Journal of
Nanoscience and Nanotechnology
Vol. 7, 1–11, 2007

Grouping of Ferritin and Gold Nanoparticles Conjugated to pRNA of the Phage phi29 DNA-Packaging Motor

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The bacteriophage phi29 DNA-packaging motor, which translocates and compresses the DNA genome of the phage into its procapsid during virion assembly, involves an essential ring formed by the packaging RNA (pRNA). We attached electron-dense nanoparticles to pRNA by hybridizing a DNA oligonucleotide with a biotin or thiol modification to a 3′-extension of core pRNA, and by coupling streptavidin and biotinylated ferritin, or 5 nm and 10 nm colloidal gold particles, to these modifications. The pRNA conjugates bound to RNA-free phi29 procapsids, and such nanoparticle-bearing procapsids were isolated by ultracentrifugation and analyzed. Electron microscopy showed that ferritin and gold particles were specifically attached to the side of the phi29 procapsid harboring the connector, which is the pRNA binding site. The pRNA-ferritin conjugates bound to procapsids with the same efficiency as pRNA which lacked the high molecular mass label. However, full DNA packaging efficiency in an *in vitro* phage assembly assay was only reached after the label of such isolated procapsid-pRNA complexes had been released with RNase H. The results provide approaches to assembled ferritin or gold-containing nano-complexes via pRNA mediated assembly.

Keywords: Bacteriophage phi29, DNA-Packaging, Supramolecular Nanoparticle Organization, Site-Specific RNA Labeling, RNA Conjugate.

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1. INTRODUCTION

The DNA-packaging motor of bacteriophage phi29 involves an RNA molecule called packaging RNA (pRNA).[1–3] DNA-packaging is a step in the assembly of double-stranded (ds) DNA viruses including the tailed bacteriophages, during which the dsDNA genome is inserted into a viral procapsid. Procapsids are assembled as protective protein shells that are initially DNA-free.[4–6] Since DNA is packaged to high density inside procapsids, the DNA packaging process generally requires an ATP driven biomolecular motor.[4, 7–12] At present the precise molecular mechanism of these motors is still elusive, but bacteriophage phi29 has come to serve as a model virus for dsDNA packaging studies. Phi29 is a comparatively small and well characterized bacteriophage (reviewed in Ref. [13]) that infects *Bacillus subtilis*. The presence of an RNA component in the DNA packaging motor is one of the characteristics of the phi29 family of phages. A defined *in vitro* DNA packaging system is available,[14–16] and the phi29 DNA packaging motor was studied by structural, biochemical, and single-molecule approaches (reviewed in Refs. [3, 4]). pRNA functions by forming an oligomeric ring at the unique vertex of the procapsid that harbors the connector through which DNA is inserted. These pRNA rings can be assembled on procapsids *in vitro* with wildtype or engineered pRNA.[1, 17–19] Even though pRNA is transcribed as a 174-nucleotide molecule, the first 120 nucleotides were found to be sufficient for *in vitro* DNA packaging.[17, 20] The structure of this core region of pRNA and its intra- and intermolecular interactions were characterized by a variety of approaches including phylogenetic analysis, nuclease probing, mutation analysis, computer modeling, and chemical methods including chemical modification and modification interference, crosslinking and photoaffinity crosslinking (reviewed in Ref. [2]). pRNA dimers and trimers can function as assembly intermediates in the formation of the oligomeric pRNA ring.[21–24] Dimer, trimer, and ring formation is enabled by four intermolecular basepairs that form between two loops of pRNA.[21, 22, 25, 26] Since each "left hand" loop pairs with a "right hand" loop of a neighbouring molecule, and vice versa, this pseudoknot interaction was called "hand-in-hand" interaction.[22, 26] The goal of the present work was to establish groundwork for a possible application of phage phi29 for *in vitro* assembly of nanocomponents and operation of hybrid biological/non-biological

