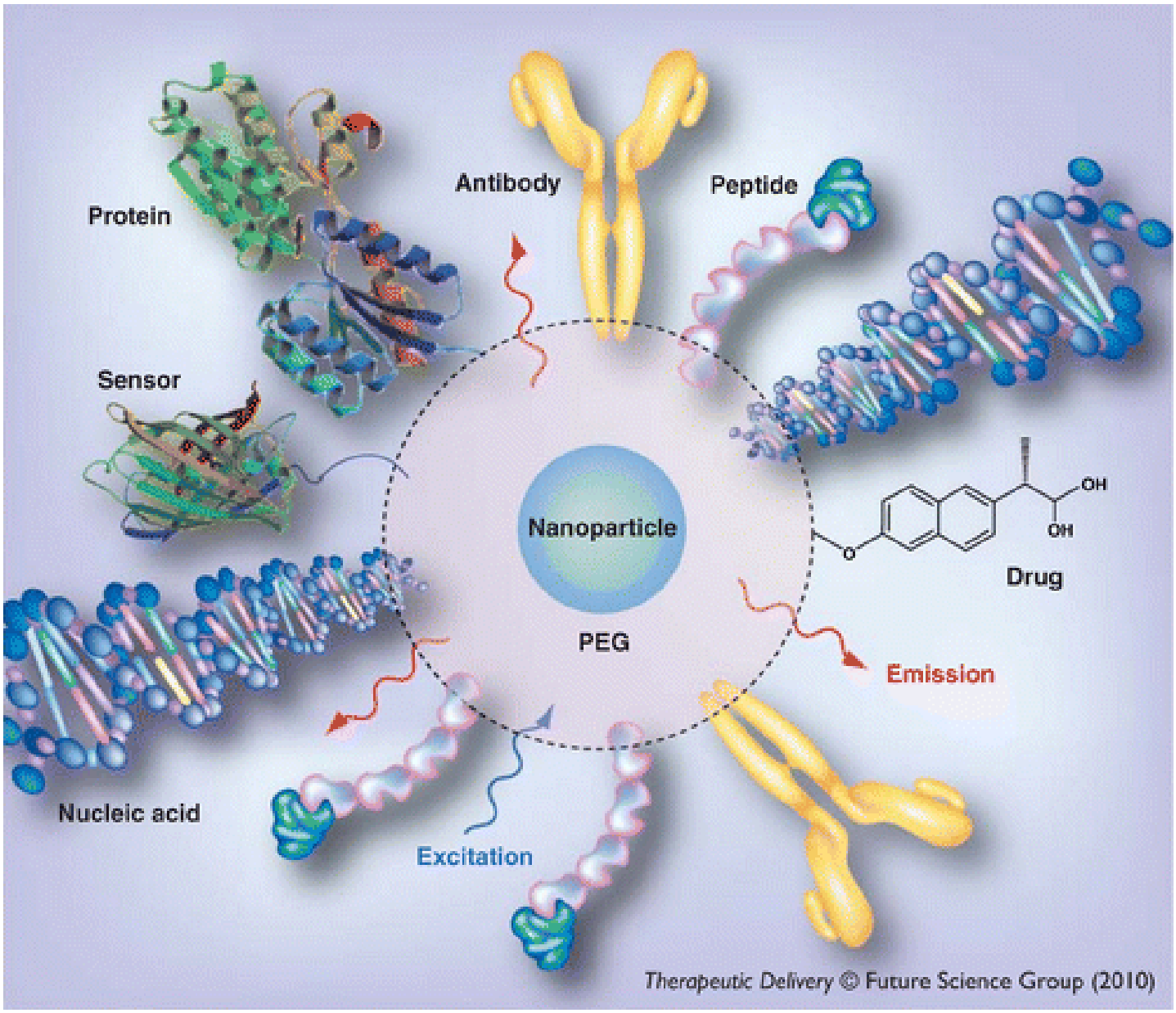
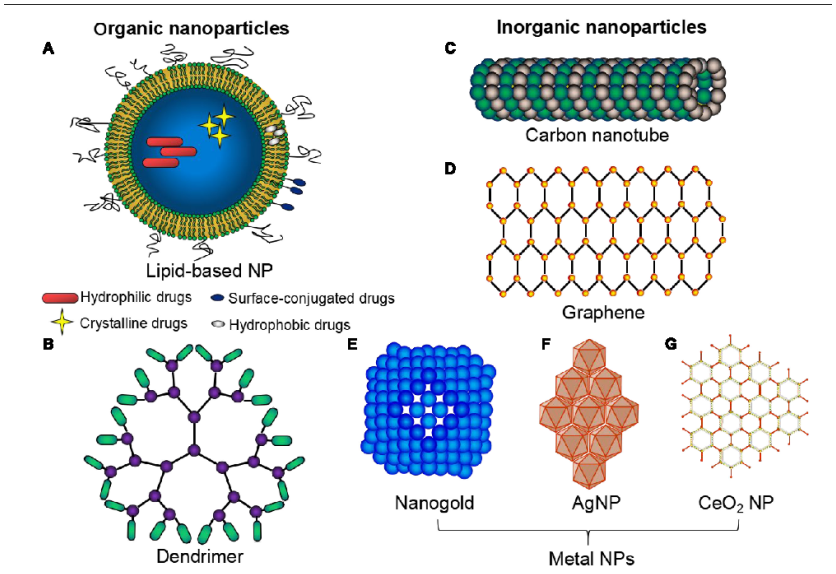


Figure 1. Bioconjugation of peptides to nanoparticles. Multifunctional nanoparticle assembly. Shown is a representative nanoparticle decorated with multiple disparate functional molecules (e.g., nucleic acids, proteins, drugs and peptides). Robust conjugation of biomolecules to the nanoparticle surface is critical for the development of 'value-added' constructs that can serve multiple functions within one nanoparticle platform. PEG: Polyethylene glycol.

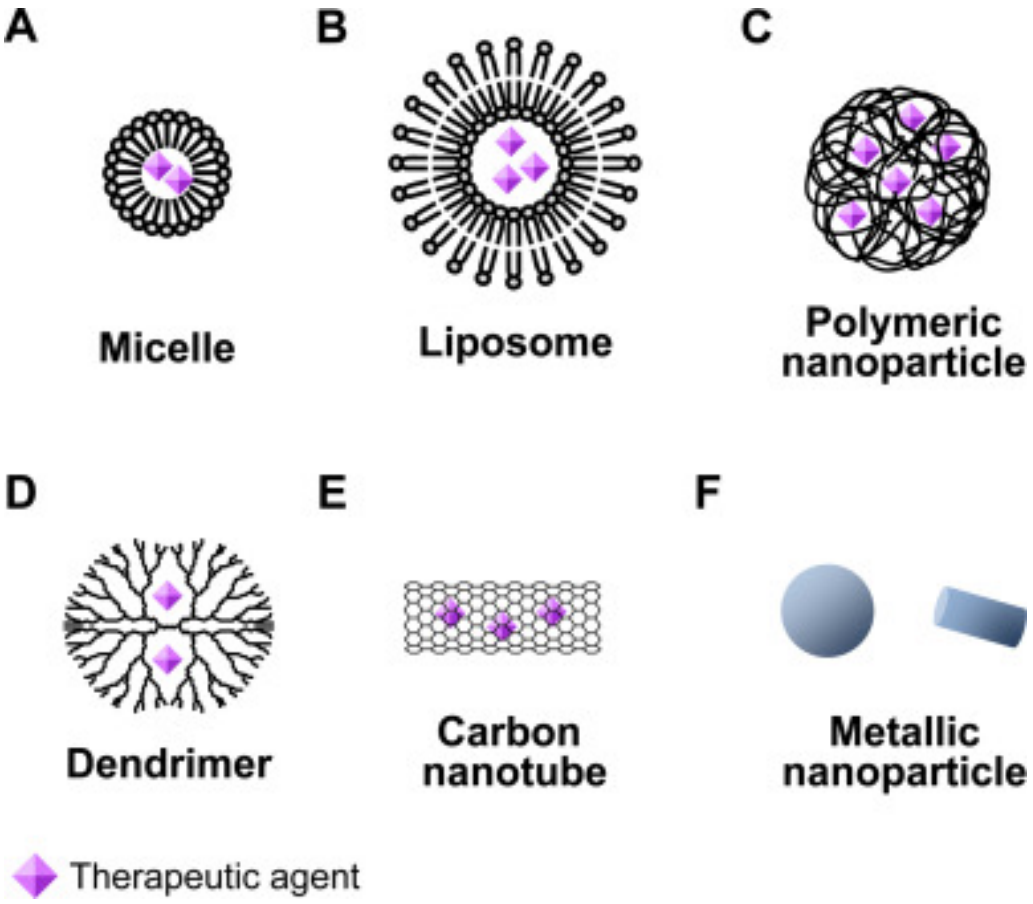
Excerpts Pages -

Nanoparticles in therapeutic applications. Therapeutic delivery & the role of nanoparticles The goal of pharmaceutical research has traditionally focused on the development of new drug formulations and novel therapeutic compounds to treat an array of diseases. Coupled with this pursuit is the need to develop effective delivery modalities.



For example, many drugs are compatible with only a limited number of delivery methods and are typically designed for systemic delivery, where they are susceptible to metabolic breakdown [1–3]. Furthermore, systemic delivery requires high dosage levels as the drugs are distributed and partitioned throughout the body. As a result, systemically administered drugs are often hampered by nonspecific toxicity and side effects in nontargeted cells and tissues, often limiting the number of doses that can be administered to patients. Thus, the need to identify more specific and targeted delivery modalities to increase the therapeutic indexes of drugs remains a key roadblock in the development of the next generation of therapeutics [4].

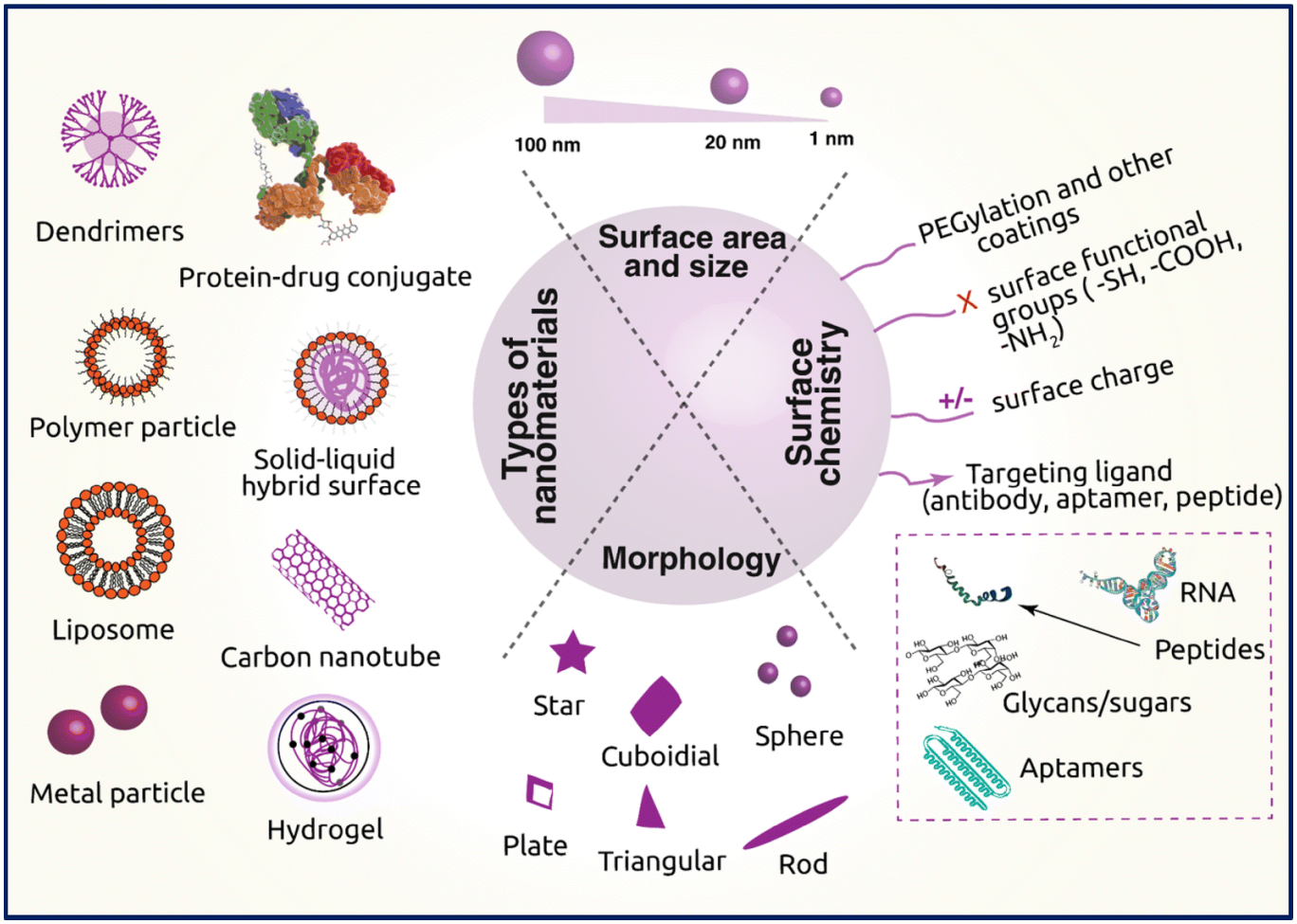
It is against this backdrop that the burgeoning field of nanoparticle (NP)-mediated drug delivery (NMDD) has emerged, to potentially address a number of the critical issues facing the delivery of pharmaceuticals.



Nanoparticles encompass a class of materials broadly defined as being 100 nm or less in size and span an array of compositions including metals, semiconductor quantum dots (QDs), oxides, polymers, vesicles (e.g., micelles/liposomes), carbon-based materials (e.g., nanotubes, fullerenes and nanodiamonds) and protein- and nucleic acid-based particles.

Examples of these materials and some of their unique properties and potential therapeutic properties are listed in Table 1. NPs also possess a number of physical attributes that make them attractive for use in therapeutic and biomedical applications.

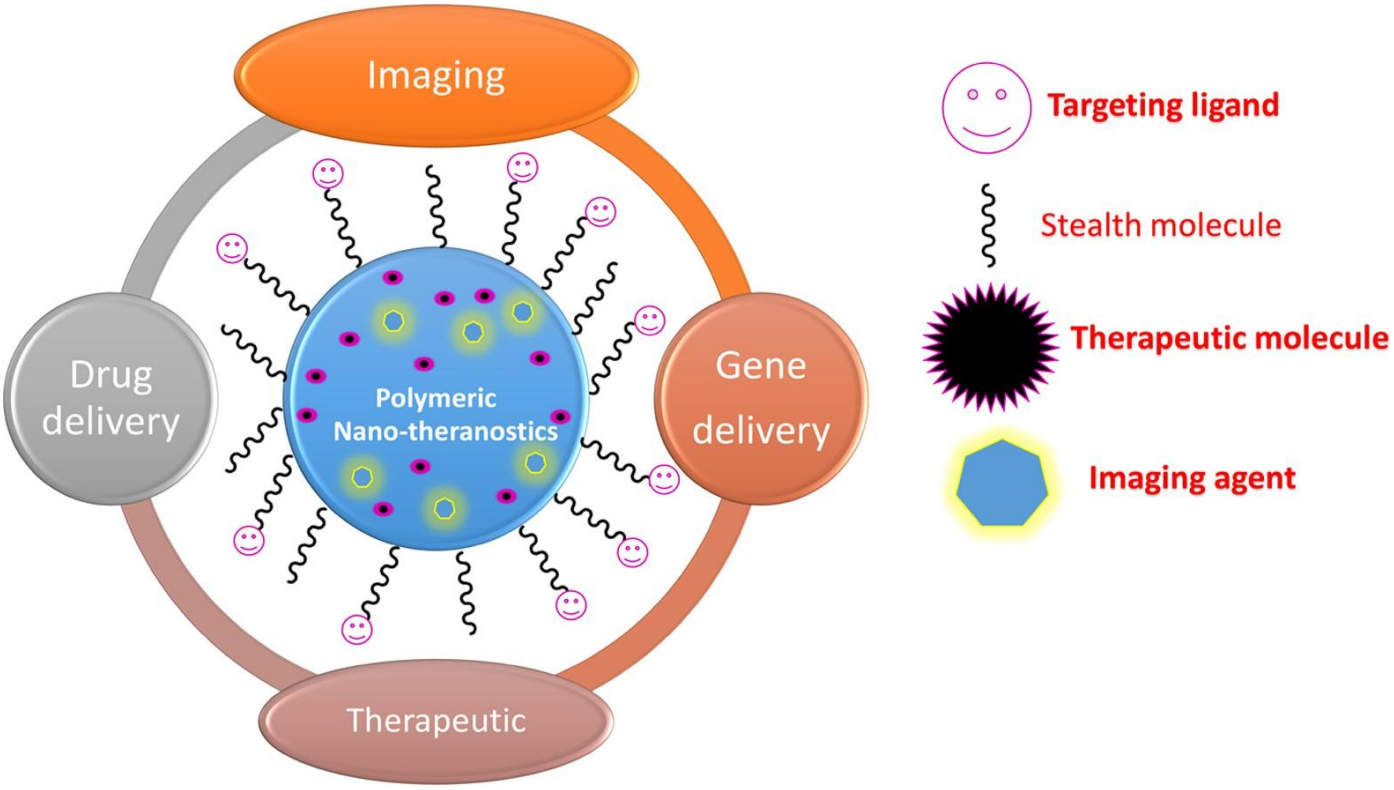
Their small size allows them to gain access to areas that are otherwise not reachable by other materials (e.g., the blood– brain barrier [BBB], the CNS, the GI tract, capillaries and the lymphatic system).



Nanoparticles

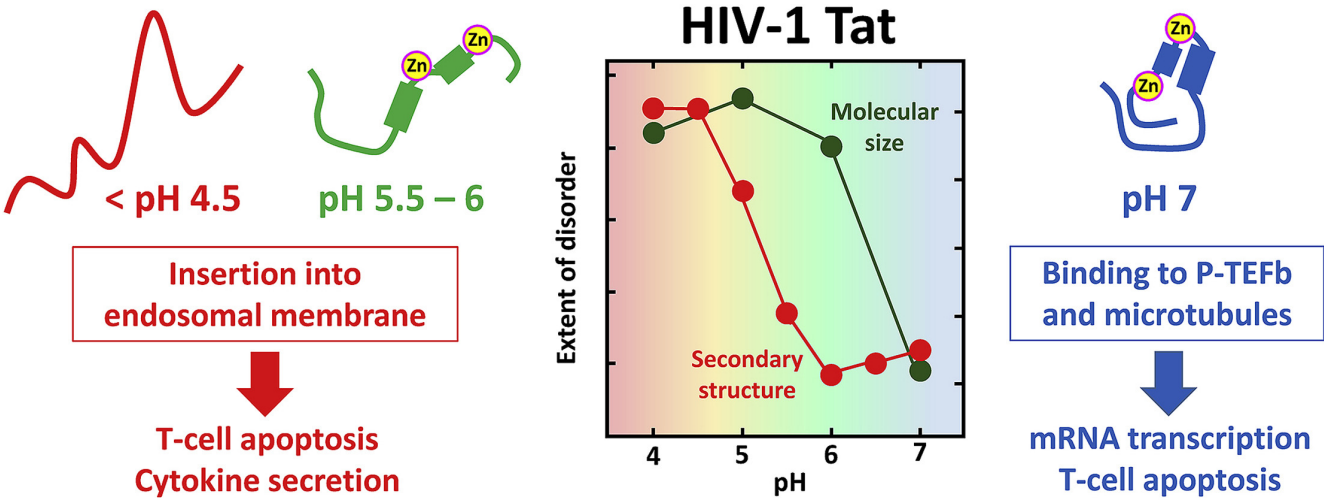
Second, their high surface-to-volume ratio affords them the ability to be decorated with a large cargo 'payload' on a relatively small frame; large numbers of cargo molecules (e.g., drugs and labels) can be loaded onto just a few particles.

Perhaps the concept driving the most interest in this area is the potential role of NPs in the development of 'theranostic' materials; materials that incorporate both a diagnostic and a therapeutic capability into a single species. This arises from NPs' unique size combined with their ability to be 'loaded' with multiple disparate functional moieties (Figure 1).



When these NPs are then further conjugated to small targeting peptides (typically <40 amino acids), 'value-added' constructs that are capable of far more than each individual component can be realized. Select examples from the literature have already demonstrated the feasibility of generating hybrid NP–peptide constructs in which the peptide adds a critical new function not inherently possessed by the NP (e.g., sensing and homing to cells).

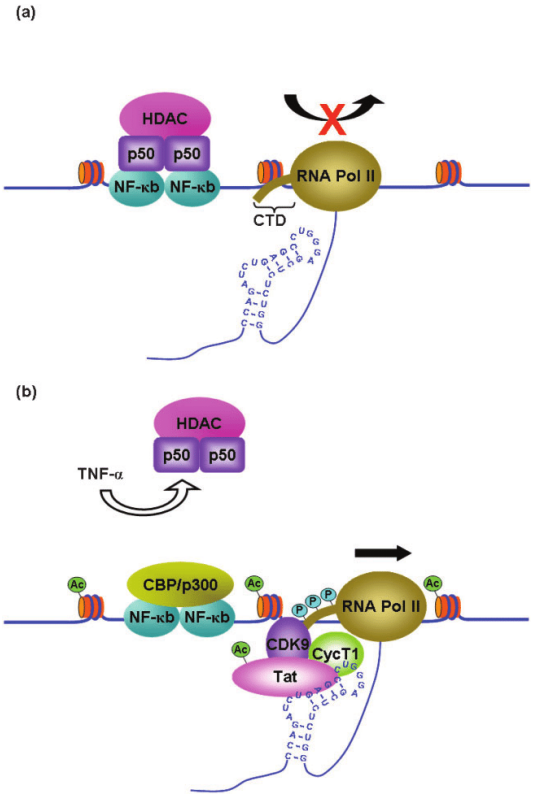
For example, micelles and liposomes that are functionalized with cell-penetrating peptides (CPPs) derived from the HIV-1 Tat protein yield hybrid 'smart' materials that can respond to the acidic pH environment of the tumor interstitium, allowing for cellular uptake of the drug-loaded vesicles only after they are appropriately targeted.



This is achieved by the 'shielding' of the CPP with either a pH-sensitive binding partner or by burying the CPP within a pH-sensitive layer of poly(ethylene glycol) (PEG) [5–8]. In this review we examine the current trends in the use of peptides for the cellular delivery of a range of NP materials of biomedical relevance.

