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Authors
Molino, Nicholas M
Wang, Szu-Wen

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Caged Protein Nanoparticles for Drug Delivery

Nicholas M. Molino and **Szu-Wen Wang***
Department of Chemical Engineering and Materials Science, University of California, 916 Engineering Tower, Irvine, CA 92697-2575

Abstract

Caged protein nanoparticles possess many desirable features for drug delivery, such as ideal sizes for endocytosis, non-toxic biodegradability, and the ability to functionalize at three distinct interfaces (external, internal, and inter-subunit) using the tools of protein engineering. Researchers have harnessed these attributes by covalently and non-covalently loading therapeutic molecules through mechanisms that facilitate release within specific microenvironments. Effective delivery depends on several factors, including specific targeting, cell uptake, release kinetics, and systemic clearance. The innate ability of the immune system to recognize and respond to proteins has recently been exploited to deliver therapeutic compounds with these platforms for immunomodulation. The diversity of drugs, loading/release mechanisms, therapeutic targets, and therapeutic efficacy are discussed in this review.

Introduction

Nanoparticles have the potential to address important issues related drug delivery, such as (i) reducing drug toxicity, (ii) protection from drug degradation/sequestration, (iii) increasing circulation times, (iv) targeting, and (v) increasing bioavailability [1–3]. Key particle variables, including size, surface charge, geometry, and the susceptibility to opsonization, all affect pharmacokinetic profiles [4]. Additionally, small size distributions of the nanomaterial allow for uniformity in drug dosing, with the size range of ~25–50 nm being optimal for receptor-mediated endocytosis (RME) and membrane wrapping kinetics of target cells [5]. Also critical for drug delivery success using nanoparticles is the capability to functionalize with multiple elements in a uniform and precise manner.

Conventional materials investigated for drug delivery include synthetic polymeric and liposomal nanoparticles [3]. These, however, may have limitations such as wide size distributions, difficulty in site-specific functionalization, low drug loading, and instability. Caged proteins represent a class of nanomaterial that may address many of these concerns [2,6–8]. Since the earliest example in drug delivery [9], advances have been made in understanding the architecture, assembly, physical properties, and biomedical applicability

*Corresponding author. phone: 949-824-2383, wangsw@uci.edu.

of these nanoparticulate systems. Recent progress towards drug delivery using caged protein scaffolds will be covered in this review.

Advantages of Caged Protein Nanocapsules for Drug Delivery

Caged protein complexes are hollow structures comprised of self-assembling protein subunits that produce nanocapsules with a nearly monodisperse size distribution. The individual asymmetrical subunits may comprise a single protein, as with pyruvate dehydrogenase E2 [10], or multiple proteins, such as with cowpea mosaic virus (CPMV) [11]. Typical sizes of protein nanocages range from 10–100 nm, and they display repetitive symmetrical features, both of which are ideal structural features for RME [5].

Protein cages are often produced in living hosts (*e.g.*, *E. coli*, plants, mammalian cells), and therefore the tools of protein engineering may be applied to impart functional elements at three distinct interfaces (*i.e.*, internal, external, and inter-subunit) [7]. This permits fine control over surface charge, drug encapsulation, ligand display, and particle stability. Classes of protein cages used in drug delivery include those derived from viruses, enzymes, the ferritin superfamily, and heat shock proteins [6,7]. While virus-like particles (VLPs) have found early applications as vehicles for gene therapy [12] and vaccines for infectious agents [13], this review will focus on delivery of therapeutic drugs.

Drug Loading and Release

Key aspects in nanoparticle drug delivery technologies are the containment and release of drugs within the particle, and these mechanisms are often related. The strategies available for a particulate system are dictated by its structure and dynamics, the type of drug loaded, and environment the nanoparticle is expected to encounter. Many avenues exist for functionalizing a protein cage, and the primary approaches are described below (Figure 1).