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nanodevices. The *in vitro* assembly of pRNA rings can be programmed by mixing pRNA molecules with complementary loop mutations.[21,22] If pRNA rings are assembled from mixtures of two, three, or six complementary pRNA mutants according to Guo et al.,[22] and each of the involved mutants is conjugated to a different nanomaterial, the pRNA moiety of conjugates may be able to direct the targeted assembly of defined groups of nanocomponents. pRNA mediated assembly has previously been suggested and tested for the formation of defined nanocomplexes as nano-building blocks or for biomedical targeting and delivery applications.[27–33] The same principle may be useful to complement other approaches[34–39] of using biomolecular self-assembly for precisely arranging particles on a nanometer scale. Furthermore, if motors incorporating pRNA conjugates retain their biological DNA packaging activity, a new approach to powering nanodevices by a biological motor may be possible. Such hybrid biological/non-biological devices and their potential applications have previously been described.[40,41] A second goal of the present report was to work towards a single particle approach of studying pRNA stoichiometry, which was recently reported.[42]

In the present paper, we report the preparation of conjugates between pRNA and ferritin or colloidal gold. These conjugates were prepared by extending pRNA at the 3′ end, hybridizing a synthetic DNA oligonucleotide with a biotin or thiol modification to the pRNA extension, and reacting biotin with streptavidin and biotinylated ferritin, or the thiol group with colloidal 5 nm or 10 nm gold. Colloidal gold was chosen as a readily available model nanoparticle. Ferritin is a natural iron storage protein with a 12 nm protein shell and an iron core.[43] Although it is a biological particle, the iron core gives it enough electron density for good observation by electron microscopy. Ferritin was also described as a template for the assembly of semiconductor nanoparticles.[44,45] We obtained preparations of both pRNA-gold and pRNA-ferritin conjugates. We also prepared phage procapsid preparations that were free of detectable RNase activity in order to prevent degradation of the conjugates during *in vitro* assembly. Both conjugates showed pRNA-mediated procapsid binding activity. Procapsids with bound pRNA-ferritin could be separated from free conjugates by ultracentrifugation. Phage assembly studies with these procapsids revealed that pRNA-ferritin conjugates formed functionally correct pRNA rings on procapsids. Our results suggest that pRNA conjugates may be useful for single particle studies of phage assembly and for the programmed self-assembly of defined multi-component nanostructures.

2. MATERIALS AND METHODS

2.1. Preparation of pRNA and pRNA-Conjugates

pRNA was synthesized by *in vitro* transcription with T7 RNA polymerase from a PCR-generated template as previously described.[19,46,47] Radiolabelled pRNA was prepared by including [^{3}H] UTP (Amersham) in the transcription reaction. The 120 nucleotides core region of pRNA was wild-type sequence[17] except for the mutations U1G and C2G, which were made for efficient *in vitro* transcription by T7 RNA polymerase, and the complementary mutations G116C and A117C, which were made to restore basepairing in the helical stem.[48] pRNA-3′-ext. was transcribed as a 143 nucleotides molecule with an altered sequence in pRNA bases 118–120 and a 23 bases extension of sequence originally derived from a plasmid vector (Fig. 1). This 118–143 sequence was 5′-AAUCCCGCGGCCATGGCGGCCGGGAG-3′. After transcription, pRNA was purified by electrophoresis