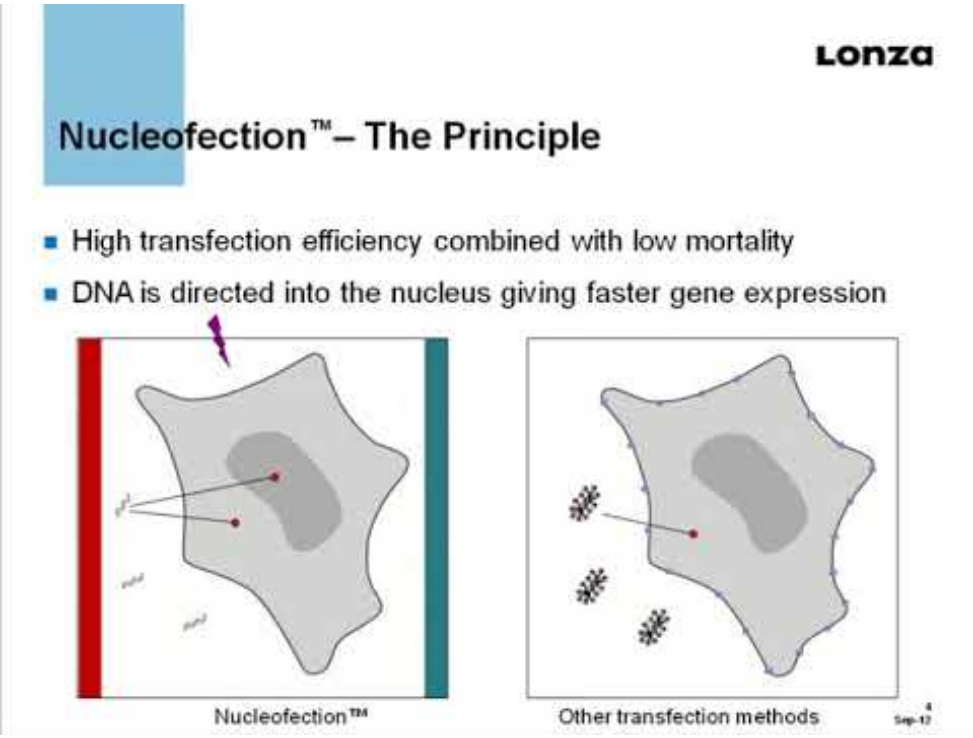
The HIV Tat protein

- Tat is absolutely required by the virus for productive infection
- Tat protein is made by the virus only in infected cells
- Tat is secreted by infected cells and is available to interact with and/or be taken up by un-infected by-stander cells
- Internalization of Tat by un-infected cells has been proposed to occur via interactions with cell surface integrins
- In un-infected cells Tat affects expression of important cellular genes:
  1. Decreases production of superoxide dismutase, glutathione peroxidase and γ-glutamyl synthetase
  2. Increases expression of cytokines and cell-adhesion molecules in human endothelial cells



The role of HIV-1 Tat in the synthesis of full-length viral mRNAs.

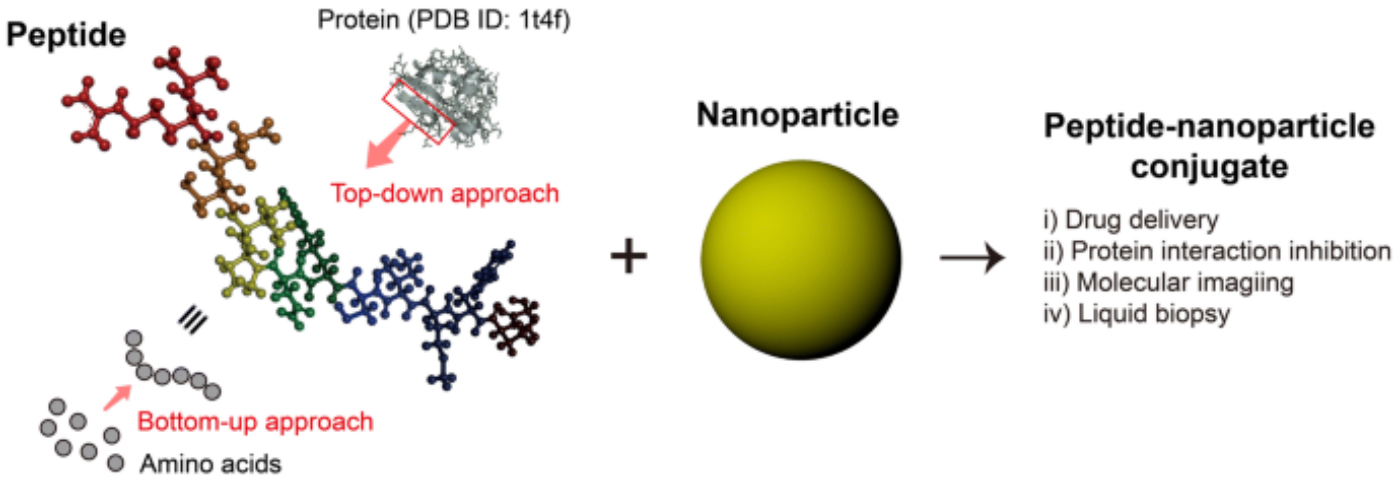
Active delivery involves the direct physical manipulation of the cell (specifically, the plasma membrane) to introduce NPs into the cell. Techniques such as electroporation and nucleofection, originally developed for the cellular delivery of nucleic acids, utilize a brief electrical pulse to permeabilize the phospholipid bilayer of the plasma membrane to allow entry of membrane-proximal materials into the cellular cytosol. Nucleofection further utilizes a transfection reagent to direct the delivered materials to the nucleus. These techniques have been demonstrated for a variety of NP materials, including QDs [12], silver NPs [13], poly(lactic-co-glycolic acid) (PLGA) NPs [14] and various nucleic acids [15].



Facilitated delivery involves the decoration of the NP surface with biological (e.g., peptides, proteins or nucleic acids) or chemical (e.g., lipidbased transfection reagents, drugs or small nutrients) moieties aimed at targeting specific cell surface receptors.

Within this group, peptides offer several unique advantages that make them rather attractive for therapeutic delivery applications. Their small size minimizes the overall radius of the resulting peptide–NP conjugate while still affording a high valence (number of peptides per NP). Furthermore, their size reduces immunogenicity in vivo.

Peptides are economical and facile to produce as they can be easily synthesized commercially or expressed recombinantly in the laboratory. From a functional perspective, peptides are biocompatible, derived from naturally occurring protein precursors and can be very specific and bind with high affinity to their cognate receptors, often with affinities comparable to those of full-length antibodies [18].

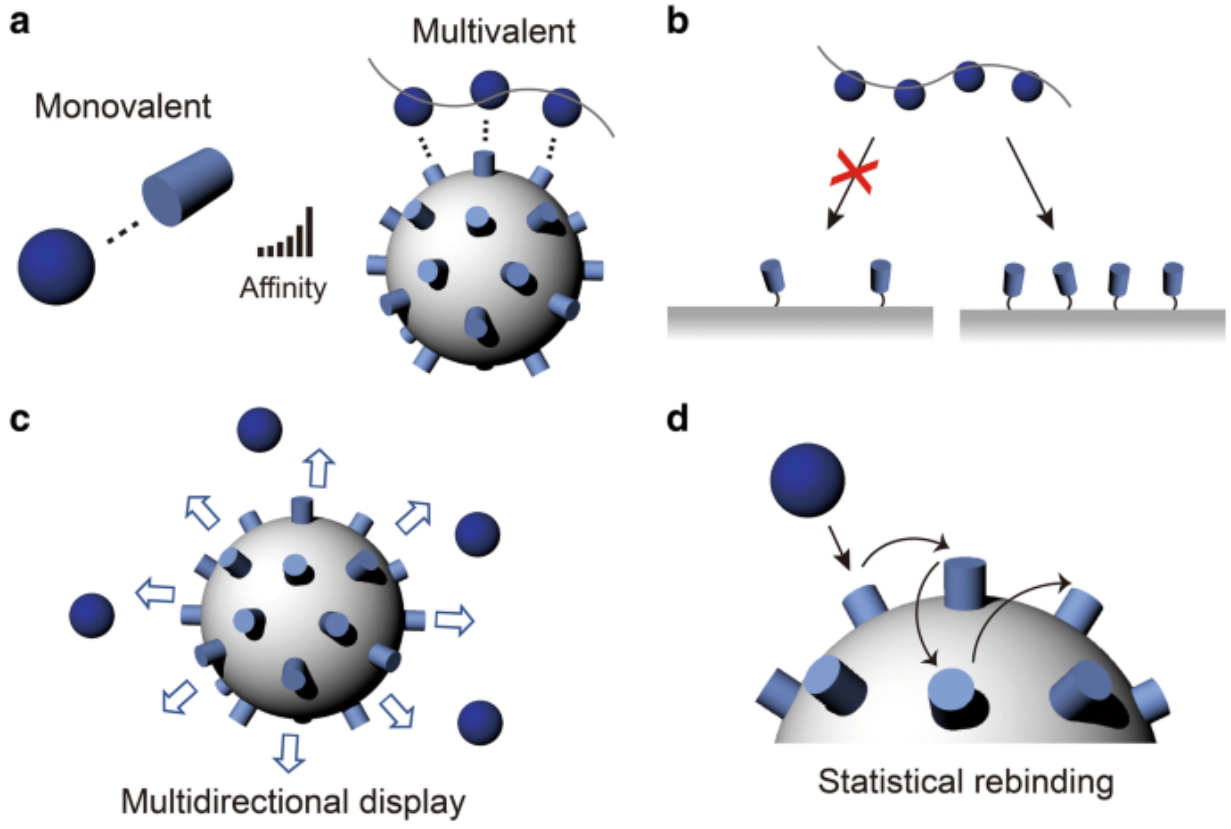


Finally, multiple different peptide species can be arrayed around the NP to incorporate multifunctionality or produce a 'value-added' material that serves multiple purposes in one NP. For these reasons, peptides represent a very attractive and useful class of molecules for the development of NPs for therapeutic delivery.

Bioconjugation of peptides to nanoparticles Beyond the individual properties of the NPs and peptides to be exploited in a bioconjugate, perhaps the next most relevant issue is the chemistry utilized to join them together as this has direct ramifications for subsequent function. Before briefly discussing the currently available NP biofunctionalization chemistries, it is quite helpful to visualize six ideal criteria or properties desired from such bioconjugations as they illuminate the potential impact on final utility [19].

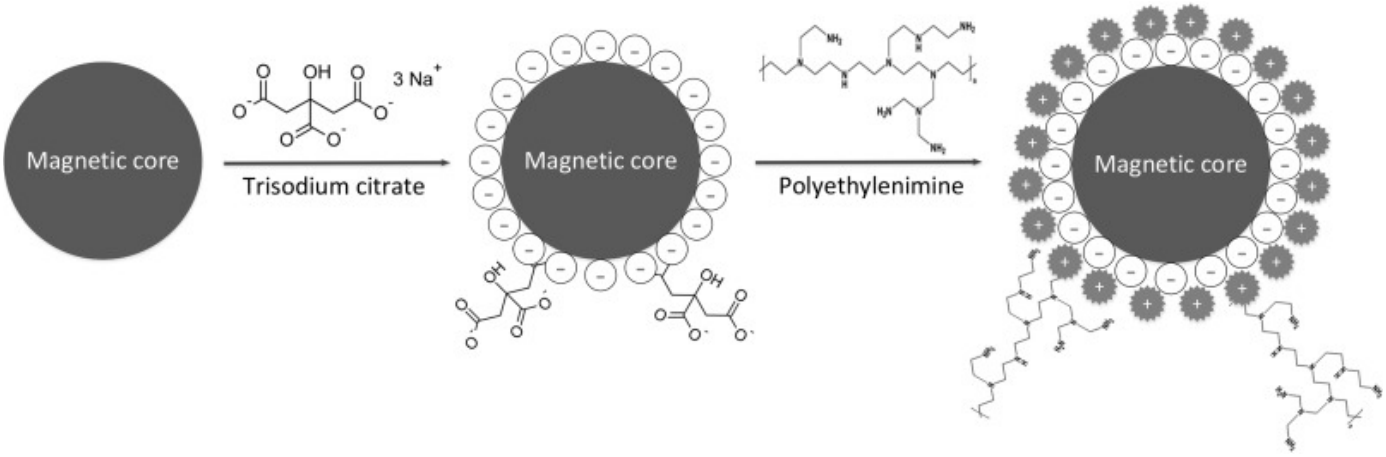
Ideally, the chemistry would attach the peptide to a NP in one of the following ways: n In a homogenous manner; n With control over its final orientation; n With control over its distance from the NP surface; n With control over ratio or valence on the NP surface.

The goal is to uniformly display the peptides on the NP surface with their active regions all clearly extended away and available for activity



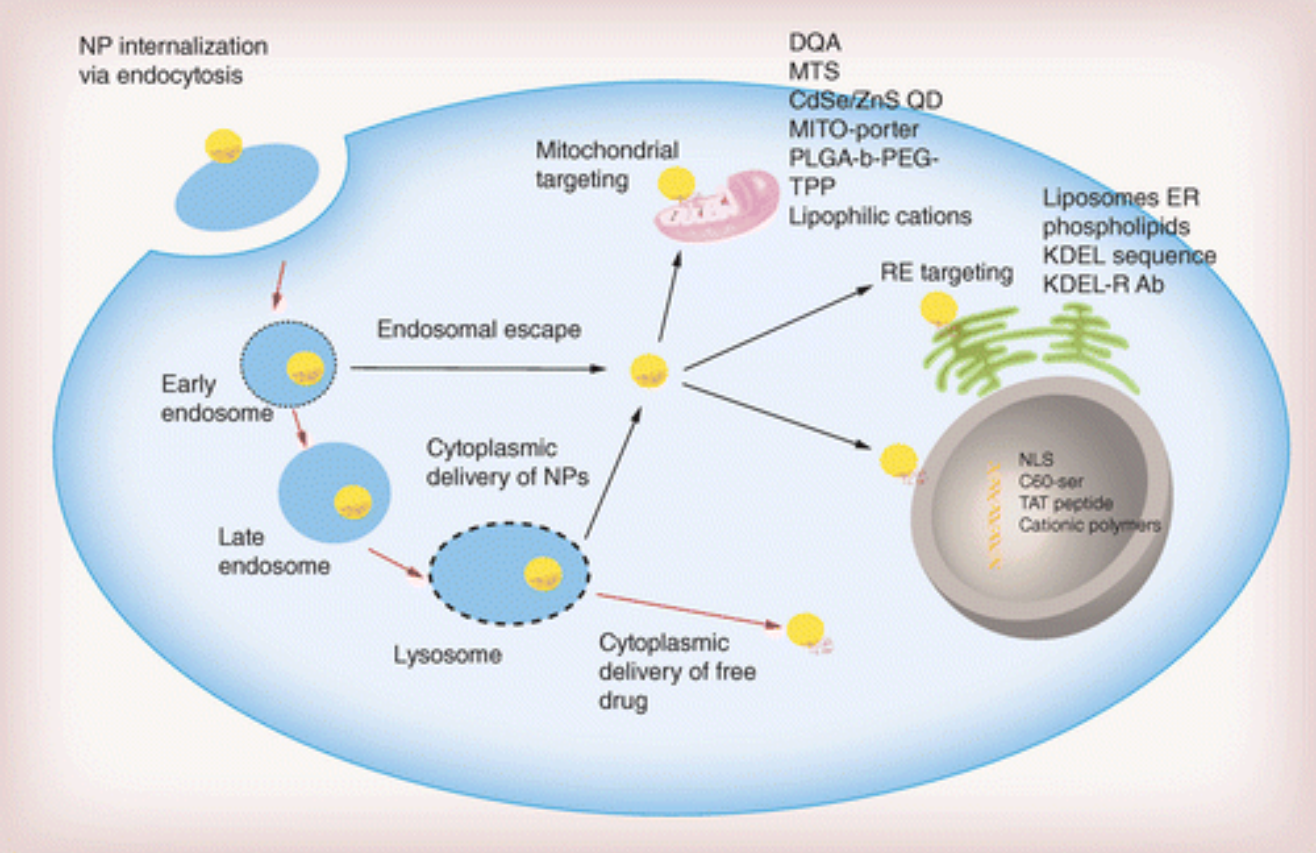
Nucleic acid delivery TAT and TAT-like peptides have also been employed for the cellular delivery of therapeutic nucleic acids. Han and co-workers functionalized bacterial magnetic nanoparticles with poly (amido amine) (PAMAM) and native TAT peptides and complexed them with siRNAs specific for the downregulation of human EGF receptor (EGFR), which is often overexpressed in many cancers.

Song et al. constructed ternary NPs consisting of polyethylenimine-coated magnetic iron beads that were noncovalently decorated with native TAT peptides and plasmid DNA encoding a luciferase reporter construct. Both in vitro (human NT2 neural stem cells) and in vivo (rat spinal cord injection), the presence of the TAT peptide increased gene expression fourfold [74]. Furthermore, the magnetofection complexes in the cerebrospinal fluid responded to a moving magnetic field; shifting away from the injection site and mediating transgene expression in a remote region (Figure 3F). This type of combinatorial approach has implications for the development of TAT-mediated targeted gene therapies that are controllable in vivo.

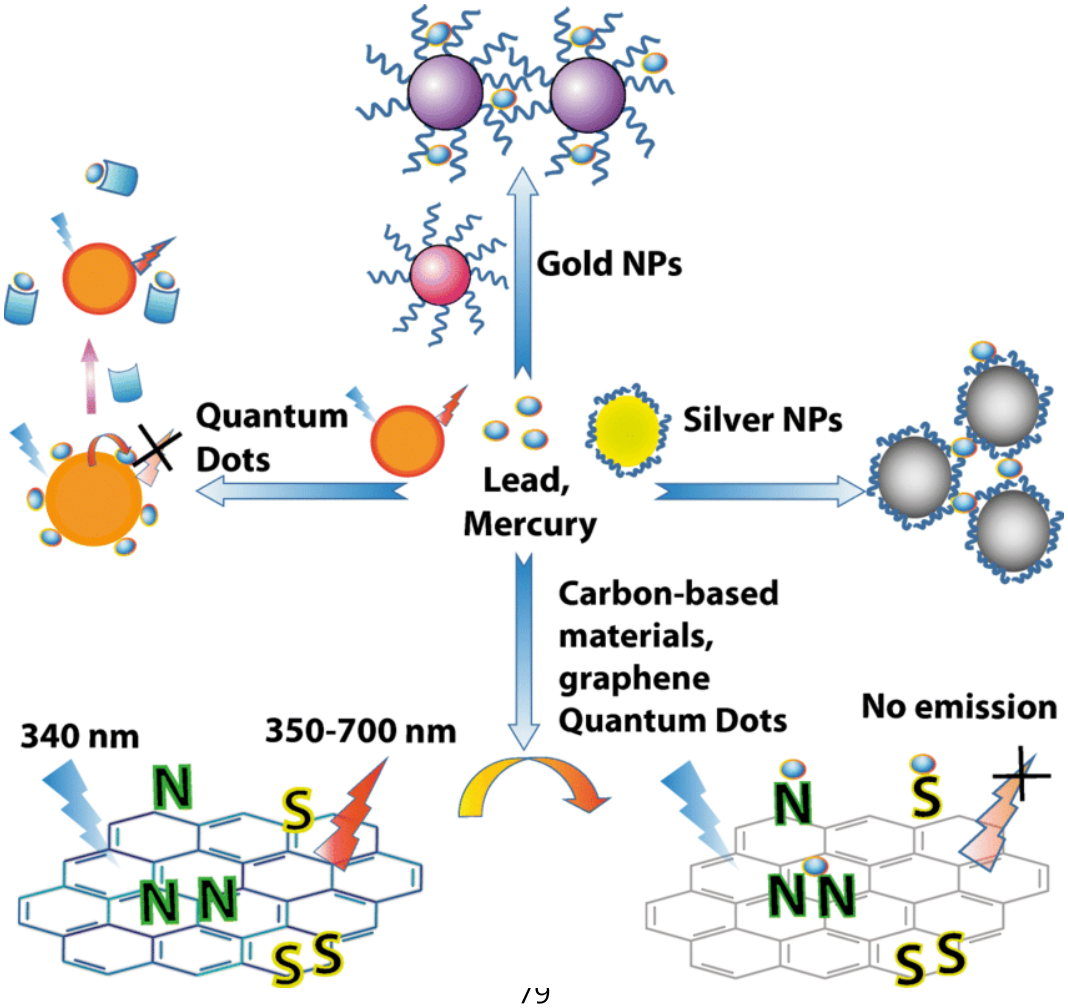


RGD peptides have also been used to target DNA- and siRNA-containing NPs to specific cells or disease sites. Cellular assays have focused primarily on delivering DNA encoding luciferase or fluorescent proteins as model systems to visualize gene delivery [99,100].

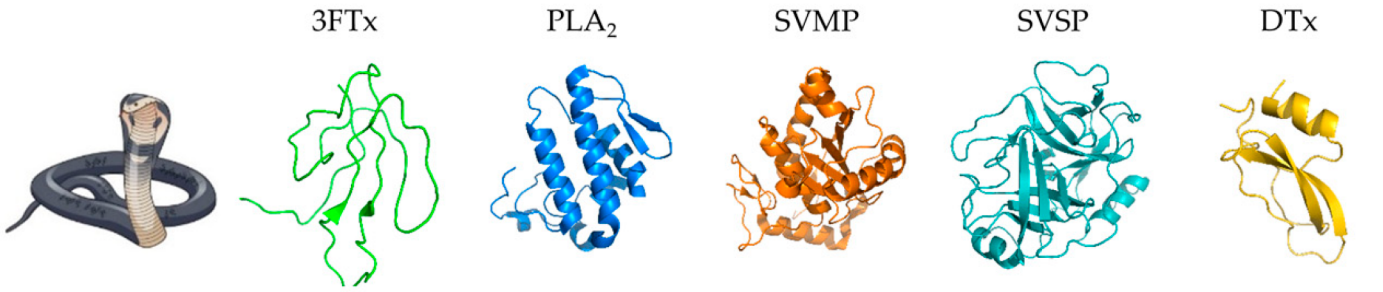
Several groups have induced endocytosis with reagents such as chloroquine to release the DNA from the endosome [100]. Therefore, current research efforts also include developing improved methods to release the NP-bound DNA from the endosome and into the nucleus for optimal expression.



Toxicity of peptide-delivered NPs A key issue to be addressed is the interaction of the peptides, NPs and NP–peptide conjugates with cells and tissues, and the corresponding toxicity of the delivered materials both in vitro and in vivo. As illustrated herein, the NP materials for which peptide-mediated delivery has been demonstrated to date encompass a range of constituent materials from noble metals, such as gold and semiconductor metals in QDs, to lipidbased materials and full-sized proteins. A thorough characterization of the resulting in vitro and in vivo toxicities is clearly necessary for each newly generated NP–peptide assembly.