Protein Engineering of Nanoparticles

One main advantage of using protein nanoparticles over other systems is the fine precision afforded by genetic engineering of functional sites at distinct locations on the nanoparticles, such as introduction of non-native amino acids [8]. For instance, a single cysteine point mutation introduces an exact number of new attachment sites, providing unique positions for drug conjugation and additional control in loading amounts [10]. The physicochemical character of the nanoparticle's hollow interior cavity can also be re-engineered to accommodate non-native hydrophobic [*14] or charged molecules [*15]. Knowledge of the protein crystal structure allows recombinant incorporation of peptides and/or entire proteins as N- or C-terminal chimeras or within loop regions [16–20]. Recombinant incorporation of peptide/protein therapeutics may prove to be one of the most effective loading mechanisms; once the genetic code for the cage-drug chimera has been modified, the nanoparticle requires little or no additional processing for use as a drug delivery vehicle. Complexities may arise, however, if recombinant incorporation of these peptides or proteins affects nanoparticle stability or assembly.

Chemical Immobilization and Release

Covalent protein-drug conjugates are a common method of drug loading, and we refer to a prior review with thorough discussions of these strategies [8]. The tools of chemistry allow post-translational bioconjugation of small molecules to amino acid side chains (either native or recombinantly engineered), such as amines [11,*21–23], carboxyls [11,24–26], cysteines [10,23,27,28], tyrosines [29], and non-native side chains through click chemistry [8,*21]. Not all chemistries applied to drug conjugation are straightforward or facile. Therefore, while providing novel and interesting drug delivery platforms, chemical strategies should be carefully chosen to avoid difficult conjugation schemes that may result in low yields, protein denaturation or disassembly, and a downstream requirement for lengthy purification steps.

Covalent attachment also allows for control over release kinetics. For example, molecules immobilized to adenovirus and ferritin through stable amide or thioether bonds are retained during physiologic conditions, but may be released during biodegradation [11,26,30]. Labile disulfide linkages permit drug release in reducing environments, and hydrazone bonds enable release in mildly acidic environments, both reported to occur during endocytosis [10,11,27,28]. The selection of release mechanism depends on the chemical properties of the drug-protein conjugate (*e.g.*, chemical stability, release kinetics) and physiological needs (*e.g.*, the microenvironment encountered by nanoparticles).

Non-Covalent Interactions in Drug Loading and Release

Protein particles often possess internal cavity surfaces with natural affinities for molecules such as nucleic acids or metals, and drugs with similar physical properties may be retained within the core. For example, single-stranded DNA can be encapsulated in hepatitis B VLPs (HBV) and Qβ nanoparticles through electrostatic interactions [31], and ferritin can chelate metal-based drugs like cisplatin [32]. Alternatively, the interior of protein nanoparticles may be redesigned to bind non-native molecules, such as in the genetic engineering of the internal E2 cavity to create a hydrophobic pocket for drug adsorption [*14], introduction of siRNA binding motifs in HBV [33], or the directed evolution of lumazine synthase to increase the affinity for infectious HIV protease through electrostatic interactions [*15].

Drugs may also interact through non-specific physical interactions with a secondary carrier molecule, which has an affinity for the protein cage interior. For instance, after diffusing through natural pores, DOX and proflavin associate with the native RNA of cucumber mosaic virus and CPMV, respectively [*34,*35]. Forming a complex between drug and negatively-charged polymeric or metallic carriers may yield encapsulation of drug within the protein [36–*39]. More stable encapsulation can be achieved through covalent ligation of drug to the secondary carrier molecules (*e.g.*, MS2 bacteriophage RNA) [**40–44], or of the protein capsule to the carrier molecule [45].

Utilizing non-specific interactions for encapsulation usually allows the slow non-triggered release of drug over time under physiologic conditions. Specific environmental conditions, however, can facilitate increased release rates. For example, the mildly acidic environment of the endosome increased drug solubility and accelerated release of hydrophobically-

associated DOX [*14], and glutathione reduction of cyclodextrins conjugated to protein nanoparticles enabled the release of the paclitaxel-cyclodextrin complexes [45].