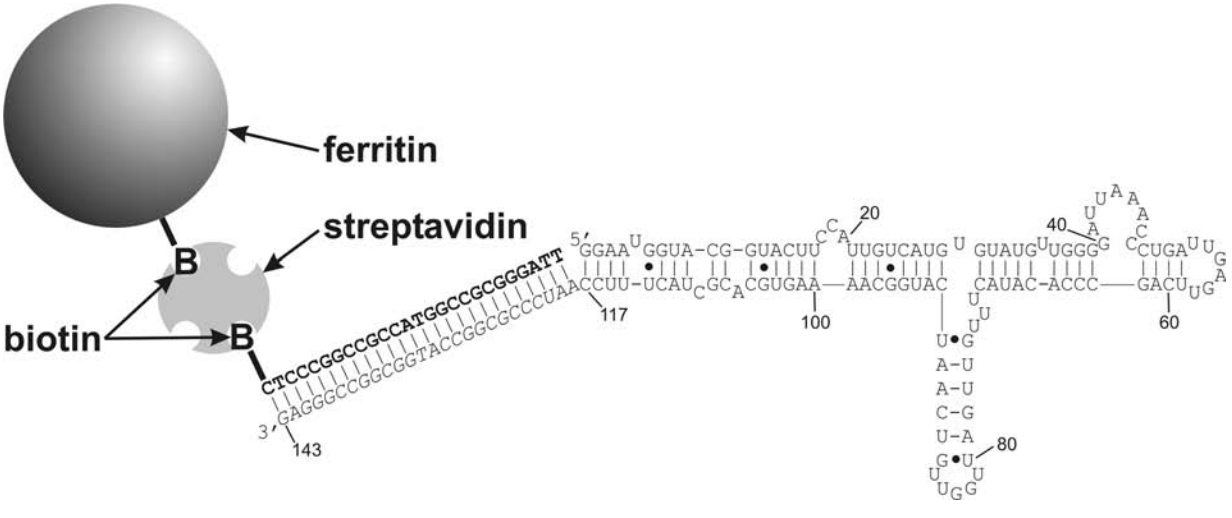

Fig. 1. Schematic drawing of pRNA-ferritin conjugate. The core region of pRNA (bases 1 to 117) is shown in the secondary structure proposed by Bailey et al. 1990.[74] Bases 118–143 are an extension with artificial sequence. A synthetic DNA oligonucleotide (bold face) bearing a biotin modification was hybridized to the pRNA extension. Streptavidin and biotinylated ferritin were bound as described in the Materials and Methods section.

through 8% polyacrylamide 8 M urea gels and isolated by passive elution in SDS-buffer and ethanol precipitation as previously described.[19, 46]

A biotin- or thiol-group was incorporated into pRNA by annealing a complementary DNA oligonucleotide, bearing the chemical modification, to bases 118–143 of pRNA-3′-ext. (Fig. 1). The DNA oligonucleotides were purchased from IDT (Integrated DNA Technologies, Coralville, IA, USA) in HPLC-purified quality. Both modifications were tethered to the 5′ end of the DNA oligo through a C6 spacer arm. Annealing was performed by heating a mixture of 10 μM pRNA-3′-ext. and 50 μM DNA oligonucleotide in phosphate buffered saline for 2 min at 75 °C, and cooling to 4 °C at a rate of 2 °C/min in a thermocycler. The annealed pRNA-DNA hybrid was purified by polyacrylamide gel electrophoresis in an 8 M urea gel at room temperature as above. The 26 bp hybrid region was not denatured under these conditions.

pRNA-streptavidin was prepared by rapidly mixing streptavidin (Sigma) with pRNA-biotin on a vortex. Final concentrations were 2.2 μM pRNA-biotin and 13.2 μM streptavidin in TMS buffer (50 mM Tris-Cl pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$). 1 μl Superasin (Ambion) per 250 μl reaction was added for protection from enzymatic RNA degradation. After 1 h incubation at 4 °C, the sample was separated on a 0.7% agarose gel in TAE buffer. Under these conditions, pRNA-streptavidin migrated slower than streptavidin with two bound pRNA molecules, which formed as a by-product. The top band was cut out and the conjugate was electroeluted into a dialysis bag. After filtration through a 0.45 μm cellulose acetate spin filter (Life Science Products Inc.) the sample was concentrated in a YM-30 centrifugal ultrafiltration unit (Millipore) to a final concentration of 0.36 μM.

Ferritin (Sigma) was biotinylated with sulfo-NHS-XX-biotin (Molecular Probes) according to the manufacturer's instructions. After the reaction, the sample was dialysed against saline at 4 °C over 4 days with several changes of saline. Aggregated ferritin was removed by centrifugation (2 min 16000× g), and traces of free biotinylation reagent were removed by ultrafiltration and 5× washing with phosphate buffered saline in a microcon YM-100 centrifugal ultrafiltration unit (Millipore). The retentate was preserved with 0.01% sodium azide and stored at 4 °C. Before use, an aliquot was sonicated and traces of aggregates were removed by centrifugation (2 min 16000× g). In preliminary experiments, the amount of this preparation of biotinylated ferritin required to completely react pRNA-streptavidin to a slower migrating band was determined. The determined amount was added to pRNA-streptavidin (final concentration: 120 nM), and after 15 min incubation at ambient temperature, this pRNA-ferritin sample was used without further purification.