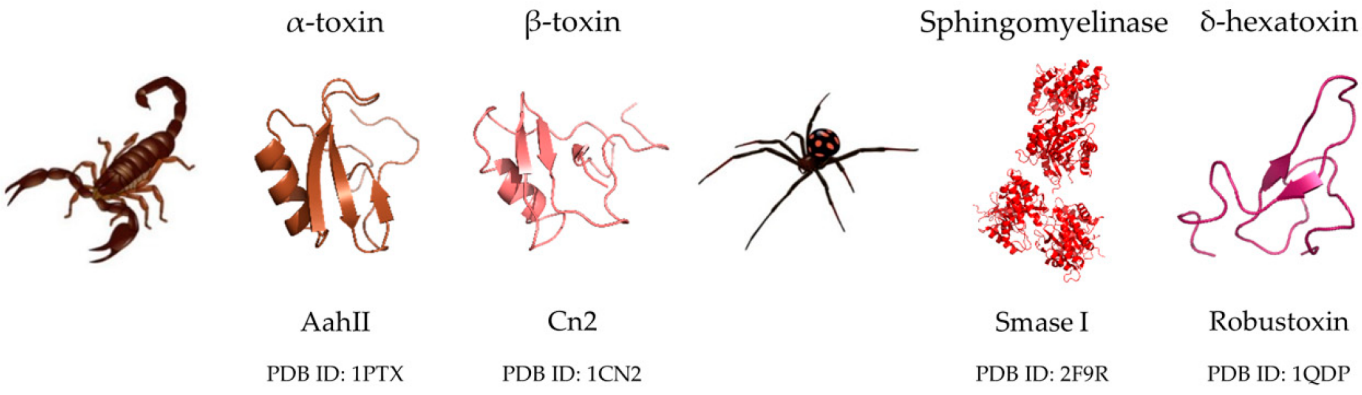


(a) Snake toxins



(b) Scorpion toxins

(c) Spider toxins



Nanobodies as novel therapeutic agents in envenomation

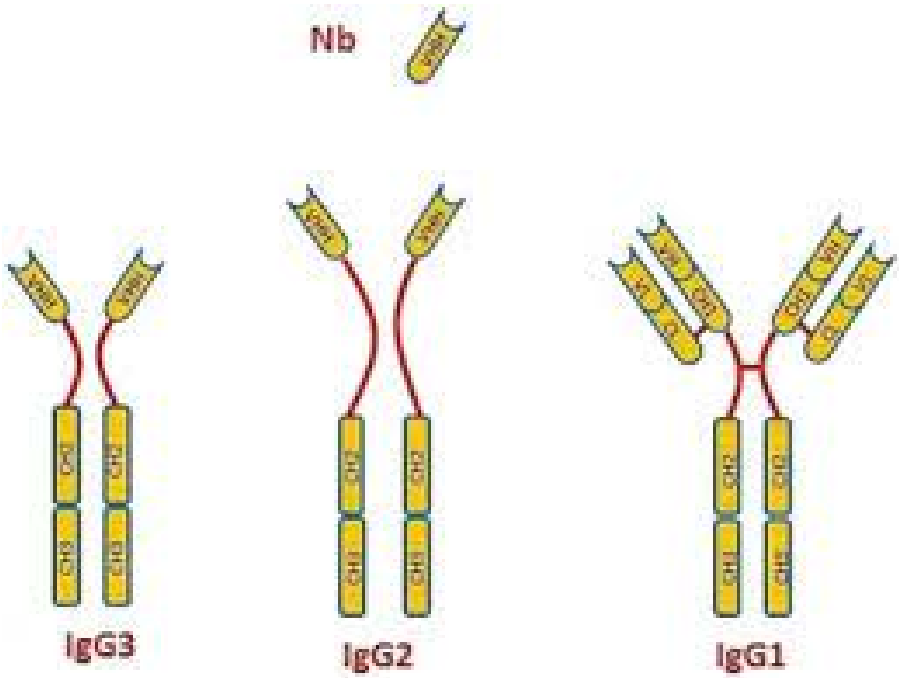
Ehsan Alirahimi[1], Fatemeh Kazemi-Lomedasht[1], Delavar Shahbazzadeh[1], Mahdi Habibi-Anbouhi[2], Mohammad Hosseininejad Chafi[1], Nazli Sotoudeh[1], Hajarossadat Ghaderi[1], Serge Muyldermans[3], Mahdi Behdani[4]

2018 Dec;1862(12):2955-2965.doi: 10.1016/j.bbagen.2018.08.019.Epub 2018 Aug 30.

Abstract

Background: An effective therapy against envenoming should be a priority in view of the high number scorpion stings and snakebites. Serum therapy is still widely applied to treat the envenomation victims; however this approach suffers from several shortcomings. The employment of monoclonal antibodies might be an outcome as these molecules are at the core of a variety of applications from protein structure determination to cancer treatment. The progress of activities in the twilight zone between genetic and antibody engineering have led to the development of a unique class of antibody fragments. These molecules possess several benefits and lack many possible disadvantages over classical antibodies. Within recombinant antibody formats, nanobodies or single domain antigen binding fragments derived from heavy chain only antibodies in camelids occupy a privileged position.

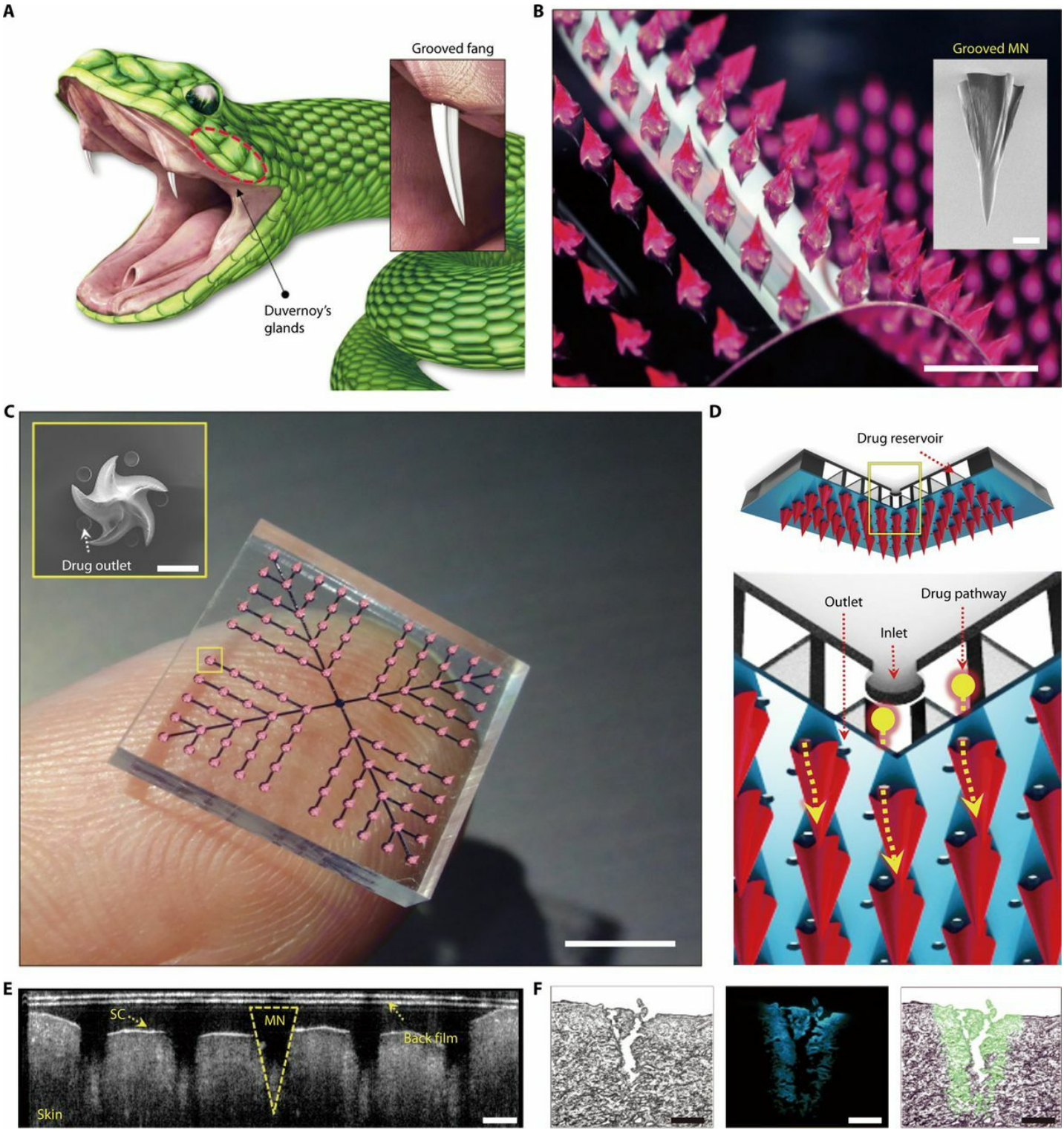
Scope of review: In this paper we will briefly review the common methods of envenomation treatment and focus on details of various in vivo research activities that investigate the performance of recombinant, monoclonal nanobodies in venom neutralization.



Recombinant monoclonal nanobodies in venom neutralization

Major conclusions: Nanobodies bind to their cognate target with high specificity and affinity, they can be produced in large quantities from microbial expression systems and are very robust even when challenged with harsh environmental conditions. Upon administering, they rapidly distribute throughout the body and seem to be well tolerated in humans posing low immunogenicity.

General significance: Scorpion and snake envenomation is a major issue in developing countries and nanobodies as a venom-neutralizing agent can be considered as a valuable and promising candidate in envenomation therapy.


A
Grooved fang
Duvernoy's glands
B
Grooved MN
C
Drug outlet
D
Drug reservoir
Outlet
Drug pathway
Inlet
E
SC
MN
Back film
Skin
F

Recent advances in microneedles-mediated transdermal delivery of protein and peptide drugs

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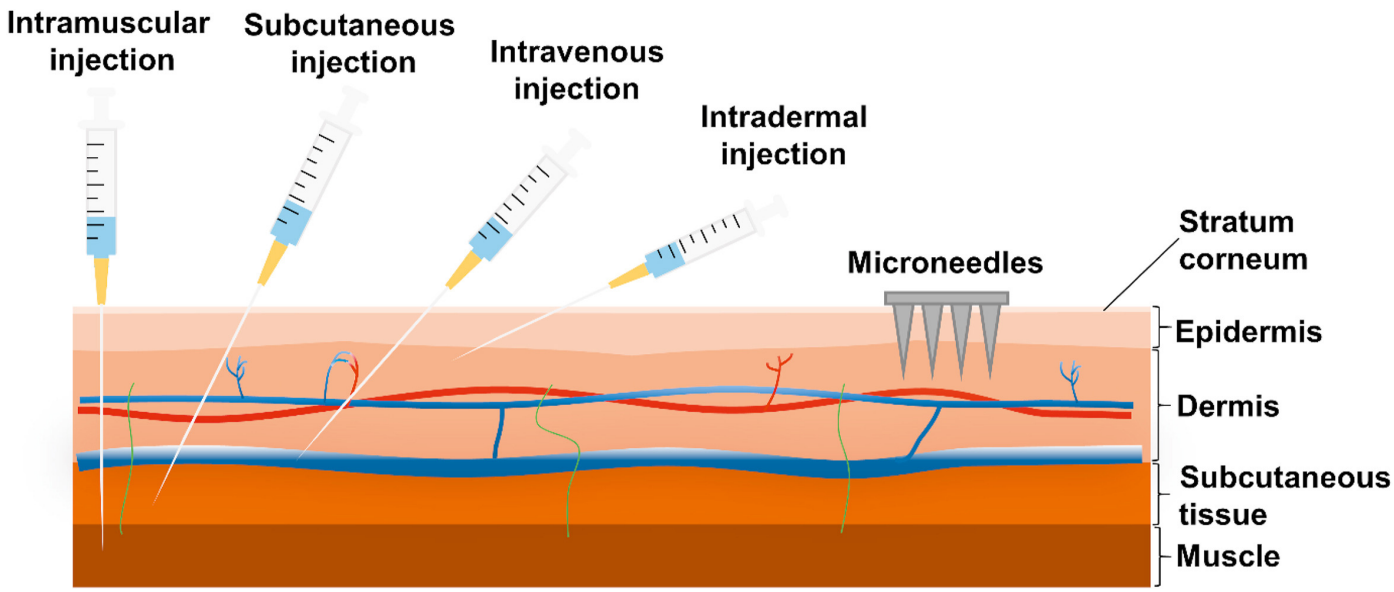
Abstract

Proteins and peptides have become a significant therapeutic modality for various diseases because of their high potency and specificity. However, the inherent properties of these drugs, such as large molecular weight, poor stability, and conformational flexibility, make them difficult to be formulated and delivered. Injection is the primary route for clinical administration of protein and peptide drugs, which usually leads to poor patient's compliance. As a portable, minimally invasive device, microneedles (MNs) can overcome the skin barrier and generate reversible microchannels for effective macromolecule permeation.

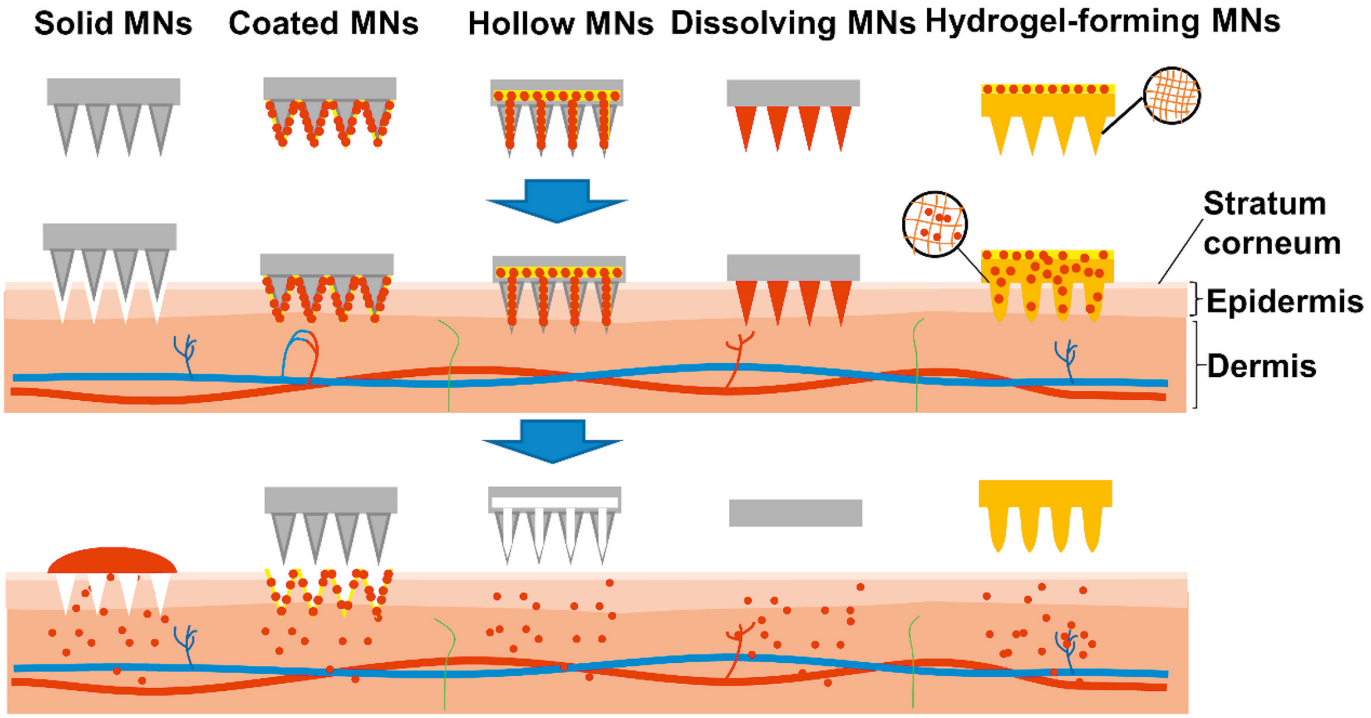
In this review, we highlighted the recent advances in MNs-mediated transdermal delivery of protein and peptide drugs. Emphasis was given to the latest development in representative MNs design and fabrication. We also summarize the current application status of MNs-mediated transdermal protein and peptide delivery, especially in the field of infectious disease, diabetes, cancer, and other disease therapy. Finally, the current status of clinical translation and a perspective on future development are also provided.



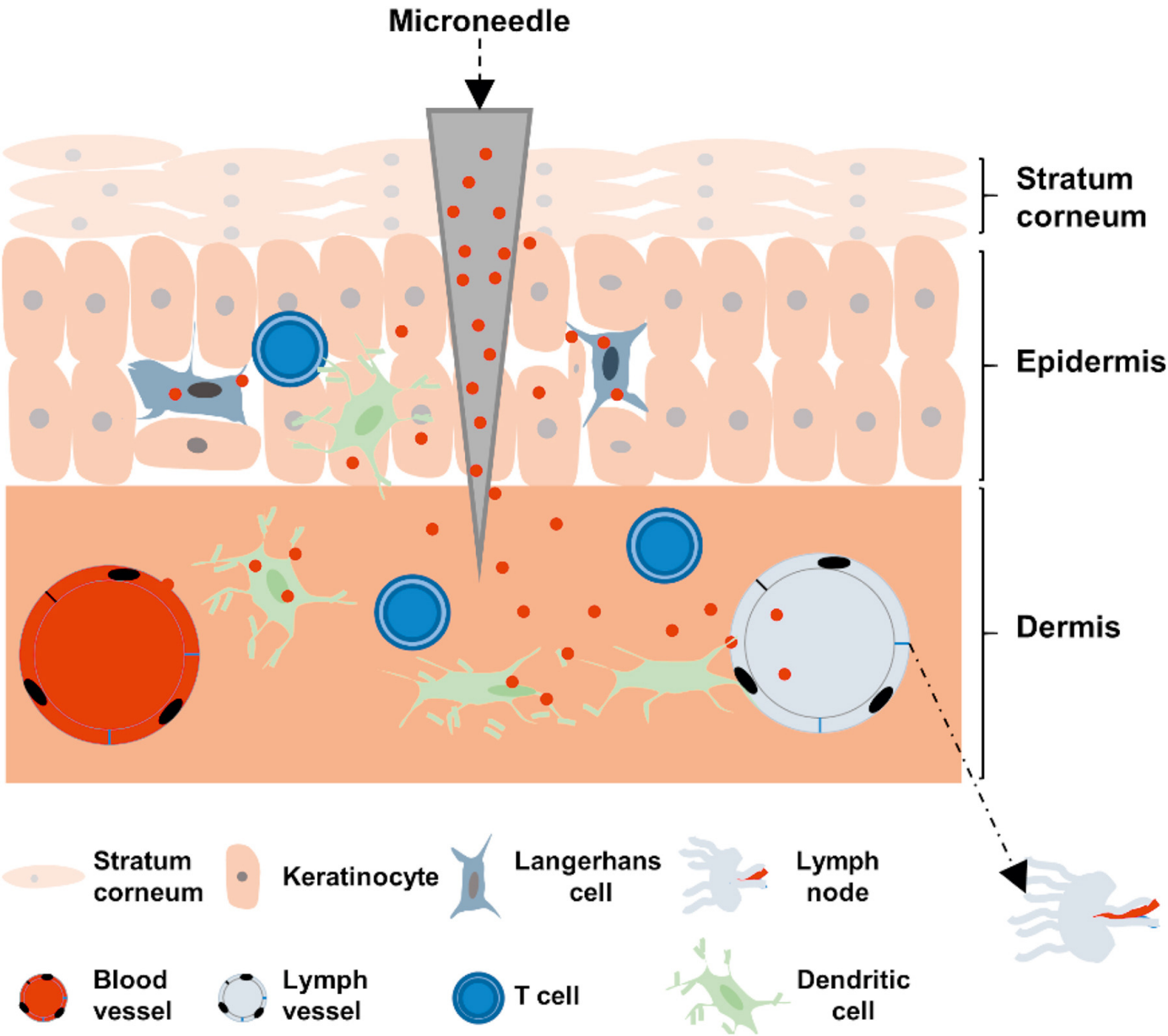
Excerpts Pages -

Proteins and peptides exhibit the most prominent effects in human body, such as molecular transportation, biological scaffold, cellular regulation, and enzymatic catalysis, which have played an important role in almost every medical field [1-4].

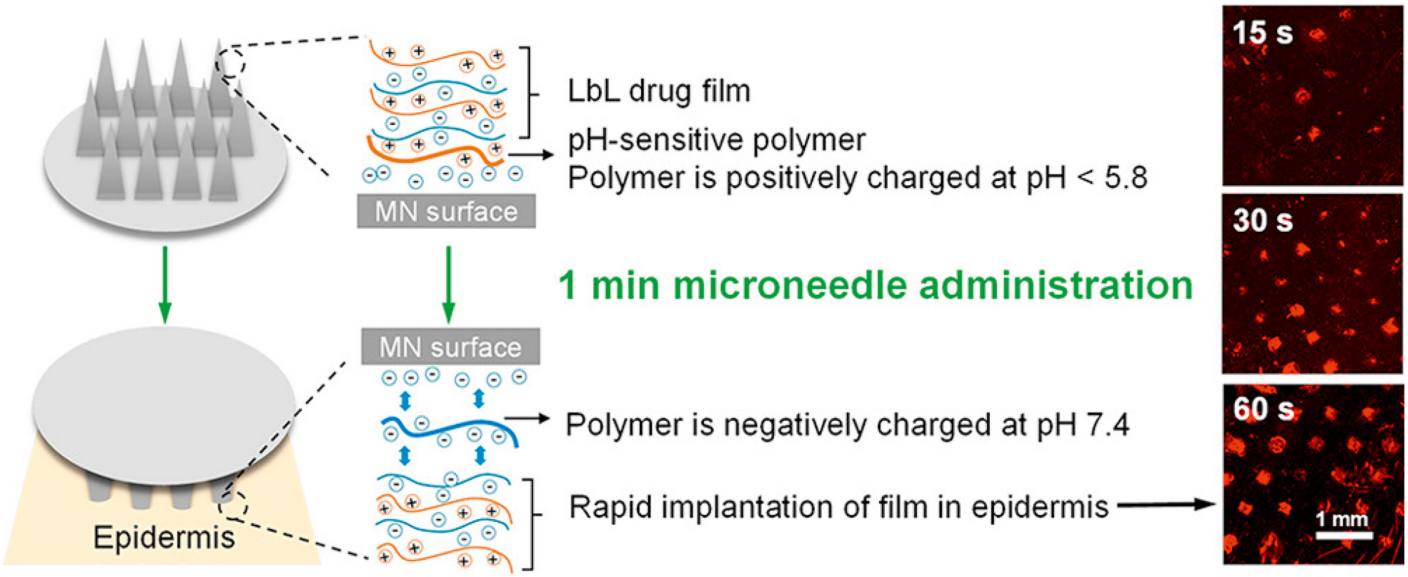
However, the application of protein and peptide drugs is commonly restrained by certain limitations. The large molecular weight of these drugs substantially decreases their permeability capacity across biological barriers such as skin and mucous membranes. Besides, loss of biological activity in response to external conditions (moisture and temperature) and endogenous proteolytic enzyme put a high difficulty on formulation and delivery technologies [7].