Environmentally-Triggered Structural Changes of Nanoparticles

In loading mechanisms requiring diffusion of drug molecules, often native pores of the caged structure enable access to the hollow interior. Some VLPs have gated pores, whereby low salt concentrations [46], basic pH [7], or osmotic shock [47] can cause the pores to swell open, allowing entry of the drug. Reversal of conditions then retains drug, preventing outward diffusion. In red clover necrotic mosaic VLPs, low magnesium and calcium levels, like that of the cytosol, promote opening of pores for intracellular drug release [46].

Native disassembly and reassembly of the protein cage under specific conditions also allows for drug encapsulation [32,33,**40,41,48]. Protein nanoparticle assembly behavior can also be manipulated by reengineering the protein subunit interfaces [49]. Alternatively, VLPs may be isolated as subunits and assembled following purification, although this method can produce particles with size heterogeneity [25]. For drug release, this type of encapsulation may require biodegradation or introduction of repulsive interactions at inter-subunit interfaces to induce environmentally-triggered disassembly [**40,41,49,50]. For downstream applications, loading mechanisms should be relatively fast, straightforward, and require little additional protein cage manipulation that may cause increased formulation heterogeneity or loss of product. Disassembly-reassembly loading mechanisms may prove to be particularly challenging; for example, Yang *et al.* obtained higher product recovery when platinum-containing drugs were generated *in situ* within the intact apoferritin core, compared to a strategy of simultaneous assembly of protein with drug [32].

Targeting Drugs to the Therapeutic Site

While protein cages have a tremendous degree of flexibility for drug containment and release, a potential drawback of their small size is loading capacity. However, accumulation of drug-containing nanoparticles at the therapeutic site may reduce the amount of drug-protein complexes required for therapeutic effect. Biodistribution studies with intravenously-administrated CPMV, CCMV, and heat shock protein have demonstrated a broad *in vivo* distribution and rapid clearance in a non-pathogenic model [51,52], and subcutaneous injection of Qβ VLPs show passive transport to the lymphatic system for interaction with immune components [53,54]. In the biodistribution for cancer, the enhanced permeation and retention effect (EPR) may allow accumulation of nanoparticles within the tumor vicinity through the leaky vasculature [2]. Although it is likely that the protein nanoparticle source and administration route may affect biodistribution, one conclusion from these studies is that protein cages can preferentially accumulate in distinct locations.

To overcome the natural biodistribution, attachment of targeting ligands can increase tropism and avidity for target tissues. This can be particularly effective with protein cages because of their unique geometry, allowing repeated and patterned display of targeting ligands. For example, the RGD amino acid motif, naturally present on adenoviruses, has affinity for upregulated integrin receptors of endothelial cells of tumor vessels; incorporation of this peptide to the surface of protein nanoparticles was shown to target drugs to tumor

[*39,55]. Certain cancer cells are known to upregulate their expression of folate, glycan, or growth factor receptors, and therefore attachment of receptor-specific ligands on the nanoparticle surface has also been investigated [*21,**22,25,38]. Other ligands for biological targeting have included peptides [**40,46], DNA aptamers [56], and antibodies [30]. Ligand density also plays an important role in targeting, and this can easily be controlled on protein cage surfaces [**40].

Effectiveness of Protein Cage-Delivered Therapeutics

A diverse collection of virus-like and non-viral particles has been examined for delivery of a variety of drugs (summarized in Table 1). Therapeutic efficacy can vary widely, even when the same drug molecule is used. As a point of comparison, doxorubicin (DOX) has been often used as a model drug. Many *in vitro* studies show that cytotoxicity of protein cage-delivered DOX is similar to or even less effective to that of the free drug, but are comparable with IC_{50} values from DOX-loaded polymeric nanoparticles [10]. When combining active targeting, protein nanocages were shown to specifically deliver DOX *via* the folate or integrin receptors. In some cases, increased cytotoxicity and enhanced anti-tumor effects were observed, compared to free drug [*35,*39]. However, others have demonstrated no cytotoxicity increase *in vitro* of folate-targeted DOX-loaded protein nanoparticles despite a significantly higher cellular uptake due to folate; this is likely due to the mismatch between rates of *in vitro* uptake and of intracellular drug release [57].