RNase H treatment of pRNA conjugates was performed by adding 0.25 u RNase H (Promega) to 50 ng pRNA in RNase H buffer (75 mM KCl, 50 mM Tris-Cl pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol) and incubating 1 h at 37 °C. Digested pRNA samples were analyzed by agarose and polyacrylamide gel electrophoresis following standard procedures.

pRNA-thiol was prepared as pRNA-biotin above. Directly before use, the thiol group was reduced by adding dithiothreitol (final concentration 10 mM) to a 0.7 μM pRNA-thiol sample. After 20 min incubation at room temperature, pRNA-thiol was recovered by using the QIAEX II kit (QIAGEN). A protocol by Zanchet et al. 2001[49] was modified for coupling of pRNA-thiol to 5 nm and 10 nm colloidal gold (British Biocell International). To 1100 μl gold colloid (5 nm: 5.0×10^{13}; 10 nm: 5.7×10^{12} particles/ml), 45 pmol (5 nm) or 12 pmol (10 nm) pRNA-thiol were added in a volume of 100 μl, which corresponds to molar ratios of pRNA to gold of 1:2 and 1:1, respectively. With 150 μl 5× TBE (Tris-borate buffer, pH 8.3) and 150 μl 500 mM NaCl, the solution was brought to 1500 μl (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, 50 mM NaCl). After 8 to 12 h incubation at 4 °C, 20 μl 5% BSA (ultrapure, Ambion) were added and incubation was continued for another 8 to 12 h. The sample was loaded on top of 3.6 ml 10% glycerol in $0.5 \times$ TBE, 50 mM NaCl, and centrifuged at 35000 rpm for 2 h 30 min at 20 °C in the SW55 rotor of a Beckman L-80 ultracentrifuge. Under these conditions, only the gold particles and pRNA-gold conjugates, but not pRNA-thiol sedimented. Most of the buffer was removed by aspiration, and the gold particles were resuspended in the bottom 75 μl of buffer. 25 μl glycerol was added and the conjugates were stored at −20 °C. Typical final molar ratios of pRNA-thiol to 5 nm and 10 nm gold were 1:5 and 1:2.5, respectively. These ratios were calculated from scintillation counting of radiolabelled pRNA, assuming complete recovery of the gold colloid. pRNA-gold conjugates were analyzed by electrophoresis in 2% agarose gels in $0.5 \times$ TBE buffer. Red bands of colloidal gold were visible directly, and pRNA was visualized by ethidium bromide staining.

2.2. Preparation of RNase-Free phi29 Procapsid

A conventional preparation of phi29 procapsid was prepared as previously described.[50] Briefly, the procapsid protein genes on plasmid pARgp7-8-8.5-10 were expressed in *E. coli*, and procapsid was isolated by ultracentrifugation of cell lysate. To remove DNA, RNA, protein impurities, and RNase activity from this conventional preparation, 200 μl were diluted to 1 ml with TMS buffer (50 mM Tris-Cl pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$), 0.3 μg DNase I (Sigma) and 0.3 μg RNase A (Sigma) were added, and the sample was incubated for 16 h at ambient temperature. The sample was loaded on top of 4 ml 10% sucrose in TMS buffer, and procapsids were sedimented by ultracentrifugation (3 h, 35000 rpm, 20 °C; SW55 rotor; Beckman

L-80 ultracentrifuge). The buffer was removed by aspiration, and the procapsids were resuspended in 200 μl TMS buffer. This sample was sonicated briefly, aggregates were removed by a 2 min spin at 16000× g, and the sample was loaded on top of 4.8 ml 10% sucrose in TMS buffer and ultracentrifuged as above. The sedimented procapsids were resuspended in 75 μl TMS buffer and sonicated briefly. Aggregates were removed as above, and 25 μl glycerol were added. Such purified procapsid samples were aliquoted and stored frozen at −70 °C.