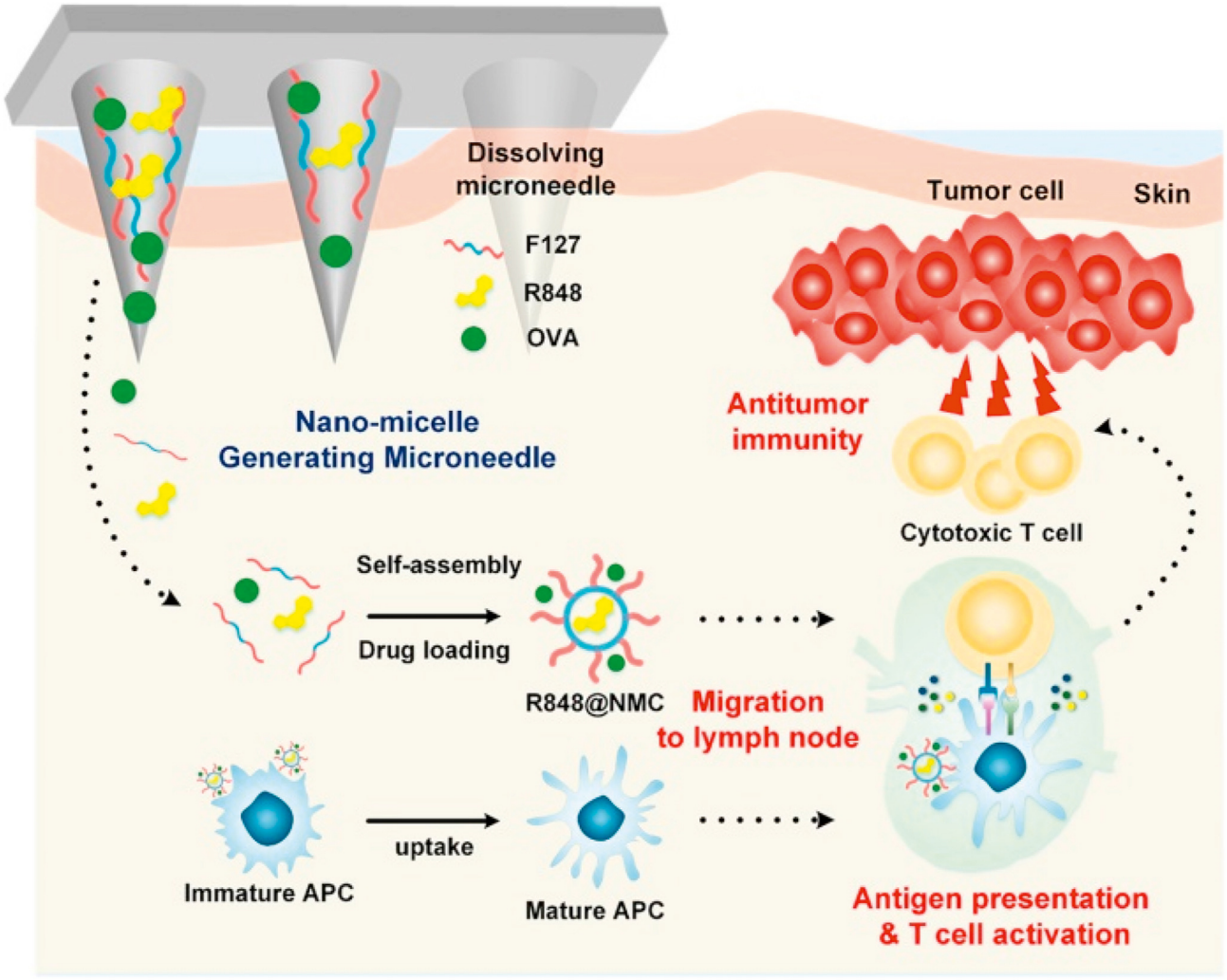
Regardless of the injection method, most protein and peptide drugs are easily degraded by various metabolic enzymes in the body, resulting in short half-life in vivo, which means frequent injections are required. Furthermore, injection therapy is inconvenient and unfriendly, especially for patients with chronic diseases such as rheumatoid arthritis and diabetes.



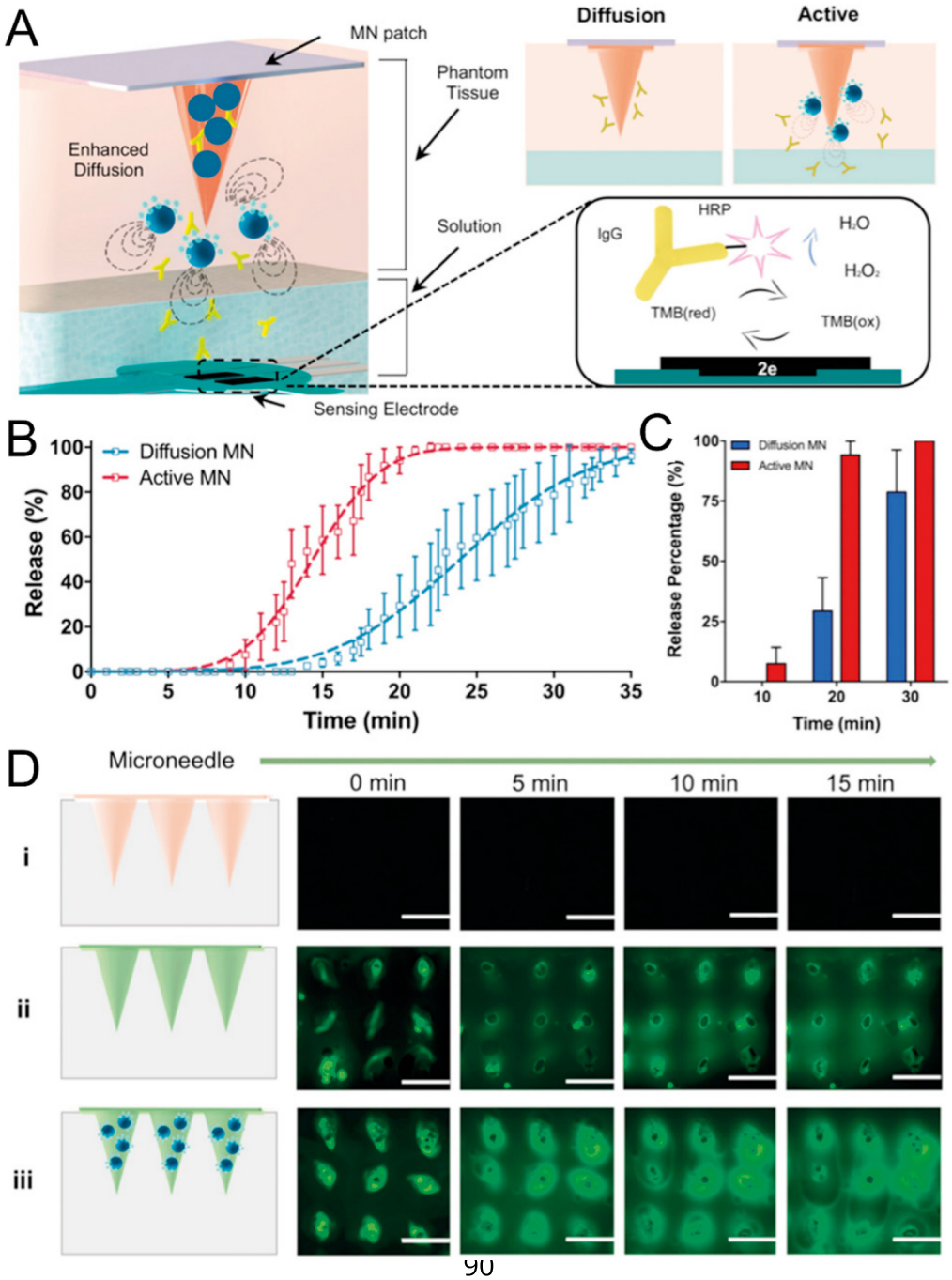
Transdermal drug delivery is a choice that delivers biologically active agents through skin portals for local or systemic effects, which is noninvasive and can be self-administered [13]. There are some requirements for the drugs suitable for transdermal administration, such as a maximum molecular weight of 1000 Da (Dalton), and a balance between hydrophobicity and polarity due to the stratum corneum barrier [14]. Most protein and peptide drugs are hydrophilic and macromolecular in nature, and therefore they cannot easily penetrate into the skin.



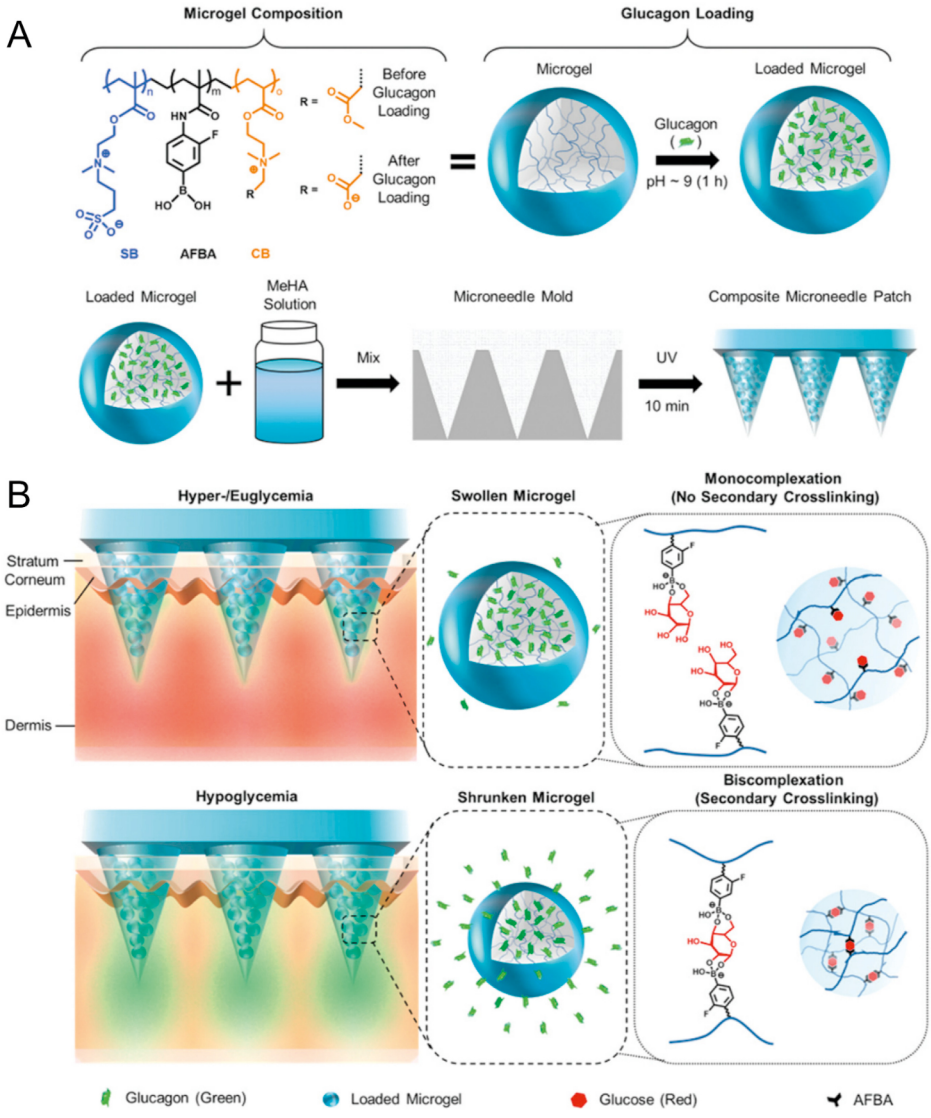
Recently, microneedles (MNs) have become a new type of drug delivery technique, and the applications of MNs have been extended to various aspects, including small chemical molecules [21,22], vaccines [23,24], genes [25], proteins [4,26], and nanoparticles [27]. Particularly, MNs provide a great prospect for the transdermal delivery of proteins and peptides [28,29]. MNs are minimally invasive device with needles (<1 mm) arranged orderly on the base. They can directly penetrate the stratum corneum by generating reversible microchannels in the skin.



These microchannels can grant access of drugs to the dermal microcirculation located in the interior layers of the skin (Fig. 1). Compared with injection, MNs will not contact with blood vessels and nerves in the deep dermis, which provide better patient compliance and favorable safety profile. Moreover, the mild fabrication condition of MNs will not impact the biological activity of proteins and peptides.

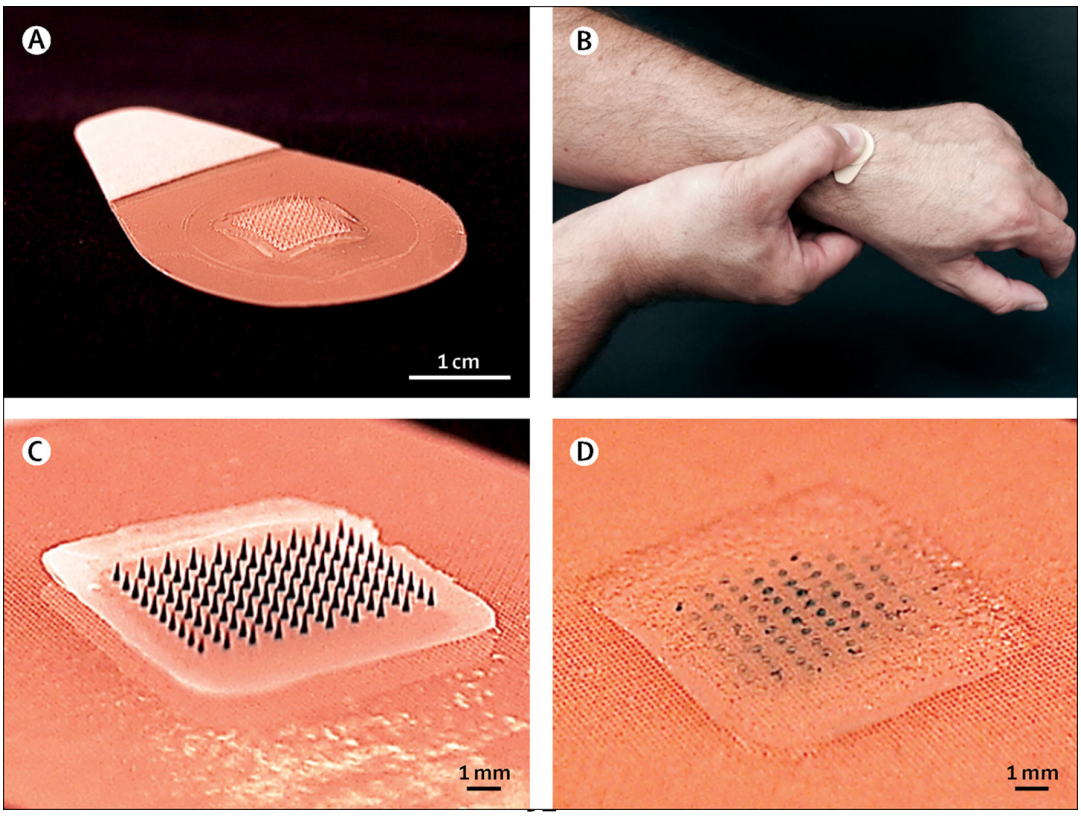


This review provides comprehensive updates on MNs mediated transdermal delivery of protein and peptide drugs. Emphasis was given to the latest development and advance in representative MNs design and fabrication. Additionally, we summarized the recent studies about the applications of MNs mediated protein and peptide delivery, particularly focusing in the field of infectious disease, diabetes, cancer, and other disease therapy. Finally, the current status of clinical translation and a perspective on future development were also provided.

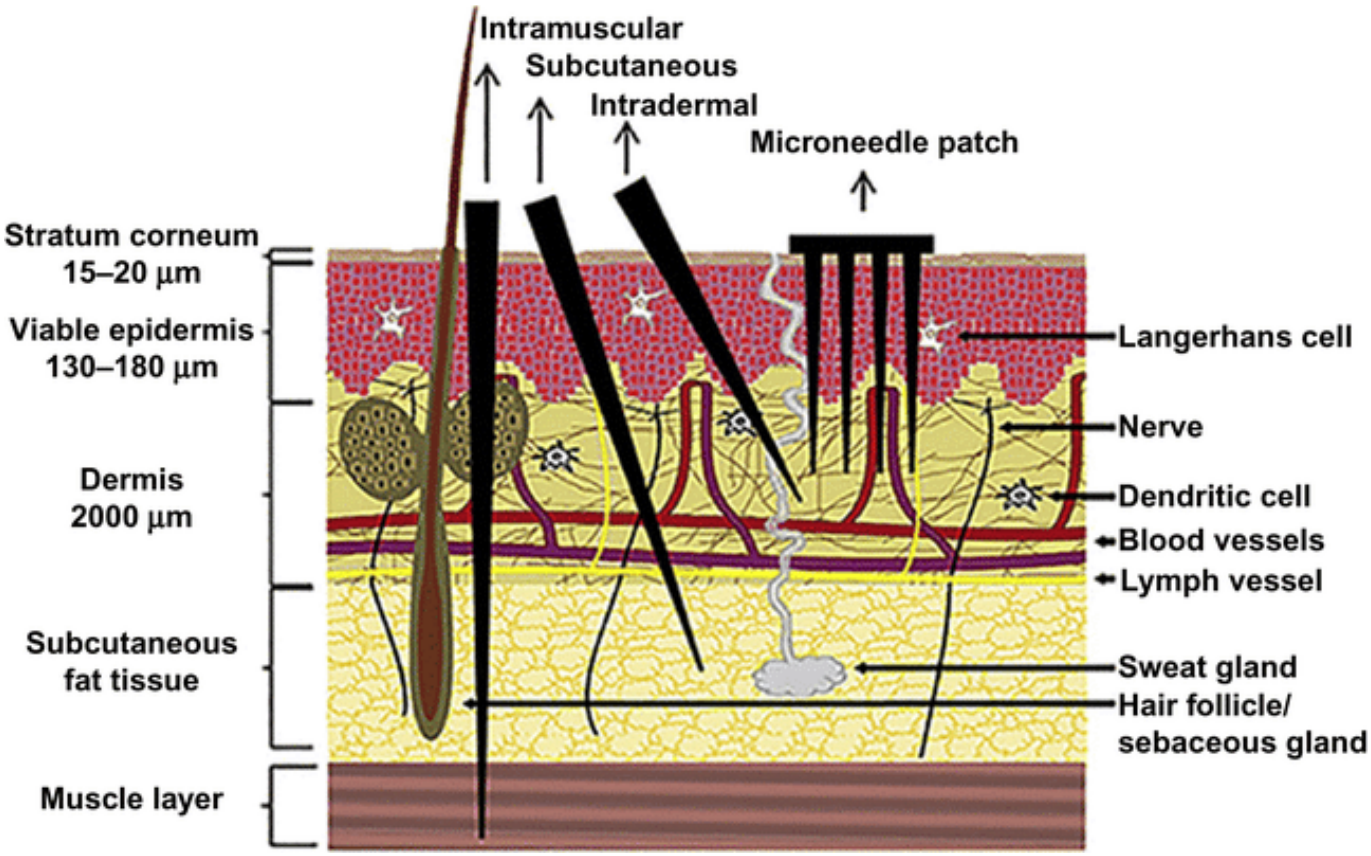


3.

Application of MNs-mediated protein and peptide delivery Proteins and peptides have become significant therapeutic modalities for various diseases, which continue to enter the market at a steady pace99e101. This can be attributed to their target specificity, high potency, and favorable safety compared with traditional small-molecule drugs. As a minimally invasive device, MNs can improve the patient's compliance and offer a multifunctional platform to overcome the skin barrier for hydrophilic and macromolecular drugs [32]. Moreover, the mild fabrication condition and solid state nature are a major advantage of MNs compared to traditional injection of the aqueous solution, which can improve drug stability and reduce the use of cold chain [80].

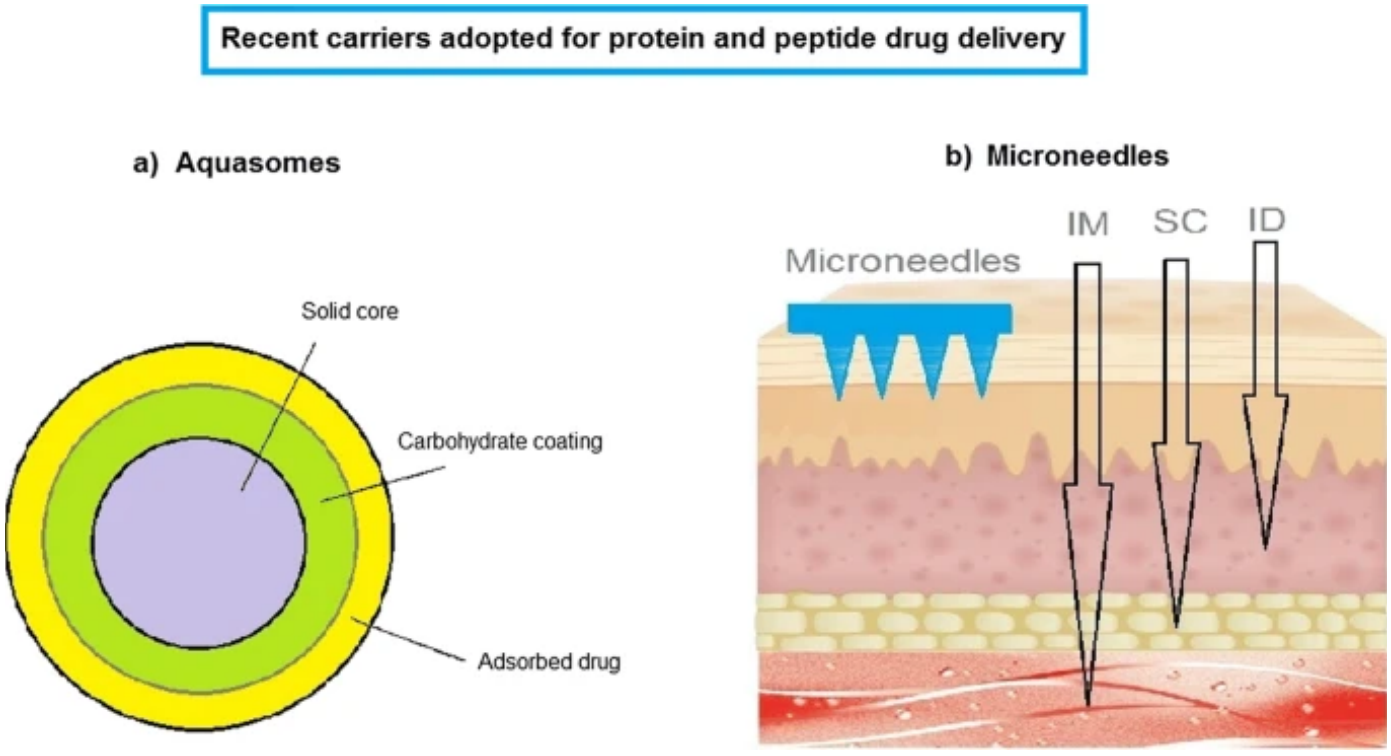


With the progress of material science and microfabrication technology, many MNs-mediated protein and peptide delivery strategies have been developed. Typically, MNs have been utilized to deliver various forms of cargoes, from native drugs to the nanoparticle or microparticle-based formulations [27]. In this section, we summarized the recent advances in MNs-mediated protein and peptide delivery, especially focused on their application for infectious disease therapy, diabetes therapy, and cancer therapy.