The variability observed in experimental results between protein particles appears to be affected by the different combinations of conditions, such as drug loading capacities and mechanisms, drug release kinetics, and rates of cellular uptake. This suggests that conditions need to be tested and optimized for each system. We note that this range of results is not unique for protein-based nanoparticles, but is also observed for experiments utilizing conventional polymeric nanoparticle systems.

Drugs with relatively poor passage through cellular membranes, such as interfering RNA [**40,42,43,47,55], bleomycin [26], and proteins [**19,**40], can exhibit a clear enhanced therapeutic benefit when delivered within a protein cage, even in the absence of specific targeting. For these types of drugs, the increased propensity for RME of protein cages enhances intracellular accumulation and therefore the potency of the drug. Other drugs with low efficacy in free unbound formulation, like peptides and photosensitizing drugs, show enhanced function when delivered with protein cages to their biological target [17,*21,23,**40,58].

Immunological aspects of protein nanoparticles

A major concern with protein nanoparticles, which exhibit repeating virus-like structural patterns, is immunogenicity. Relatively high IgG titers and B-cell numbers were observed following single administrations of cowpea chlorotic mottle virus, CPMV, and heat shock protein [51,52]. Elevated immune responses, especially following multiple administrations, could induce rapid clearance or neutralization of a drug delivery system or create potentially severe inflammatory responses [51,52].

Since the FDA approval of Doxil, the attachment of polyethylene glycol (PEG) to nanoparticles is conventionally investigated for immune evasion and enhanced pharmacokinetics. PEGylation can reduce immune cell uptake in adenovirus and other protein cages [59,60], and varying the molecular weight of the PEG modulates the extent of reduced immune recognition [59]. While the effect of PEGylation on access to the protein cage interior has not been directly investigated, *in vitro* studies did not demonstrate any inhibitory effects of polymer attachment on the intracellular release of encapsulated drug, indicating that small molecules are not blocked from passage through native pores [**40,57]. Additionally, PEGylation of CPMV and E2 cages did not alter the physical structure or stability of the nanoparticles, nor did it prevent unfolding of the E2 nanoparticle under extreme conditions [59,61]. Recent investigations, however, have also shown evidence for anti-PEG immune responses, with PEG-coated nanoparticles mediating increased complement activation and IgM antibody production [61,62]. Alternative methods are being explored, such as coating with polyketals [63] or glycan shielding [64], and the future clinical success of protein cages as drug delivery vehicles will rely on the ability to safely tune and harness immune responses.

One emerging area of research is to exploit the immune system's ability to recognize and interact with these protein platforms. Since subcutaneously-injected protein cages passively target lymphatics and are naturally recognized by many cells of the immune system, protein cages are particularly well-suited for delivering immune-modulating drugs for applications such as cancer immunotherapies or autoimmune disease treatment. For example, co-delivery of CpG DNA motifs and antigen in the E2 nanoparticle yields increased dendritic cell (DC) uptake and activation, and increased antigen cross-presentation, compared to free forms of the drug [*65]. CpG has also been non-specifically loaded to Qβ and HBV VLPs for *in vivo* delivery to DCs, and the inflammatory side-effects associated with the drug were alleviated [31]. Other immune-modulating drugs, such as IL-2 and IFN-γ, have also been explored for similar purposes [18,**19] (Table 1).