2.3. Preparation of phi29 DNA-gp3

DNA-gp3 was prepared as previously described[14] by CsCl ultracentrifugation of DNA isolated from *Bacillus subtilis* SpoA12 infected with mutant phage sus4(369)-sus8(22). Since such preparations were found to contain trace amounts of pRNA and occasionally gave rise to background plaque formation in phage assembly experiments (not shown), such preparations were subjected to size-selective precipitation with polyethylene glycol (PEG).[51] DNA-gp3 was dialysed against TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA) to remove CsCl and supplemented with (final concentrations) 0.5 M NaCl and 8% PEG 8000 (Sigma). The sample was incubated 16 h on ice and centrifuged at 4 °C and 14000× g for 30 min. The pellet was dissolved in TE buffer at a final DNA-gp3 concentration of 50 ng/μl.

2.4. Assay of Purified phi29 Procapsid Preparation

Samples of phi29 procapsid preparations were subjected to SDS polyacrylamide gel electrophoresis and Coomassie staining according to standard procedures. Phage assembly was performed according to previously published methods.[52] Each reaction contained 50 ng pRNA or the amount of pRNA-conjugate corresponding to 50 ng pRNA, determined by scintillation counting of incorporated tritium label, and 0.5 μl conventional procapsid preparation, or 1 μl procapsid preparation after additional purification and 2 mg/ml BSA (ultrapure, Ambion), in 17.5 μl volume. Dialysis of the pRNA/procapsid mixture, packaging with 50 ng DNA-gp3 (after purification by PEG precipitation) and his-tagged ATPase gp16 purified by affinity chromatography,[53] neck and tail assembly, and plating was performed as previously described.[52]

Preparation of procapsids with bound pRNA-3′-ext., pRNA-biotin, pRNA-streptavidin, and pRNA-ferritin and analysis by *in vitro* phage assembly:

One standard pRNA to procapsid binding reaction contained 2 μl purified procapsid preparation, 50 ng [^{3}H]-labelled pRNA-3′-ext. or the same molar amount of derived pRNA-conjugate, based on scintillation counting, and 3 mg BSA (ultrapure, Ambion) in 15 μl TMS buffer. Each reaction was dialysed 15 min against TBE buffer (90 mM Tris-base, 90 mM boric acid, 2 mM EDTA) followed by 30 min against TMS buffer at ambient temperature on a 0.025 μm membrane filter (Millipore). Procapsid-pRNA complexes were separated from unbound pRNA by ultracentrifugation: Five standard reactions per sample were combined and loaded on top of a 5–20% sucrose gradient in TMS buffer. Separation was performed at 35000 rpm in a SW55 rotor of a Beckman L-80 ultracentrifuge for 30 min at 20 °C. Twenty-nine equal fractions per sample were collected from the bottom, and 80 μl per fraction was subjected to scintillation counting.

In vitro DNA packaging and phage assembly was performed as previously described.[52] For each of the five samples, the three fractions of the sucrose gradient containing the peak of procapsid/pRNA complex (i.e., fractions #19–21 for procapsids with no pRNA, pRNA-3′-ext., pRNA-biotin, and pRNA-streptavidin; fractions #18–20 for procapsids with pRNA-ferritin) were pooled. 10 μl per pooled sample were used for DNA packaging either (i) without treatment, (ii) after addition of 1 μl 10× RNase H buffer (750 mM KCl, 500 mM Tris-Cl pH 8.3, 30 mM $MgCl_2$, 100 mM dithiothreitol) but no enzyme and 1 h 37 °C incubation, or (iii) after addition of 1 μl 10× RNase H buffer, 0.25 u RNase H (Promega), and 1 h 37 °C incubation. DNA packaging was performed by adding 3 μl packaging buffer (10 mM ATP, 5 mM dithiothreitol in TMS buffer), 1 μl (50 ng) purified phi29 DNA-gp3, 10 μl his-tagged gp16 in PEG buffer,[53] and incubating 1 h at ambient temperature. Phage assembly with the neck and tail components gp9 and gp11-14, and plating was performed as previously described.[16]

2.5. Formation and Analysis of Procapsid/pRNA-Gold Conjugates

Procapsid/pRNA-gold complexes were prepared as procapsid/pRNA-ferritin complexes, except that pRNA-5 nm gold equivalent to 20 ng pRNA or pRNA-10 nm gold equivalent to 5 ng pRNA were used per microliter of procapsid preparation. Sucrose gradient ultracentrifugation and phage assembly were performed as described for procapsid/pRNA-ferritin.