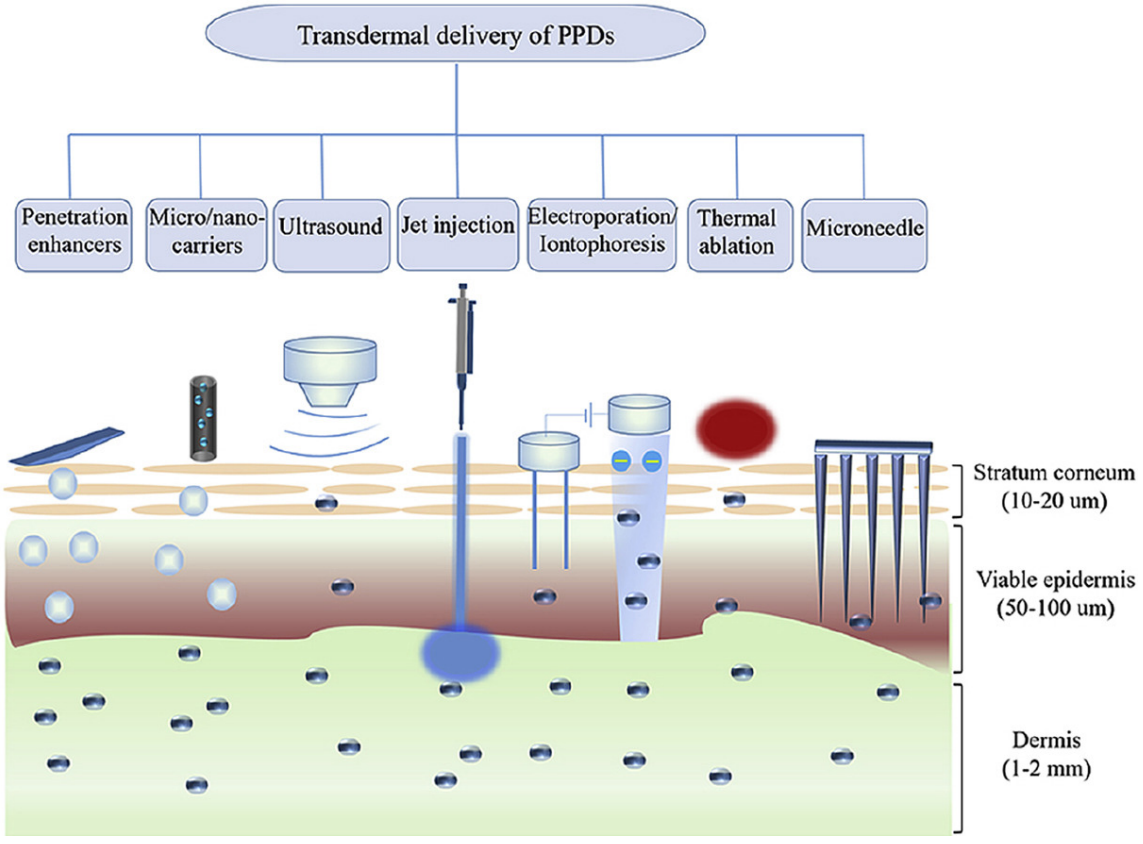
3.1.

Infectious disease therapy Infectious diseases such as influenza, measles, and hepatitis B are one of the main causes of human deaths, which is a major public health concern worldwide. Vaccination has been recognized as the most successful, and cost-effective public health intervention strategy to combat infectious diseases [47,102]. Compared with other antigen molecules, only proteins can induce both cellular and humoral immunity [103]. In addition, the versatility and customizability of proteins make protein-based vaccines one of the most effective strategies for artificially immunity induction [10].

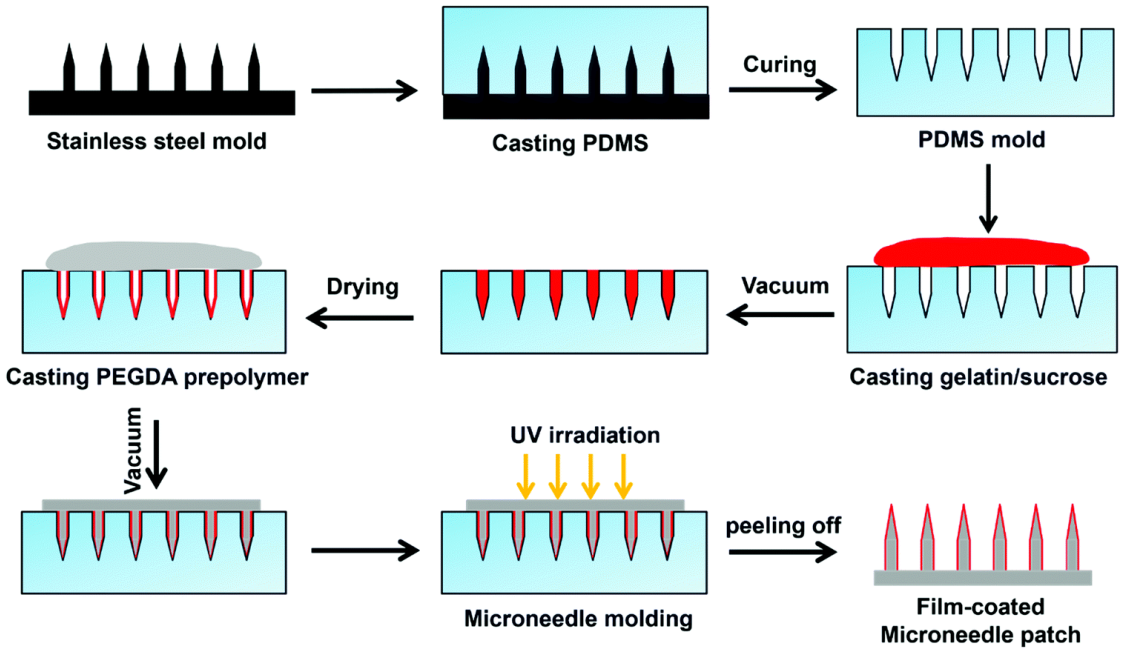


Most vaccines are administered by subcutaneous or intramuscular injection, which is relatively painful, resulting in poor patient compliance [104]. There are a large number of antigen presenting cell populations in the skin, such as macrophages, dermal dendritic cells (DCs), and Langerhans cells, making the skin a unique target for immunomodulation [59,105,-107]. MNs are easy to use with minimal pain, which provide a promising platform for transcutaneous immunization with improved efficacy [108-110] (Fig. 3). Over the past few decades, MNs have been developed successfully as an experimental delivery system for various protein and peptide vaccines (Table 1).

Dissolving MNs for influenza vaccine delivery could also improve the efficiency of virus clearance and enhance cellular recall response, compared with conventional intramuscular injection [72, 129].



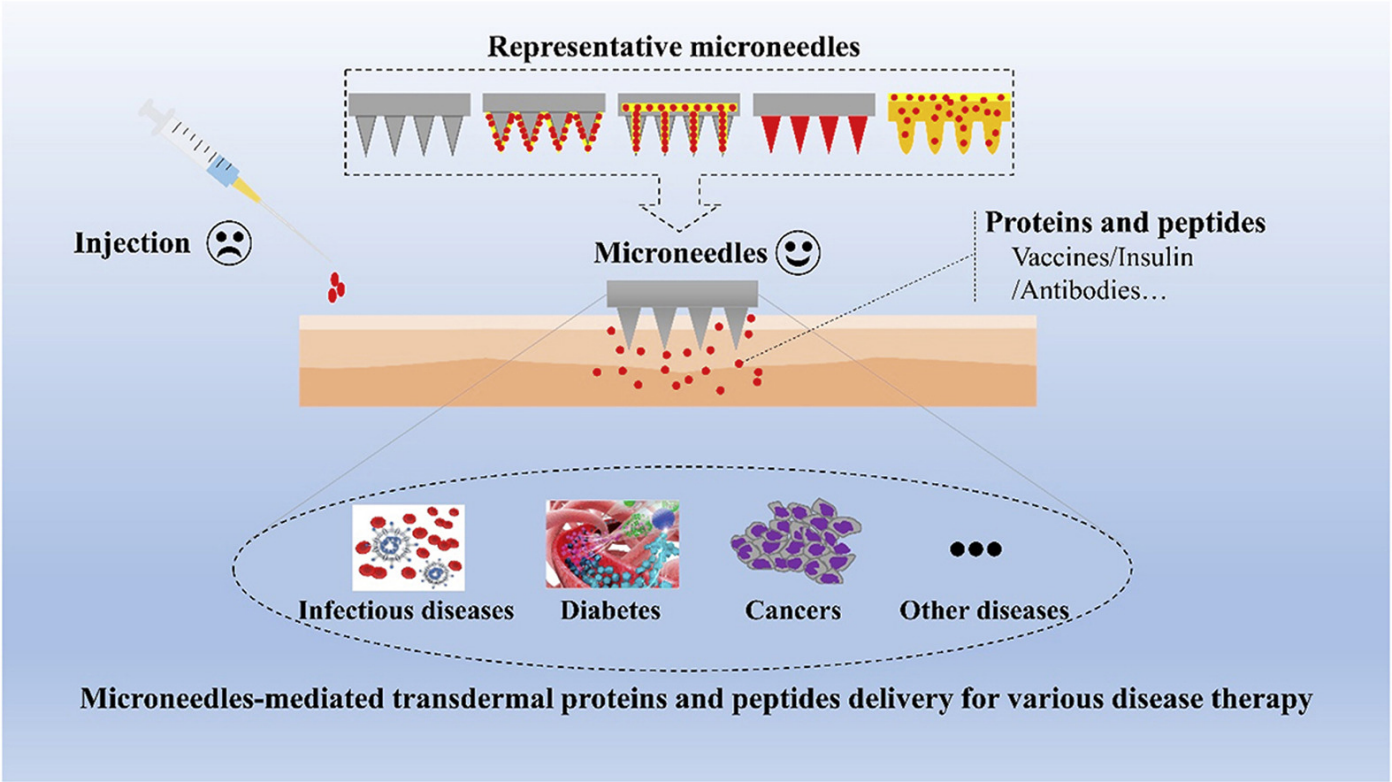
The key parameter of protein and peptide vaccine formulation is to maintain the stability of the vaccine component, which is crucial during the fabrication, transportation, and storage process. Appropriate formulation techniques using MNs can retain the long-term antigen immunogenicity and allow flexible storage conditions [145,146]. DeMuth et al. [127] found that the sucrose-coated MNs effectively delivered adenovirus into the skin and allowed storage at room temperature for several months without losing the biological activity of adenovirus vectors. Mistilis et al. [130] screened different dissolving MNs formulation combinations to stabilize a trivalent subunit influenza vaccine. After being stored at 25 C for 24 months, dissolving MNs formulated by combinations of arginine/heptagluconate, sucrose/arginine, and trehalose/sucrose still retained the vaccine immunogenicity. The mice immunization experiment also proved that the antibody titer was equivalent to the fresh liquid vaccine provided by intradermal injection [130].



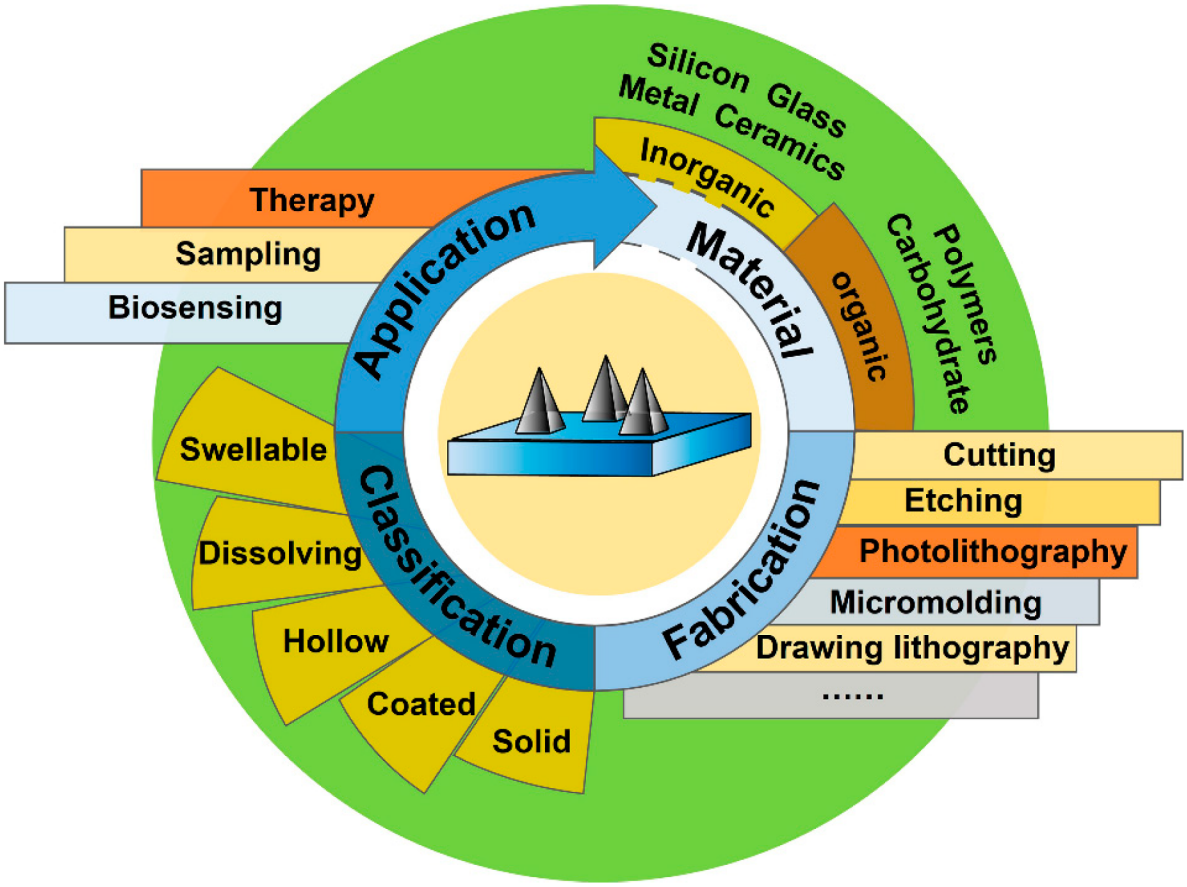
4.

MNs-mediated protein and peptide delivery in the clinic

As mentioned above, the fundamental research has proved the advantages and feasibility of MNs-mediated protein and peptide delivery. At present, many therapies based on MNs-mediated transdermal delivery of protein and peptide drugs have entered clinical use. As shown in Table 3, most currently active clinical trials focus on the vaccination of infectious diseases and insulin delivery for diabetes treatment. These clinical trials mainly utilized the hollow MNs infusion system, and a few investigated dissolving or coated MNs. This is mainly because the research on coated MNs, dissolving MNs or hydrogel-forming MNs (HFMs) started later. And they usually require more sophisticated MNs design and manufacturing

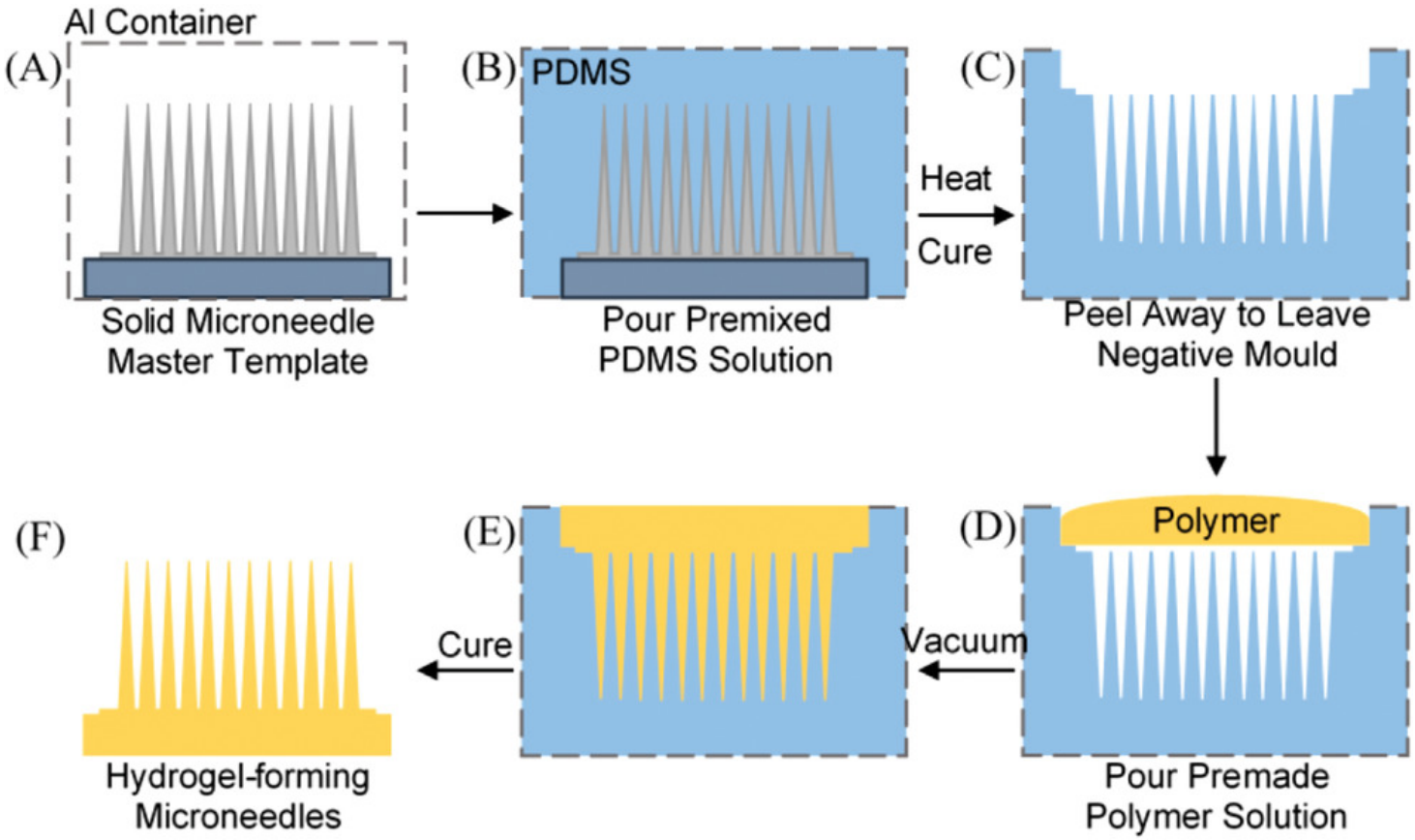


The interdisciplinary divide between microfabrication and pharmaceutical research also delayed the development of drug delivery [23]. At this stage, the field is at an important transitional point. More MNs products will be translated into clinical and medical practice in the near future.



(Ed. Note: First reported in 2012, HFMs are the newest form of MNs.[8]Consisting of swellable polymers [crosslinked hydrogels], HFMs have a different working mechanism compared to other MNs. When inserted into the skin, HFMs will swell due to the hydrophilic nature of the hydrogels, meaning they readily uptake water.)

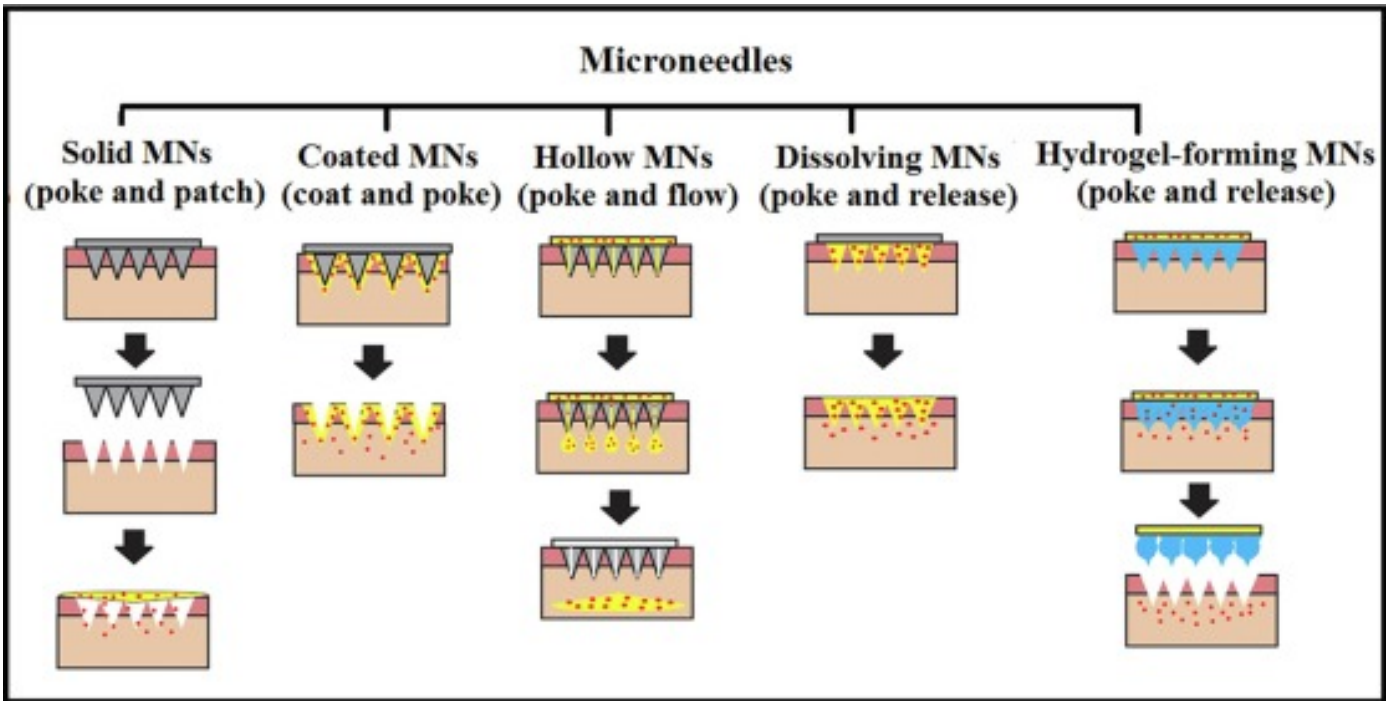
https://onlinelibrary.wiley.com/doi/full/10.1002/mabi.202000307



Hydrogel-forming Microneedles (HFMs)

Schematic to show the micro-molding method for the production of HFMs. First, A) a master structure is placed inside an aluminum container and B) premixed PDMS is poured around the mold and air bubbles removed. C) Heat curing of the PDMS allows the master structure to be removed leaving a negative mold. D) This negative mold can be used by adding premixed polymer solution and E) vacuuming to ensure complete filling. F) Curing the polymer solutions allows the mold to be peeled away, leaving HFMs.