Summary and Future Outlook

Caged protein nanoparticles allow the introduction of multiple functional elements at precise interfacial locations. This has enabled different mechanisms of drug encapsulation, controlled release, and specific targeting, the combinations of which have shown benefit to treatment of tumors *in vivo* [**22,*39]. A new strategy being explored is the rational design of protein cages *in silico* [66], which has the potential to redesign native cages or develop novel cages tailored to a specific application.

Many recent studies have shown that the effectiveness of drugs can be comparable to or even better than that of free drug while reducing side effects, especially when targeted to a therapeutic site. While immune recognition is a concern for some applications, researchers have begun to harness this natural effect by delivering immune-modulating drugs to various cell types. It may be that protein cages are particularly well-suited for immune-based treatments, and clinical trials involving Qβ VLP-mediated delivery of CpG drug molecule have emerged for application in cancer immunotherapy [*67]. We are now starting to see the

emergence of these nanoplatforms for therapeutic applications, and future studies should further reveal their niche in the clinical realm of drug delivery.

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Highlights for Molino & Wang review

- Caged proteins can overcome limitations of other nanoparticles for drug delivery.
- Unique physical, chemical, and recombinant methods allow drug loading/release.
- Simultaneously display of drug and targeting molecules can improve drug efficacy.
- Caged proteins are well-suited for immunotherapy due to similarities with viruses.