2.6. Transmission Electron Microscopy

Electron microscopy grids with formvar/carbon film (Electron Microscopy Sciences) were made hydrophilic by glow discharge and floated on drops of sample on parafilm for 5 min. The grids were transferred to drops of 2.5% glutaraldehyde in 100 mM cacodylate buffer supplemented with 10 mM $MgCl_2$ for 20 min at ambient temperature, washed on 4 drops of water, and stained with 2% uranyl acetate for 5 min. Electron micrographs were taken on a Philips CM-10 operated at 80 kV with a typical magnification of 105000× and a typical underfocus of 0.2 μm.

3. RESULTS

3.1. pRNA Conjugates

The novel pRNA conjugates (e.g., pRNA-ferritin, Fig. 1) described in this paper were prepared through a series of binding and purification steps. A modified pRNA with an extension at the 3′ end of core pRNA, called pRNA-3′-ext., was transcribed and purified efficiently with an overall yield around 1.5 μg per μl transcription reaction. Incorporation of a biotin or thiol group by hybridisation of a synthetic DNA oligonucleotide bearing the chemical modification also worked efficiently, because an excess of the DNA oligonucleotide was used in the annealing reaction. Such modified pRNAs were purified almost to homogeneity (pRNA-biotin: Fig. 2(A) lane 7, Fig. 2(B) lane 5) by polyacrylamide-urea gel electrophoresis with a recovery of about 70%. Under the conditions we used, the 26 basepair RNA–DNA hybrid region was stable in 8 M urea gels. When pRNA-biotin was mixed with a six-fold molar excess of streptavidin, two major reaction products were identified by gel electrophoresis (not shown). Since streptavidin has previously been described to bind typically up to two biotinylated DNA molecules,[54] even though it has four biotin binding sites, the two bands were attributed to streptavidin with one (pRNA-streptavidin) or two molecules ($(pRNA)_2$-streptavidin) of pRNA-biotin bound. In order to identify which of the two bands was formed by the desired pRNA-streptavidin conjugate, material from both bands was isolated, mixed with biotinylated ferritin, and resubjected to gel electrophoresis. Only one of the two samples showed reduced migration rate. Therefore it was identified as the desired pRNA-streptavidin (1:1) with ability to bind biotinylated ferritin. Presumably, $(pRNA)_2$-streptavidin could not bind biotinylated ferritin because the two remaining biotin binding sites were sterically inaccesible. Interestingly, pRNA-streptavidin migrated slower than $(pRNA)_2$-streptavidin in 0.8% agarose gels, but the two conjugates formed one band in 3% agarose gels. In 8% polyacrylamide 8 M urea gels, pRNA-streptavidin migrated faster than $(pRNA)_2$-streptavidin (not shown). The desired pRNA-streptavidin isolated from 0.8% agarose gels with about 12% recovery was almost homogeneous (Fig. 2(A) lane 5) and reacted almost completely with biotinylated ferritin (Fig. 2(A) lane 3). pRNA-ferritin was prepared by adding enough biotinylated ferritin to pRNA-streptavidin to get a complete band shift, and used without purification. In order to make pRNA-gold conjugates, pRNA-thiol was prepared in the same way as pRNA-biotin, and coupled to 5 nm and 10 nm colloidal gold particles. Purification was by ultracentrifugation so that gold particles with bound pRNA sedimented, but free pRNA-thiol was removed with the supernatant (Fig. 7). Since the pRNA was tritium-labelled, it could be quantified by scintillation counting and the molar pRNA:gold ratios were determined, assuming complete recovery of the gold particles, as 1:5 for 5 nm and 1:2.5 for 10 nm gold particles. The recovery of pRNA-thiol after reductive treatment was about 50%, and the coupling efficiency was about 35% for pRNA-5 nm gold and 44% for pRNA-10 nm gold.