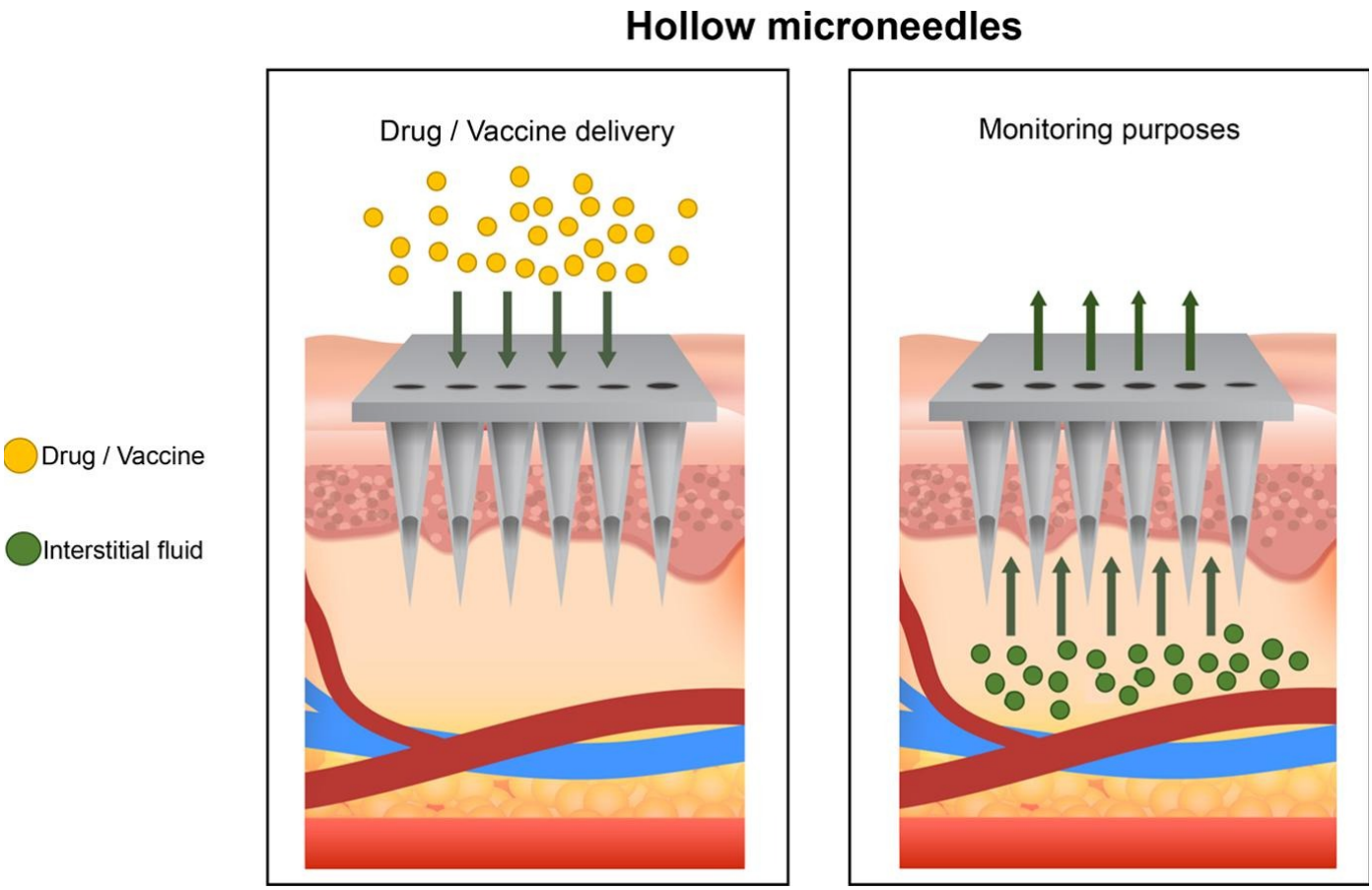
Based on different drug delivery strategies, MNs can be generally classified into five categories, including solid MNs, coated MNs, hollow MNs, dissolving MNs, and hydrogel-forming MNs (Fig. 2). Each type of MNs has been extensively studied for transdermal drug delivery. However, the protein and peptide drugs are usually sensitive to high temperature, pH value, and organic solvents compared with inert small molecules [33].



To avoid the damage of their biological activity, it is necessary to understand the properties of each type of MNs, and then select reasonable MNs types to formulate them.

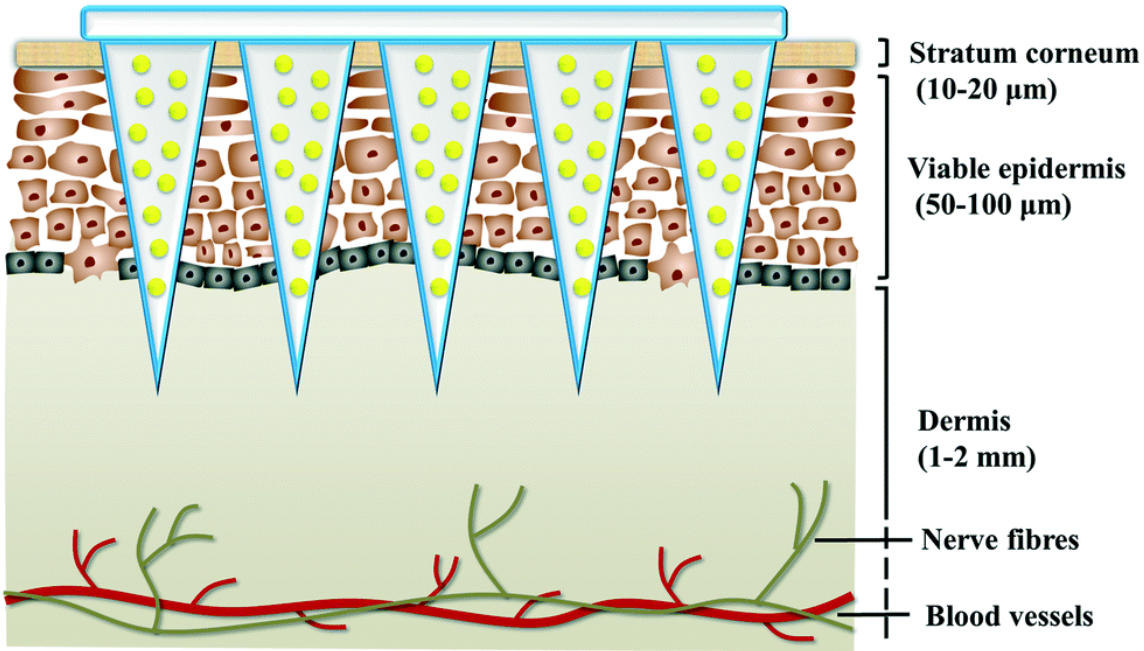
2.3.

Hollow MNs Hollow MNs are sub-millimeter devices acting like micron-scale syringes, which can penetrate the stratum corneum to allow the flow of liquid formulation into the epidermis or dermis [59]. In the simplest form, drug delivery using hollow MNs is achieved through passive diffusion. Since the passive diffusion rate in dense tissues is relatively low, faster transport rate through pressure driven flow or diffusion has been successfully achieved [21,47]. Consequently, compared with solid MNs, hollow MNs can allow the administration of larger doses, and simultaneously provide an exact transport rate [21,60-62].



2.4. Dissolving MNs

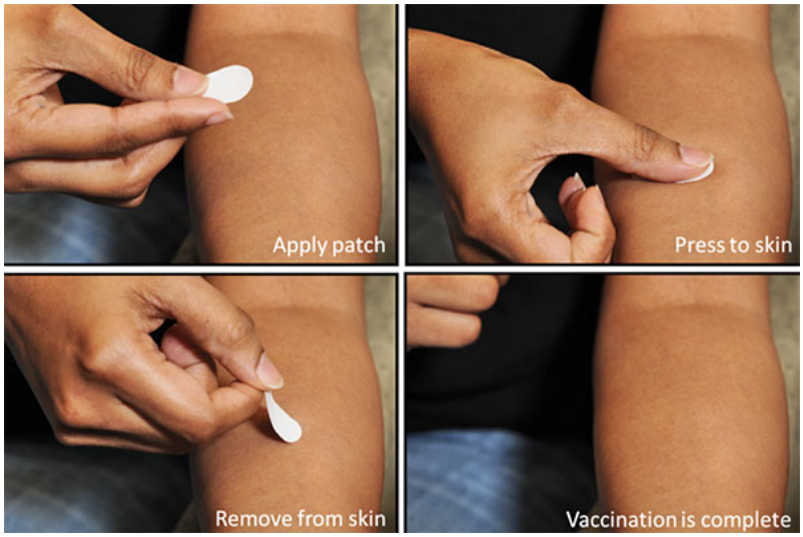
Dissolving MNs are usually prepared from dissolvable materials with therapeutic agents incorporated into the needles, which can effectively deliver drugs into the skin by the dissolution of needle matrix66e68. Many materials have been used to prepare dissolving MNs, from low molecular weight carbohydrates to high molecular biodegradable polymers, including dextran, CMC sodium, hyaluronic acid (HA), chondroitin sulfate, polyvinylpyrrolidone (PVP), and polyvinylalcohol (PVA).



The use of dissolving MNs is also a one-step administration that is pretty compliant for patients. Dissolving MNs have the unique advantages that they leave no harmful material and do not generate biohazardous sharp waste after application [69-71]. In addition, the mild preparation condition of dissolving MNs makes industrialization easier to achieve, which is quite beneficial to protein and peptide drugs.

2.5.

Hydrogel-forming MNs Hydrogel-forming MNs are usually fabricated from crosslinked polymeric materials, which can pierce the stratum corneum and absorb interstitial fluid to cause the polymeric matrix swell. The drug diffusion through the swollen matrix allows for the delivery to the dermal tissue. Hydrogel-forming MNs can be removed from the skin, leaving almost no polymeric residue behind [22]. Besides, the hydrogel-forming MNs also involve a one-step application, and its drug diffusion will not be blocked by compressed skin tissue like hollow MNs [22].



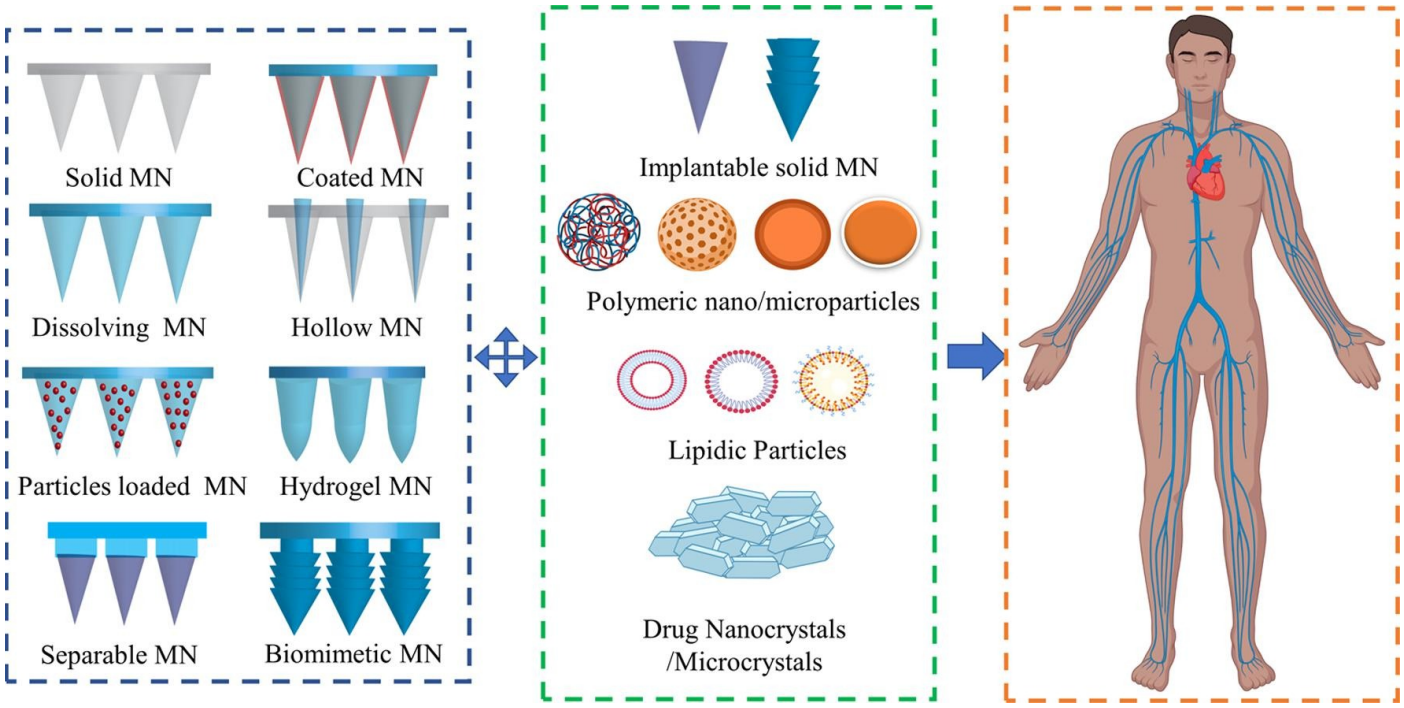
Hydrogel-forming MNs usually does not contain the drug, and instead, drugs are loaded into a matching reservoir, such as a polymeric film90. Therefore, it is not limited by the amount of drug that can be loaded into the needle or needle surface, which significantly increases the drug amount that can permeate into the skin. Recently, other forms of hydrogel-forming MNs have also appeared, in which the drug has not been loaded separately from the needles [73,91]. Novel in situ hydrogel-forming MNs were also developed using biocompatible thermosensitive copolymer.

5. Conclusions and prospects

Proteins and peptides have high specificity and potency compared to small molecules, which have been demonstrated to be effective for the treatment of various diseases. Nonetheless, because of the inherent properties of proteins and peptides, such as large molecular weight, poor stability, and conformational flexibility, they are usually administered by injection, which is inconvenient and unfriendly.

MNs can improve the patient's compliance and overcome the skin barrier for protein and peptide drugs. MNs have been developed in several designs with different delivery strategies, which can be generally classified into solid MNs, coated MNs, hollow MNs, dissolving MNs, and hydrogel-forming MNs. Skin plays a unique role in biology and immunomodulation. The active immune environment in the skin can synergize with the MNs-mediated vaccine delivery to fight infectious diseases and treat cancers. It is also an important application for MNs in diabetes treatment, and MNs also make safer closed-loop glucoseresponsive therapies possible.

MNs-mediated transdermal delivery of checkpoint inhibitors has reduced their off-target effect and achieved local targeted delivery to treat superficial cancers. In short, MNs are a very promising strategy for protein and peptide delivery to treat various diseases. The successful formulation of proteins and peptides depends on a thorough understanding of their physicochemical and biological characteristics. Notably, the formulation and handling of proteins and peptides need special attention in optimizing their stability and efficacy.



The research for addressing fundamental issues including drug loading, pharmacokinetic and pharmacodynamic profile, safety, and storage of MNs will promote transdermal protein and peptide drug delivery. With the advancement already achieved in the area of microfabrication technologies available in designing MNs, more intelligent MNs systems will gradually emerge. Proteins and peptides are potent active pharmaceutical ingredients, which may break the limit of low drug loading of MNs.

The comprehensive characterization methodologies, including both in vitro and in vivo, have been used to evaluate the ability of MNs to deliver drugs safely and effectively into the skin. The approaches currently used in the field will pave way to the development of standardized protocols for MNs evaluation in the future [97]. It is optimistically expected that extensive academic research in combination with the pharmaceutical industry will further accelerate the clinical translation of MNs mediated transdermal delivery of protein and peptide drugs.

Stabilising the Integrity of Snake Venom mRNA Stored under Tropical Field Conditions Expands Research Horizons

Gareth Whiteley[1], Rhiannon A E Logan[1], Kam-Yin D Leung[1], Fiona J Newberry[1], Paul D Rowley[1], John P Dunbar[1], Simon C Wagstaff[2], Nicholas R Casewell[1], Robert A Harrison[1]

2016 Jun 9;10(6):e0004615.doi: 10.1371/journal.pntd.0004615.eCollection 2016 Jun.

Abstract

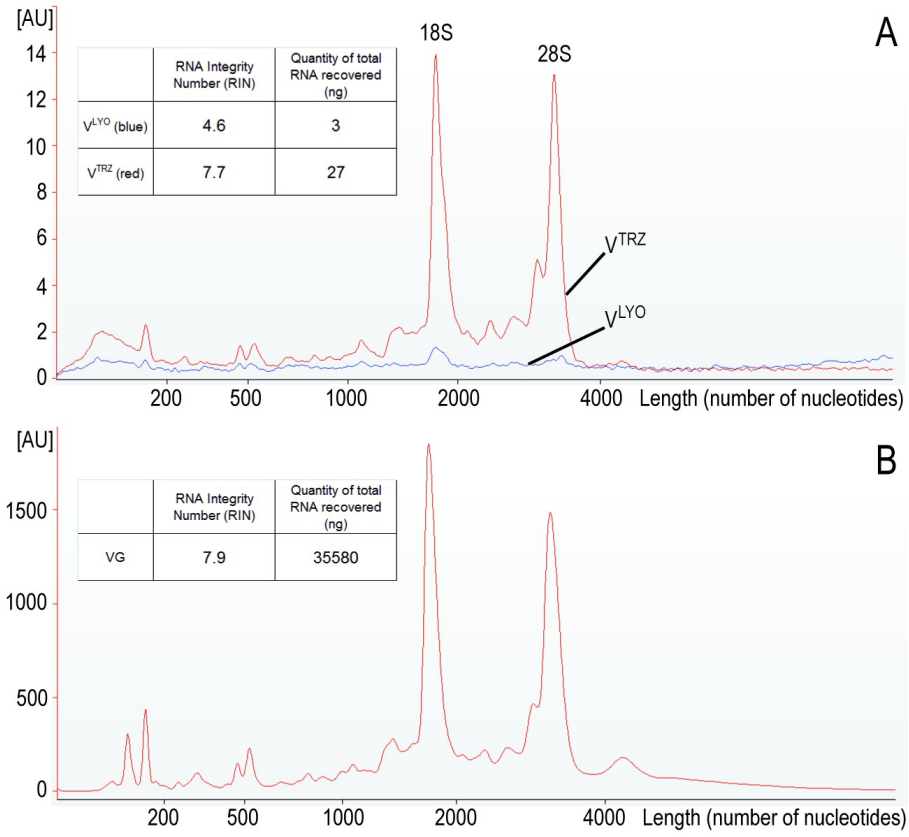
Background: Snake venoms contain many proteinaceous toxins that can cause severe pathology and mortality in snakebite victims. Interestingly, mRNA encoding such toxins can be recovered directly from venom, although yields are low and quality is unknown. It also remains unclear whether such RNA contains information about toxin isoforms and whether it is representative of mRNA recovered from conventional sources, such as the venom gland. Answering these questions will address the feasibility of using venom-derived RNA for future research relevant to biomedical and antivenom applications.

Venom gene	Forward primer	Reverse primer	Length of PCR product (base pairs)
AMP	TTGTGGAGTGGTAATTGCG	AGCCATTCTATGTTGCTCTT	2741
SVMP	CTCCAAAATGATCCAAGTTCTC	ATTTGGAAAAGGAAGCATGG	1880
LAO	ATTCCCATCCACAATCTTC	CGACATGTTTTGGCTGATATAC	1693
VEGF	GAGAGTAGACCGCAGGGGAACG	CAAAATGGCAAACAGGGAGATGAA	934
SP	GGATCCATGGTGCTGATCAGAGTG	CTCGAGTCACCGGGGGCAAGTCGC	782
CTL	CTGCCGGGAAGGAAGGAAGACCAT	GAGCGAAGGGGGCAGAGCAGAGAT	589

doi:10.1371/journal.pntd.0004615.t001

Full-length sequence Bitis arietansprimers

Methodology/principal findings: Venom was extracted from several species of snake, including both members of the Viperidae and Elapidae, and either lyophilized or immediately added to TRIzol reagent. TRIzol-treated venom was incubated at a range of temperatures (4-37°C) for a range of durations (0-48 hours), followed by subsequent RNA isolation and assessments of RNA quantity and quality.

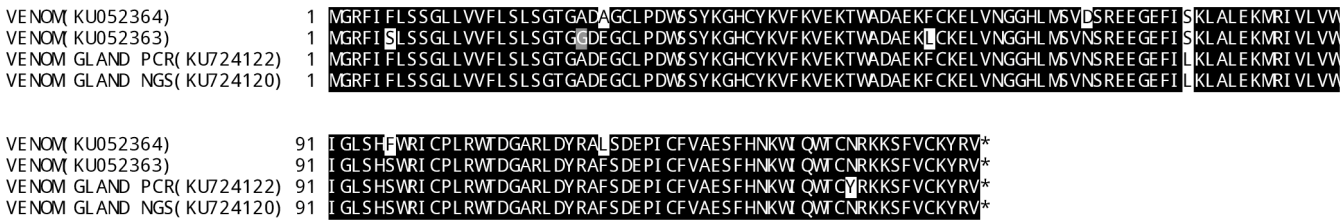


Quantitative and qualitative analysis of *Bitis arietanstotal* RNA isolated from lyophilized venom, TRizol-treated venom and venom gland.

Subsequently, full-length toxin transcripts were targeted for PCR amplification and Sanger sequencing. TRIzol-treated venom yielded total RNA of greater quantity and quality than lyophilized venom, and with quality comparable to venom gland-derived RNA in tropical conditions.

Full-length sequences from multiple Viperidae and Elapidae toxin families were successfully PCR amplified from TRIzol-treated venom RNA. We demonstrated that venom can be stored in TRIzol for 48 hours at 4-19°C, and 8 hours at 37°C, at minimal cost to RNA quality, and found that venom RNA encoded multiple toxin isoforms that seemed homologous (98-99% identity) to those found in the venom gland.

A



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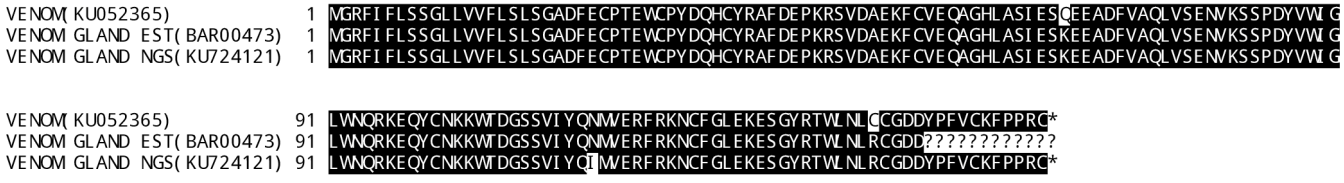


Full-length sequences

Conclusions/significance: The non-invasive experimental modifications we propose will facilitate the future investigation of venom composition by using venom as an alternative source to venom gland tissue for RNA-based studies, thus obviating the undesirable need to sacrifice snakes for such research purposes. In addition, they expand research horizons to rare, endangered or protected snake species and provide more flexibility to performing fieldwork on venomous snakes in tropical conditions.