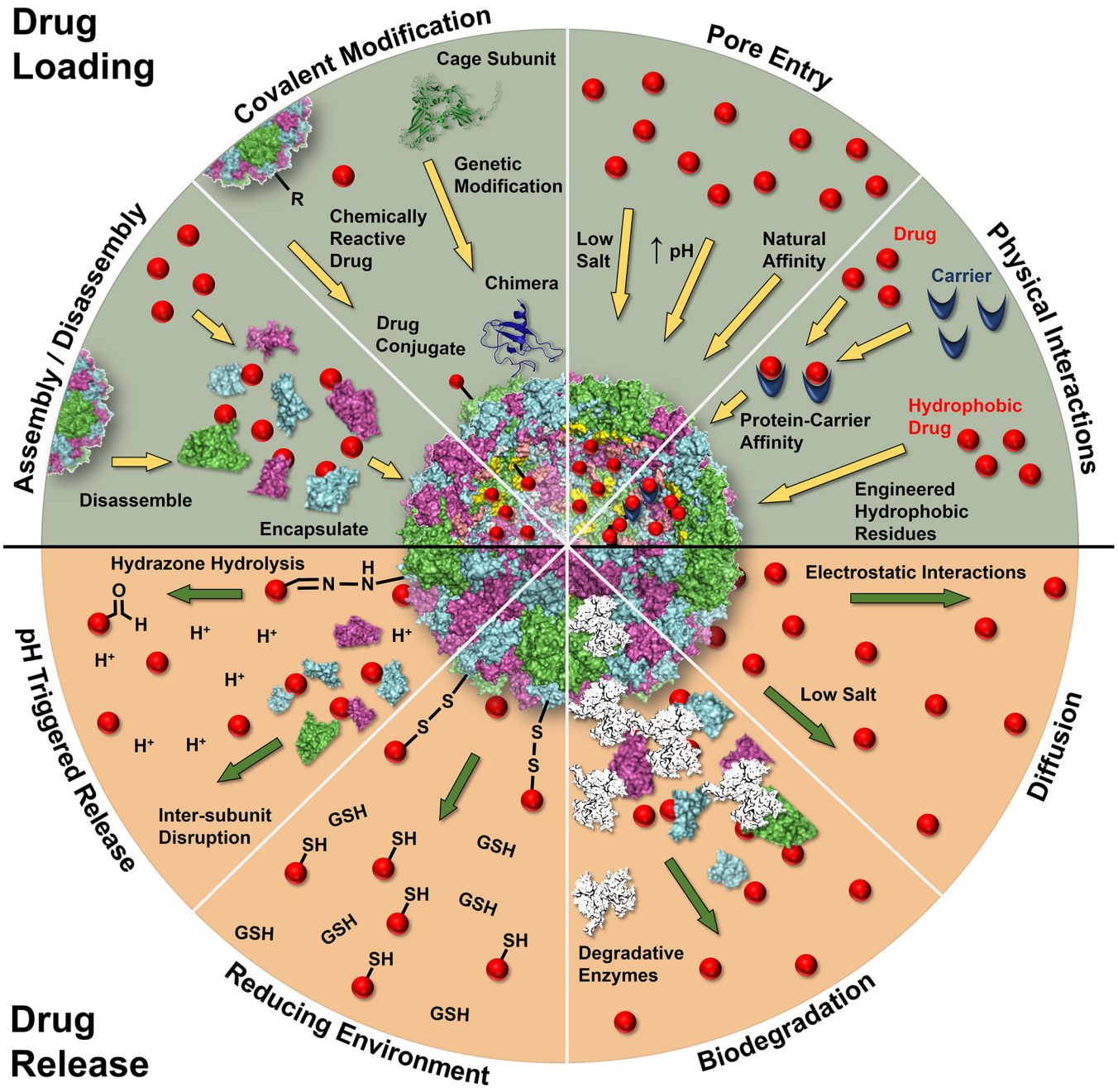

Figure 1.

Summary of methods explored for drug loading (upper panel) and drug release (lower panel) in protein nanoparticle cages. R = any reactive amino acid side chain; GSH = glutathione. Representative protein cage scaffold is cowpea chlorotic mottle virus (PDB code 1CWP, assembly from VIPERdb; http://viperdb.scripps.edu/).

Table 1

Summary of various caged protein systems, both viral and non-viral, which have been explored as delivery vehicles for therapeutics. The specific caged protein, drugs used, and the phase of research are given.

Protein cage	Drug	Phase of Recent Investigations	References
Virus-like particle			
Adenovirus	bleomycin	*in vitro*	[26]
	paclitaxel	*in vivo*	[**22]
Avian sarcoma leukosis virus	Cre recombinase	*in vitro*	[**19]
	5-FU		
	Caspace-8		
	Ifn-γ		
	TRAIL		
Cowpea chlorotic mottle virus	$Ru(bpy_2)$phen-IA	*in vitro*	[58]
Cowpea mosaic virus	DOX	*in vitro*	[11]
	proflavin		[*34]
Cucumber mosaic virus	DOX	*in vivo*	[*35]
Hepatitis B virus	CCL19	*in vivo*	[18]
	IL-2		
	CpG		[31]
	siRNA		[55]
Hibiscus ringspot virus	DOX	*in vitro*	[38]
HIV-1	CD40L	*in vivo*	[16]
JC polyomavirus	paclitaxel	*in vitro*	[45]
	siRNA	*in vivo*	[47]
MS2 Bacteriophage	siRNA	*in vitro*	[**40,41,55]
	DOX		[**40]
	5-fluorouracil		
	ricin toxin A-chain		
	miRNA	*in vivo*	[42,43]
	antisense ssDNA		[44]
Murine polyomavirus	methotrexate	*in vitro*	[24]
Qβ	EGF	*in vitro*	[68]
	porphyrin		[*21]
	CpG	*phase 2 clinical trial*	[*67]
Red clover necrotic mosaic virus	DOX	*in vitro*	[46]
Rotavirus	DOX	*in vitro*	[25]
SHIV	CD40L	*in vivo*	[20]
Non-viral particle			
E2 subunit of pyruvate dehydrogenase	DOX	*in vitro*	[10,57]

Protein cage	Drug	Phase of Recent Investigations	References
	CpG		[*65]
Ferritin	duanomycin	*in vitro*	[37]
	cisplatin		[32]
	5-fluorouracil		[36]
	trastuzumab		[69]
	AP-1 peptide	*in vivo*	[17]
	DOX		[*39]
DNA binding protein	SnCe6	*in vitro*	[23]
Heat shock protein	DOX	*in vitro*	[27,28]