Snake Venom Toxins Targeted at the Nervous System

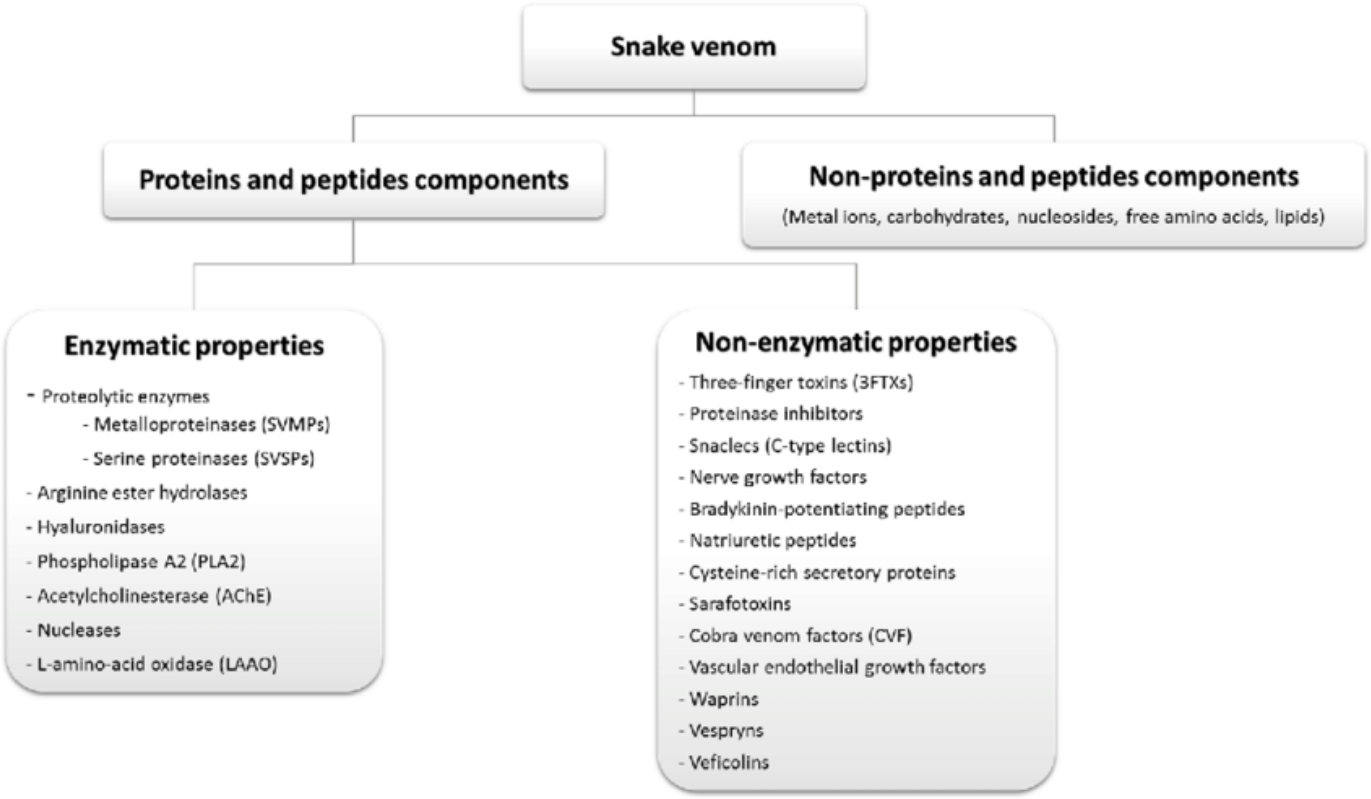
Alexey V. Osipov, Yuri N. Utkin

First Online: 27 February 2015

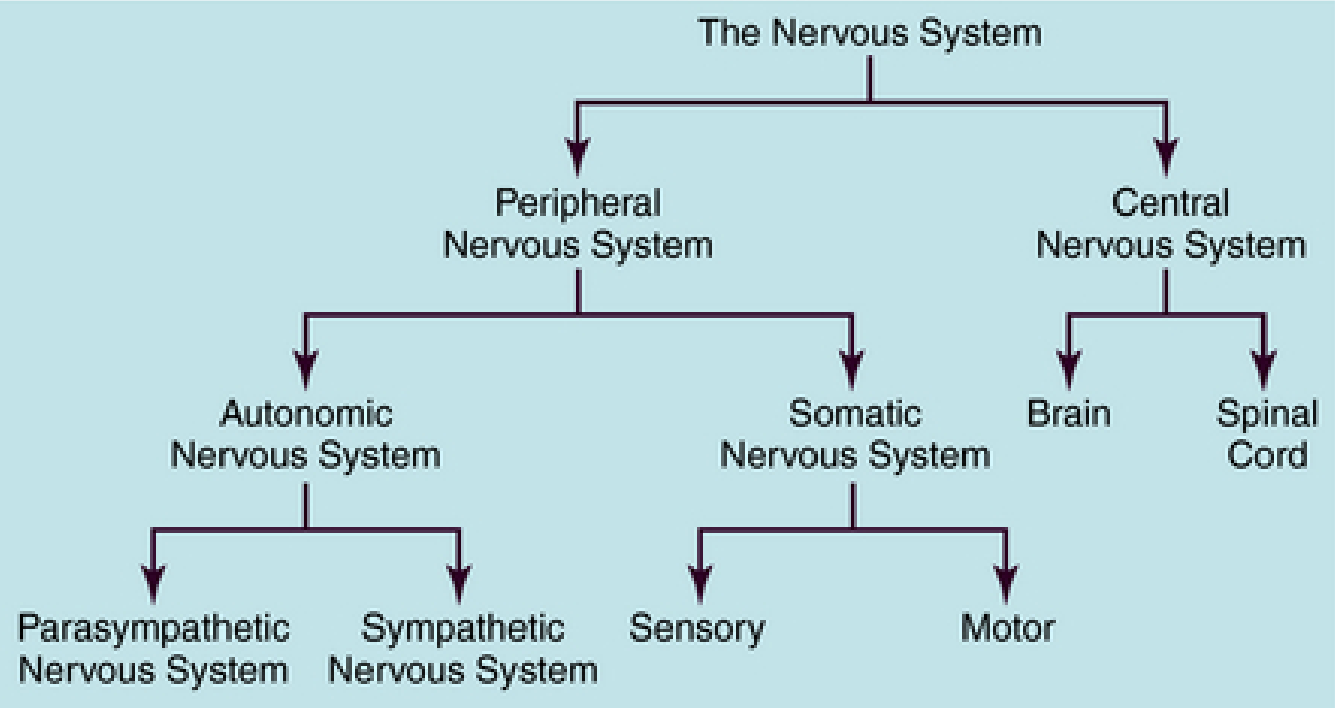
Abstract

One of the main venom targets in a prey organism is the nervous system. The disturbance of this system kills or paralyzes a prey effectively. To achieve this task, snake venoms contain an array of peptide and protein toxins called neurotoxins which belong to several structural types and possess diverse biological activities.

The most abundant neurotoxin groups are three-finger toxins and phospholipases A2, while other less represented groups include dendrotoxins of BPTI–Kunitz-type family, CRISPs (cysteine-rich secretory proteins), acetylcholine esterase, and peptide toxins.



Neurotoxins affect different departments of the nervous system including both the central nervous system (CNS) and the peripheral nervous system (PNS). The toxins impairing the somatic PNS are acting at presynaptic site (β-neurotoxicity) or postsynaptic site (α-neurotoxicity); there are neurotoxins active inside a synaptic cleft as well.



Effectors of sympathetic and parasympathetic systems of the autonomic PNS are also found in the venoms. Snake venom contains also neurotrophins and blockers of several types of ion channels, including effectors of sensory systems. Acting at different sites of the nervous system and being complementary, neurotoxins produce a cumulative effect resulting in very efficient oppression of the prey or predator.

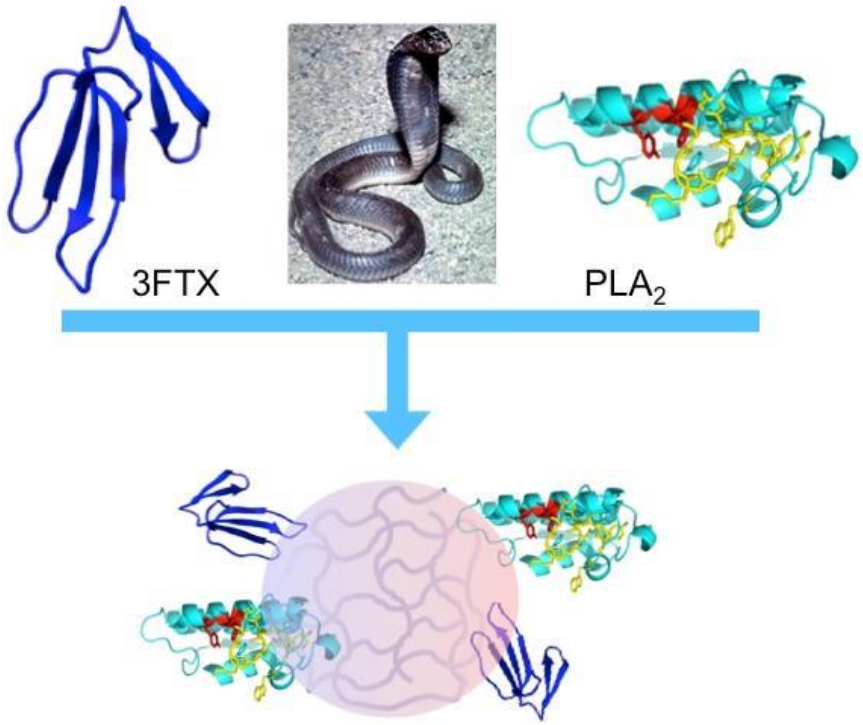
Effects of snake venom polypeptides on central nervous system

Alexey V. Osipov, Yuri N. Utkin

2012 Dec;12(4):315-28.doi: 10.2174/1871524128037606l8.

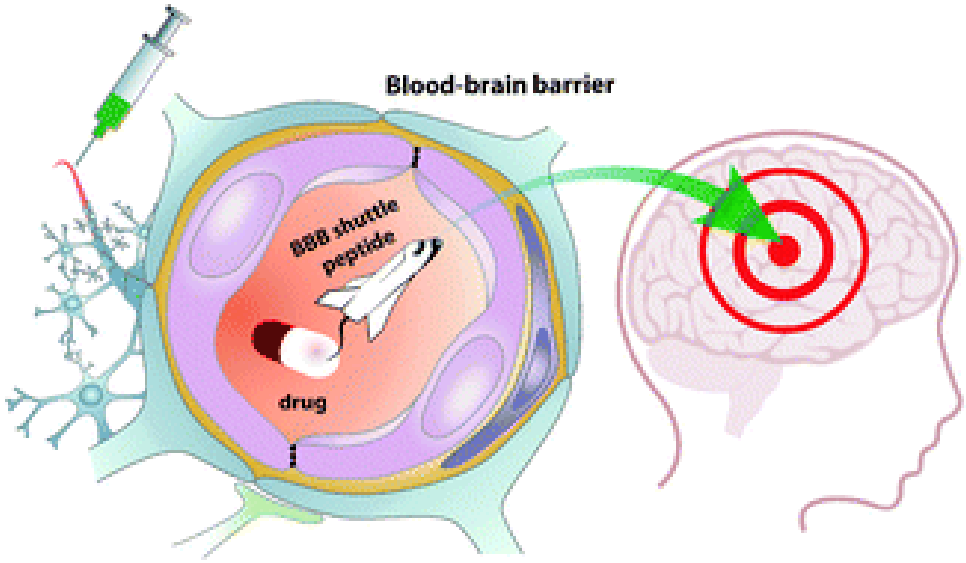
Abstract

The nervous system is a primary target for animal venoms as the impairment of its function results in the fast and efficient immobilization or death of a prey. There are numerous evidences about effects of crude snake venoms or isolated toxins on peripheral nervous system. However, the data on their interactions with the central nervous system (CNS) are not abundant, as the blood-brain barrier (BBB) impedes penetration of these compounds into brain.



Three-finger toxins and phospholipases A2

This updated review presents the data about interaction of snake venom polypeptides with CNS. Such data will be described according to three main modes of interactions: - Direct in vivo interaction of CNS with venom polypeptides either capable to penetrate BBB or injected into the brain. - In vitro interactions of cell or sub-cellular fractions of CNS with crude venoms or purified toxins. - Indirect effects of snake venoms or their components on functioning of CNS under different conditions. Although the venom components penetrating BBB are not numerous, they seem to be the most suitable candidates for the leads in drug design.



The compounds with other modes of action are more abundant and better studied, but the lack of the data about their ability to penetrate BBB may substantially aggravate the potentials for their medical perspectives. Nevertheless, many such compounds are used for research of CNS in vitro. These investigations may give invaluable information for understanding the molecular basis of CNS diseases and thus lay the basis for targeted drug design. This aspect also will be outlined in the review.

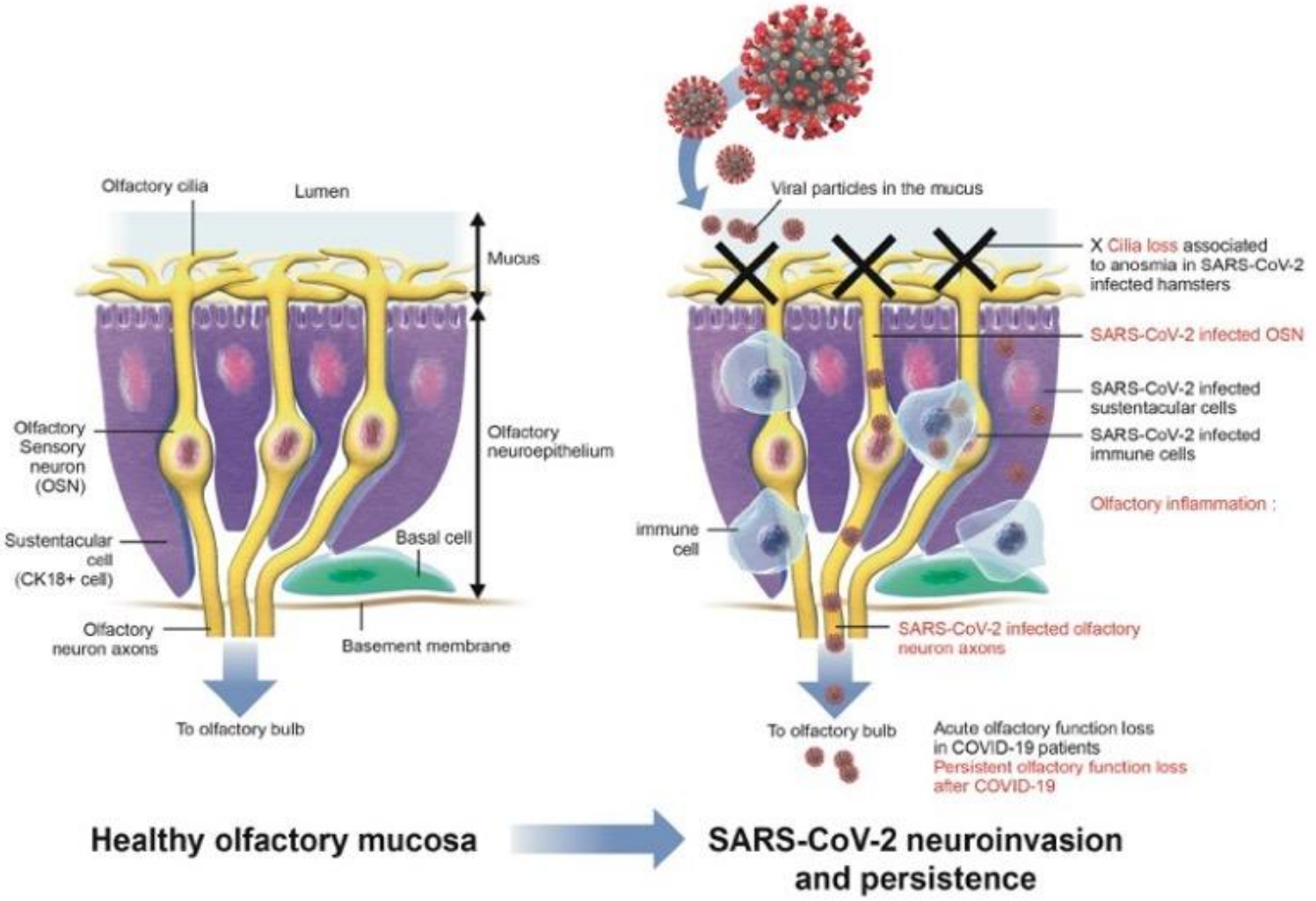
Persistent anosmia and olfactory bulb atrophy after mulga (Pseudechis australis) snakebite

Moksh Sethi[1], Mark Cook[2], Kenneth D Winkel[3]

2016 Jul;29:199-201.doi: 10.1016/j.jocn.2015.12.019.Epub 2016 Feb 17.

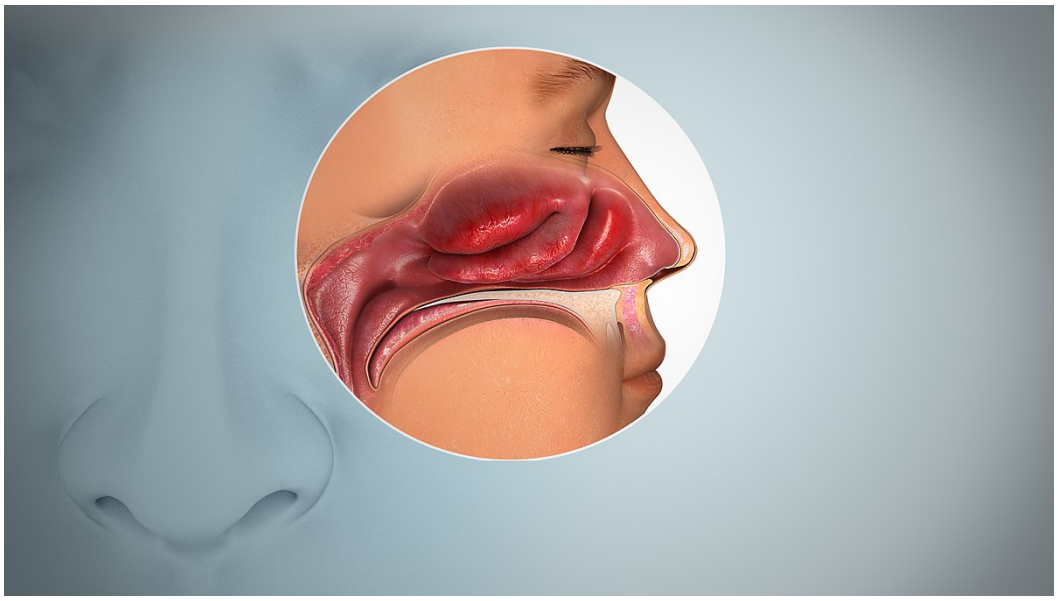
Abstract

Loss of sense of smell is an intriguing yet under-recognised complication of snakebite. We report olfactory function testing and neuroimaging of the olfactory bulbs in a 30-year-old man with anosmia persisting for more than 1 year after mulga (Pseudechis australis) snakebite. This problem was first noted by the patient 1 week after being definitely bitten in Queensland, Australia. He had then presented to a regional hospital where his envenomation was considered mild enough to not warrant antivenom administration. A week later the patient noted a reduction of sense of smell, which progressed to complete inability to smell over the ensuing weeks. On clinical review the patient's neurologic and rhinologic examination did not reveal any structural cause for anosmia.


Olfactory cilia
Lumen
Mucus
Olfactory Sensory neuron (OSN)
Olfactory neuroepithelium
Sustentacular cell (CK18+ cell)
Basal cell
Olfactory neuron axons
Basement membrane
To olfactory bulb
Viral particles in the mucus
X Cilia loss associated to anosmia in SARS-CoV-2 infected hamsters
SARS-CoV-2 infected OSN
SARS-CoV-2 infected sustentacular cells
SARS-CoV-2 infected immune cells
Olfactory inflammation :
immune cell
SARS-CoV-2 infected olfactory neuron axons
To olfactory bulb
Acute olfactory function loss in COVID-19 patients
Persistent olfactory function loss after COVID-19
Healthy olfactory mucosa
SARS-CoV-2 neuroinvasion and persistence

Formal olfactory testing was performed using ''sniffin' sticks" and the patient scored 17 on this test, indicating severe hyposmia (functional anosmia <16.5, normal score >30.3 for men aged 16-35years). MRI of the brain showed no abnormalities. The olfactory bulb volumes were then measured on a volumetric T2-weighted MRI that demonstrated significantly reduced volume of both bulbs, with the right 34.86mm(3) and left 36.25mm(3) (normal volume ⩾58mm(3), 10th centile).

The current patient represents a rare instance of a definite, untreated, elapid (mulga snake) envenomation with an intriguing disjunction between the mildness of the systemic features and the severity of the olfactory lesion. It is also unclear if early antivenom use attenuates this condition, and due to the delayed manifestation of the symptoms, awareness of this phenomenon may be lacking amongst physicians.



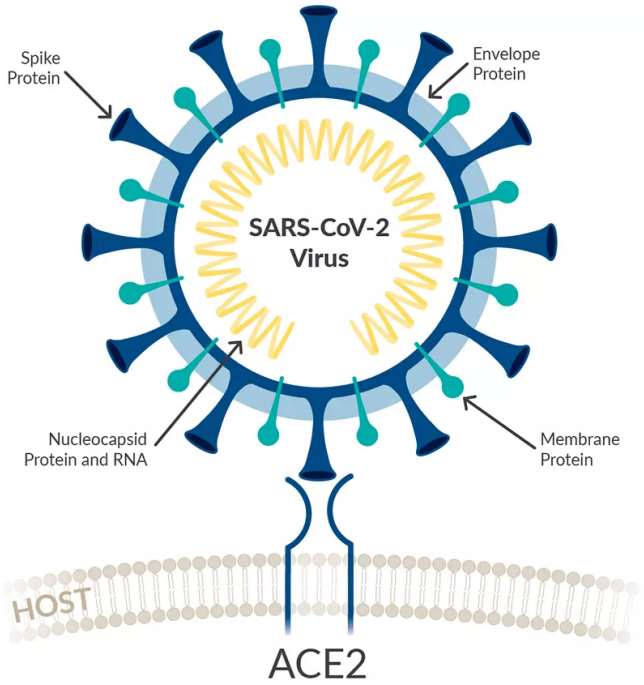
Snake venom phospholipases A2 possess a strong virucidal activity against SARS-CoV-2 in vitro and block the cell fusion mediated by spike glycoprotein interaction with the ACE2 receptor

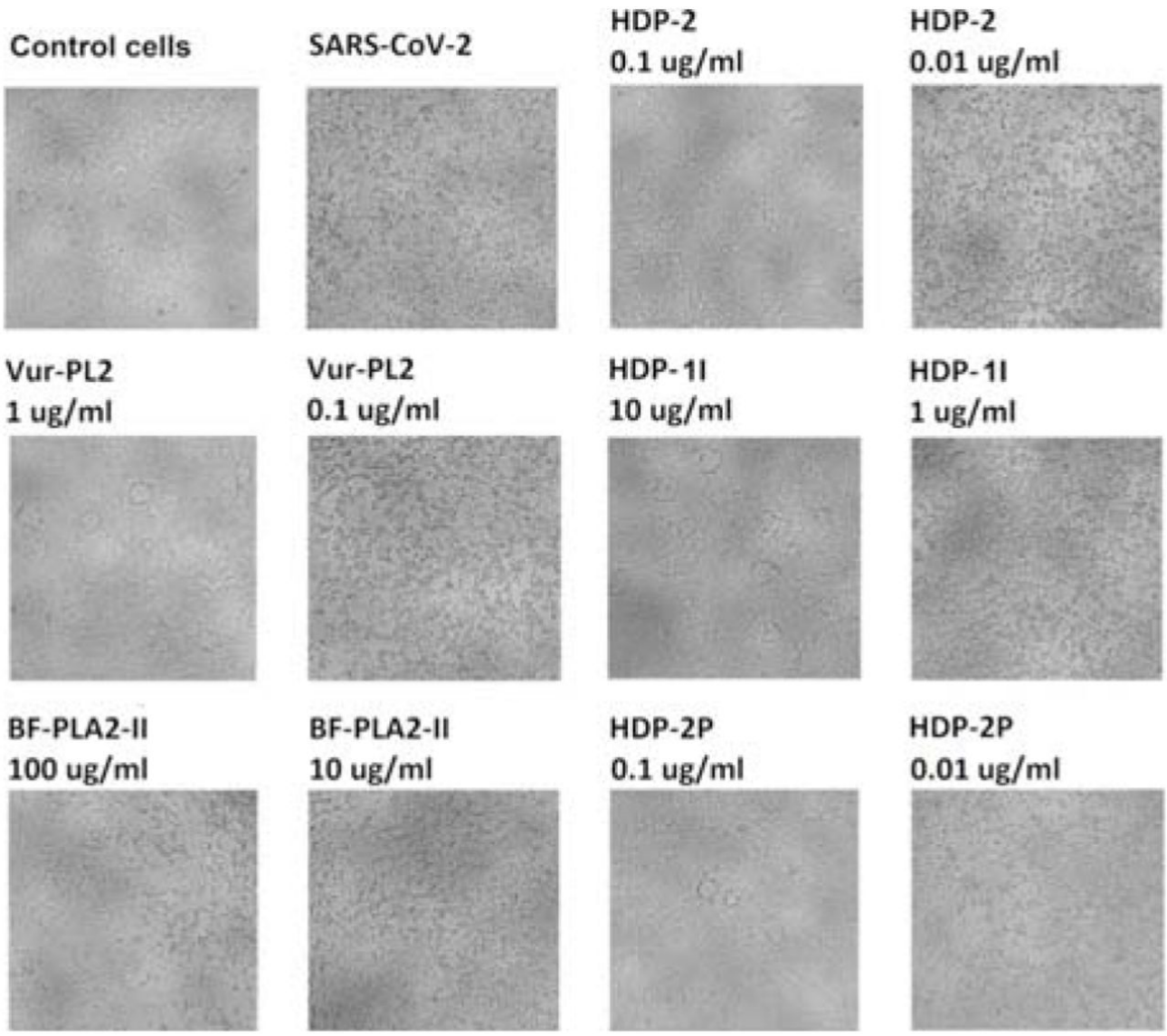
Andrei E. Siniavin, Maria A. Nikiforova, Svetlana D. Grinkina, Vladimir A. Gushchin, Vladislav G. Starkov, Alexey V. Osipov, Victor I. Tsetlin, Yuri N. Utkin

doi:https://doi.org/10.1101/2021.01.12.426042

Abstract

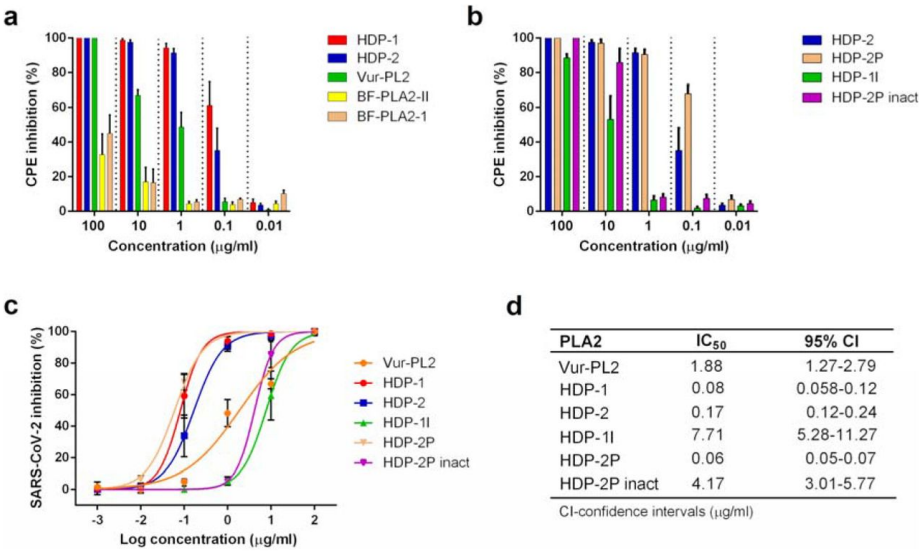
A new coronavirus was recently discovered and named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In the absence of specific therapeutic and prophylactic agents, the virus has infected almost hundred million people, of whom nearly two million have died from the viral disease COVID-19. The ongoing COVID-19 pandemic is a global threat requiring new therapeutic strategies. Among them, antiviral studies based on natural molecules are a promising approach.





Inhibition of the SARS-CoV-2 cytopathic effect by the snake venom PLA2s.

The superfamily of phospholipases A2 (PLA2s) consists of a large number of members that catalyze the hydrolysis of phospholipids at a specific position. Here we show that secreted PLA2s from the venom of various snakes protect to varying degrees the Vero E6 cells widely used for the replication of viruses with evident cytopathic action, from SARS-CoV-2 infection PLA2s showed low cytotoxicity to Vero E6 cells and the high antiviral activity against SARS-CoV-2 with IC50values ranged from 0.06 to 7.71 μg/ml. Dimeric PLA2 HDP-2 from the viper *Vipera nikolskii*, as well as its catalytic and inhibitory subunits, had potent virucidal (neutralizing) activity against SARS-CoV-2.



PLA2	IC_{50}	95% CI
Vur-PL2	1.88	1.27-2.79
HDP-1	0.08	0.058-0.12
HDP-2	0.17	0.12-0.24
HDP-1I	7.71	5.28-11.27
HDP-2P	0.06	0.05-0.07
HDP-2P inact	4.17	3.01-5.77

CI-confidence intervals (μg/ml)

Inactivation of the enzymatic activity of the catalytic subunit of dimeric PLA2 led to a significant decrease in antiviral activity. In addition, dimeric PLA2 inhibited cell-cell fusion mediated by SARS-CoV-2 spike glycoprotein. These results suggest that snake PLA2s, in particular dimeric ones, are promising candidates for the development of antiviral drugs that target lipid bilayers of the viral envelope and may be good tools to study the interaction of viruses with host cell membranes.

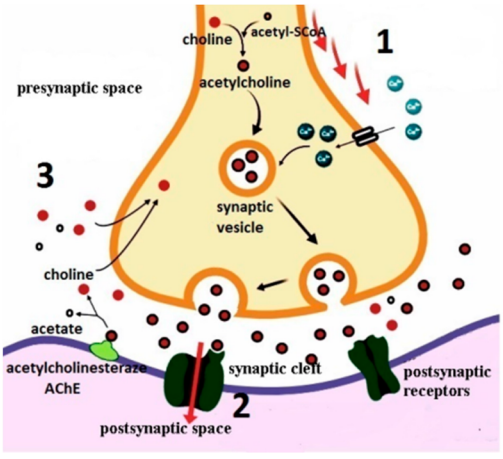
Nicotinic cholinergic system and COVID-19: *In silico* evaluation of nicotinic acetylcholine receptor agonists as potential therapeutic interventions

Nikolaos Alexandris[a1] George Lagoumintzis[ab1] Christos T. Chasapis[a1] Demetres D. Leonidas[c] Georgios E. Papadopoulos[c] Socrates J. Tzartos[d] Aristidis Tsatsakis[e] Elias Eliopoulos[f] Konstantinos Poulas[ab] Konstantinos Farsalinos[a]

https://doi.org/10.1016/j.toxrep.2020.12.013

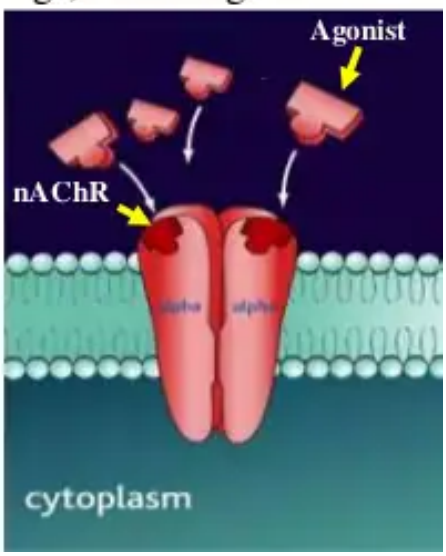
Abstract

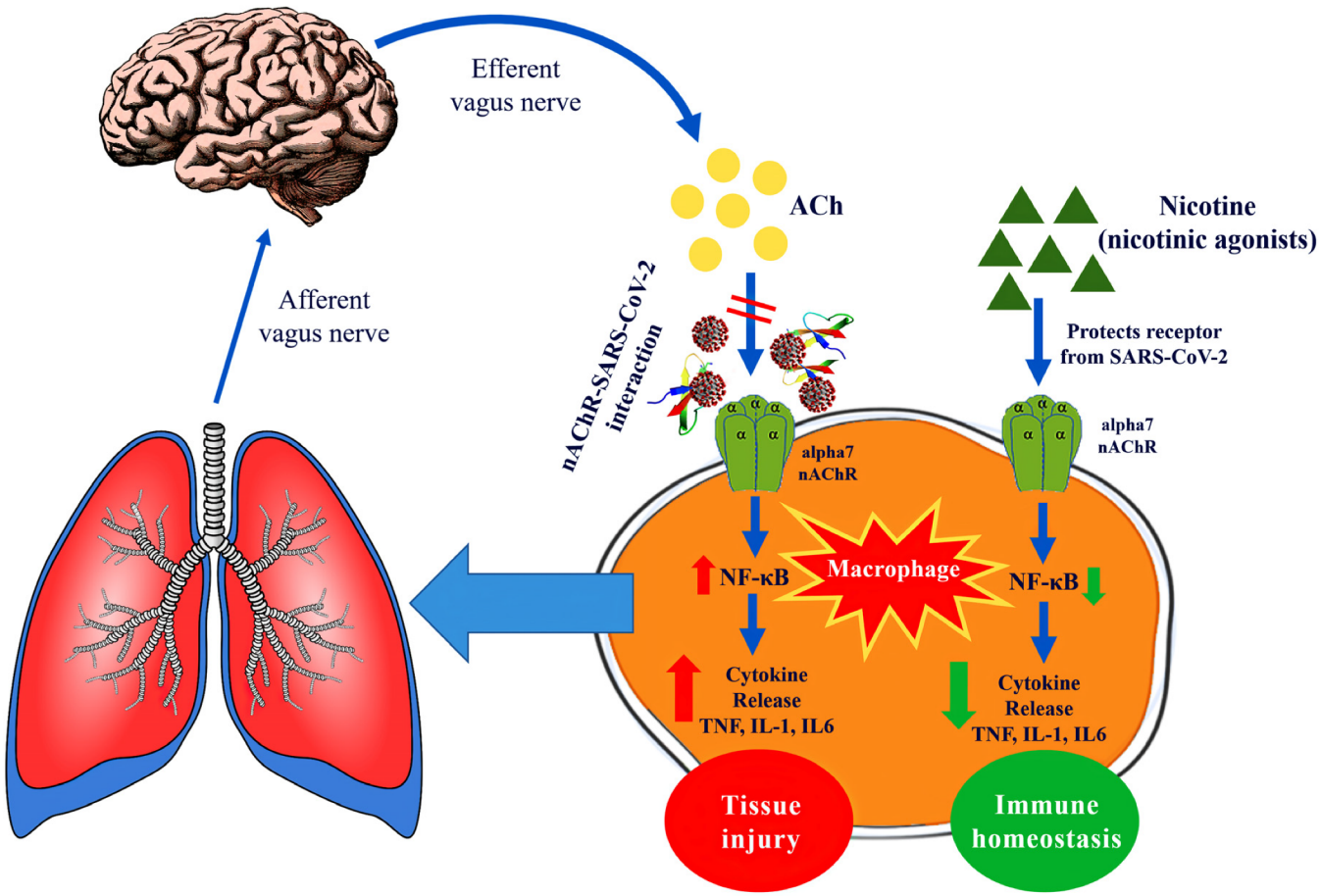
SARS-CoV-2 infection was announced as a pandemic in March 2020. Since then, several scientists have focused on the low prevalence of smokers among hospitalized COVID-19 patients. These findings led to our hypothesis that the Nicotinic Cholinergic System (NCS) plays a crucial role in the manifestation of COVID-19 and its severe symptoms. Molecular modeling revealed that the SARS-CoV-2 Spike glycoprotein might bind to nicotinic acetylcholine receptors (nAChRs) through a cryptic epitope homologous to snake toxins, substrates well documented and known for their affinity to the nAChRs. This binding model could provide logical explanations for the acute inflammatory disorder in patients with COVID-19, which may be linked to severe dysregulation of NCS.



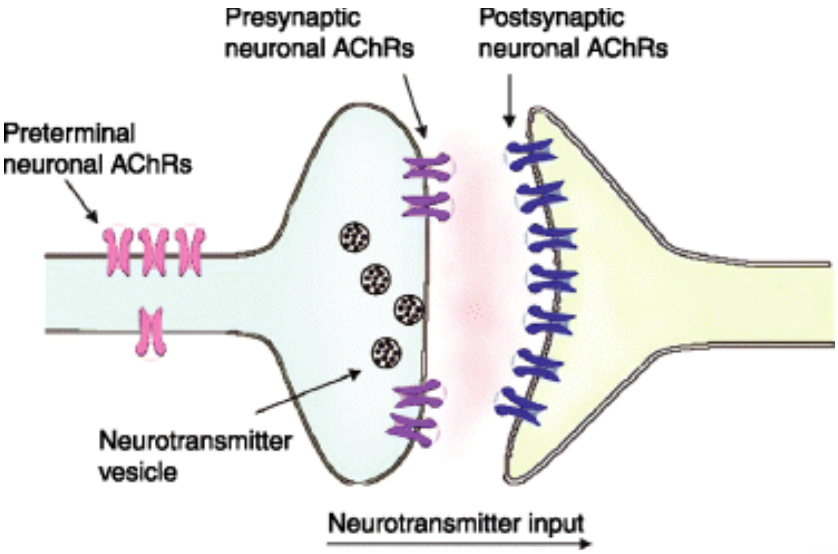
Nicotinic Acetylcholine Receptors (nAChRs)

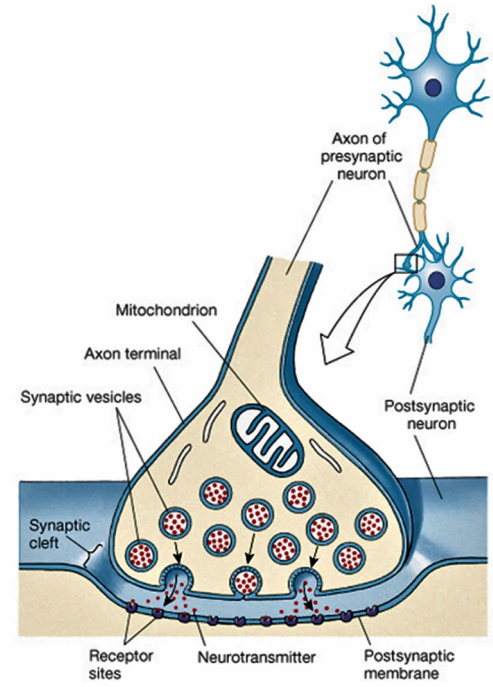
- Receptor proteins respond to the neurotransmitter, acetylcholine.
- Nicotinic Acetylcholine Receptors also respond to drugs, including the Nicotinic Receptor Agonist.
- They are found in the CNS and PNS, muscle and many other tissues of many organisms.
- In insects, limited to the central nervous system.
- Transmit outgoing signals from the presynaptic to the postsynaptic cells.





In this study, we present a series of complexes with cholinergic agonists that can potentially prevent SARS-CoV-2 Spike glycoprotein from binding to nAChRs, avoiding dysregulation of the NCS and moderating the symptoms and clinical manifestations of COVID-19. If our hypothesis is verified by *in vitro* and *in vivo* studies, repurposing agents currently approved for smoking cessation and neurological conditions could provide the scientific community with a therapeutic option in severe COVID-19.





Engineered Aptamers for Enhanced COVID-19 Theranostics

Caleb Acquah,1 Jaison Jeevanandam,2 Kei Xian Tan,3 and Michael K. Danquah 4

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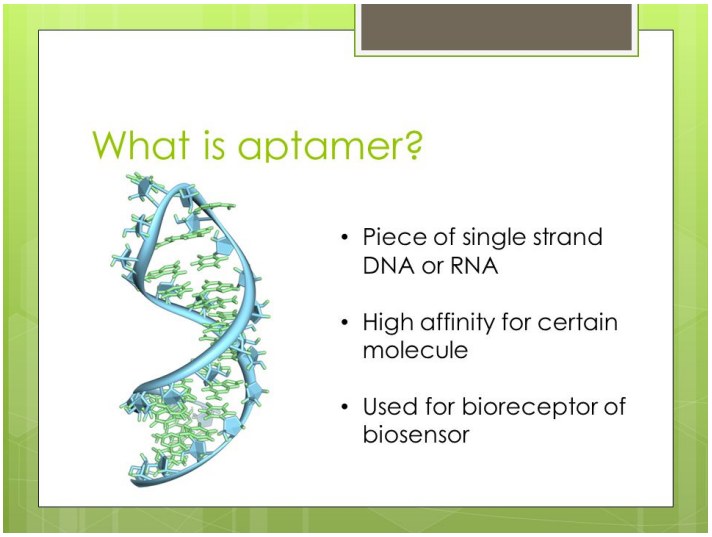
Abstract

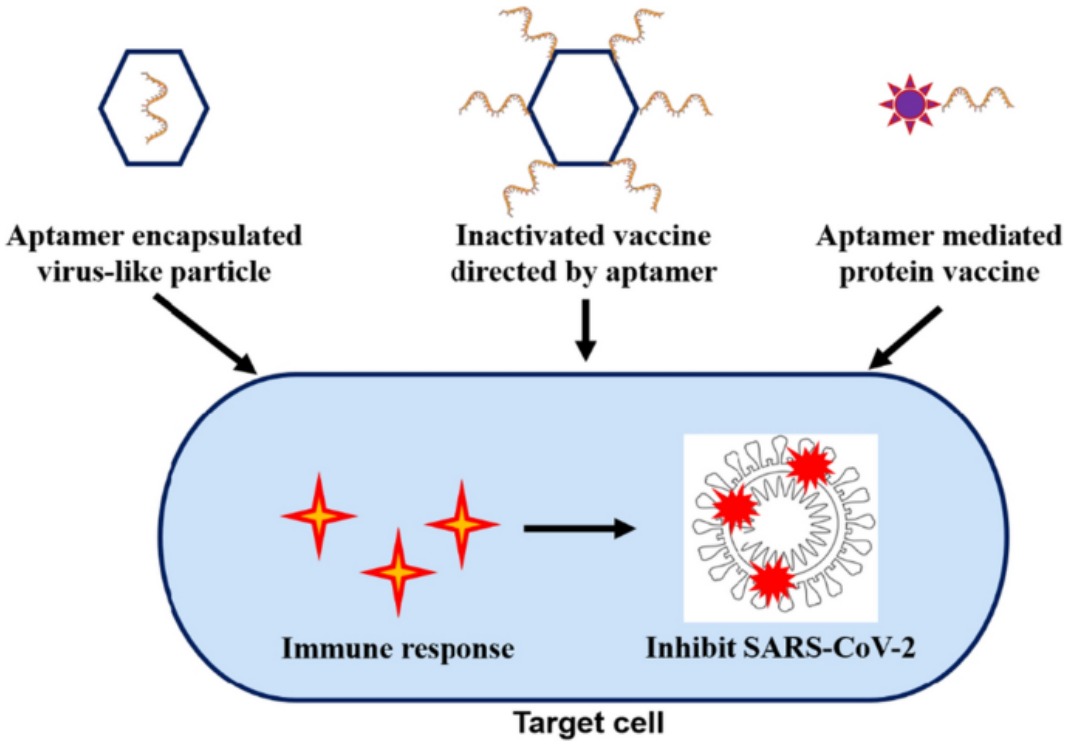
Introduction

The 2019-novel coronavirus disease (COVID-19) is an intractable global health challenge resulting in an aberrant rate of morbidity and mortality worldwide. The mode of entry for SARS-CoV-2 into host cells occurs through clathrin-mediated endocytosis. As part of the efforts to mitigate COVID-19 infections, rapid and accurate detection methods, as well as smart vaccine and drug designs with SARS-CoV-2 targeting capabilities are critically needed. This systematic review aimed to present a good mapping between the structural and functional characteristics of aptamers and their potential applications in COVID-19 theranostics (Ed. note: integration of diagnosis and therapy in a single platform using nanomaterials).



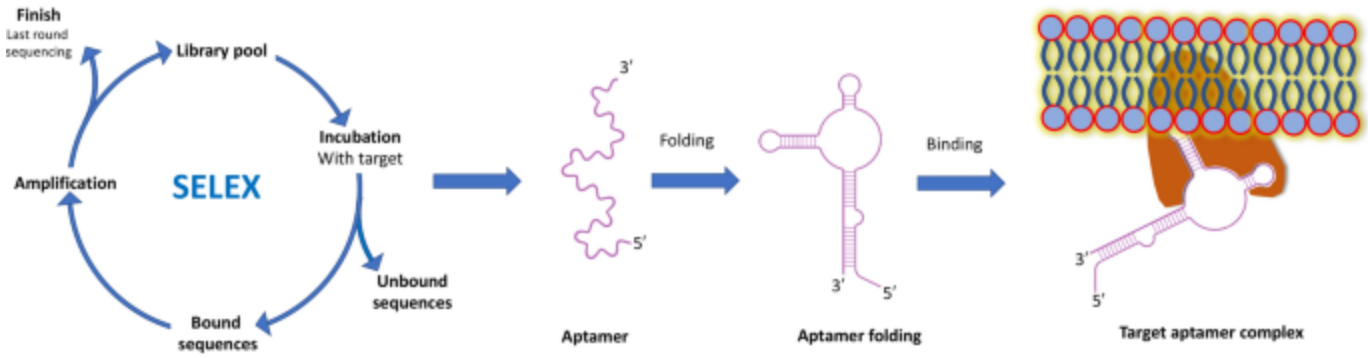
Methods

In this study, extensive discussions into the potential development of aptameric (Ed. note: Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule) systems as robust theranostics for rapid mitigation of the virulent SARS-CoV-2 was made. Information required for this study were extracted from a systematic review of literature in PubMed, SCOPUS, Web of Science (WOS), and other official related reports from reputable organisations.



Results

The global burden of COVID-19 pandemic was discussed including the progress in rapid detection, repurposing of existing antiviral drugs, and development of prophylactic vaccines. Aptamers have highly specific and stable target binding characteristics which can be generated and engineered with less complexity for COVID-19 targeted theranostic applications.



Systematic Evolution of Ligands by EXponential Enrichment (SELEX)

Conclusions

There is an urgent need to develop safe innovative biomedical technologies to mitigate the dire impact of COVID-19 on public health worldwide. Research advances into aptameric systems bode well with the fact that they can be engineered for the development of effective and affordable diagnostics, therapeutics and prophylactic vaccines for SARS-CoV-2 and other infectious pathogens.

Snakes Could Be the Original Source of the New Coronavirus Outbreak in China

A study of the virus's genetic sequence suggests similarities to that seen in snakes, but the origin must still be verified

By Haitao Guo, Guangxiang "George" Luo, Shou-Jiang Gao,
The Conversation US on January 22, 2020

Excerpt

FROM BATS TO SNAKES

The researchers used an analysis of the protein codes favored by the new coronavirus and compared it to the protein codes from coronaviruses found in different animal hosts, like birds, snakes, marmots, hedgehogs, manis, bats and humans. Surprisingly, they found that the protein codes in the 2019-nCoV are most similar to those used in snakes.

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