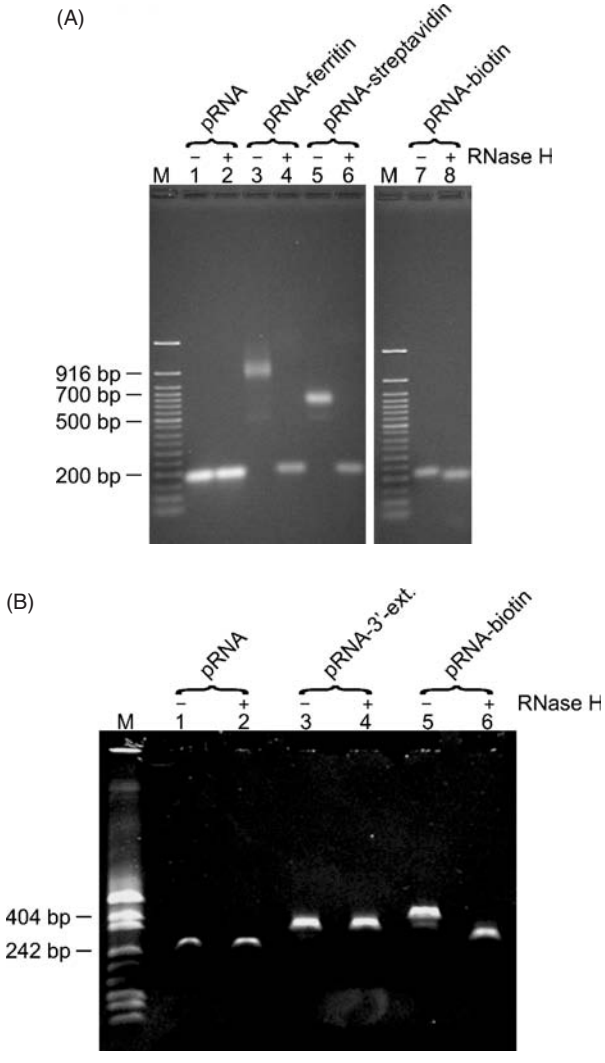

Fig. 2. Electrophoresis gels (2% agarose (A), 8% polyacrylamide 8M urea (B)) showing pRNA conjugates before (−) and after (+) treatment with RNase H. RNase H is an endoribonuclease that specifically cuts RNA that is hybridized to DNA. Digestion of RNA–DNA hybrid regions caused the release of label attached to pRNA (A, lanes 3–8; B, lanes 5–6), but pRNA without hybridized DNA oligonucleotide (A, lanes 1–2; B, lanes 1–4) was no substrate for RNase H. 50 ng pRNA was loaded per lane. The gels were stained with ethidium bromide. M, molecular mass markers: 50 bp DNA Ladder, New England Biolabs (A); pUC18 MspI digested (B).

3.2. Additional Purification of phi29 Procapsids and DNA-gp3 and Use for Phage Assembly with pRNA Conjugates

When *in vitro* DNA packaging and phage assembly experiments were performed initially, all pRNA conjugates gave the same yield of plaque forming units. Subsequently,

RESEARCH ARTICLE

procapsid preparations and procapsid-pRNA complexes were analysed by polyacrylamide gel electrophoresis. SDS-PAGE gels revealed that procapsid preparations had protein impurities (Fig. 3(A)). When procapsid preparations and pRNA conjugates were mixed and incubated to form procapsid-pRNA complexes, and checked by PAGE-urea electrophoresis, partial degradation of the labelled pRNA was observed (not shown). Apart from this contaminating RNase activity, the procapsid preparations were also found to contain nucleic acids. Therefore, procapsids were treated and purified by incubation with DNase I and RNase A, and repeated sedimentation by ultracentrifugation and resuspension. The resulting procapsid preparations were virtually free of contaminating proteins (Fig. 3(A)) and showed no nucleic acid impurities or RNase activity (not shown). In the course of purifying components of the *in vitro* phage assembly assay it was also noted that phi29 DNA-gp3 preparations had a marginal level of contamination with pRNA, which was enough to give rise to background plaque forming units in negative controls of the *in vitro* phage assembly assay. This contamination was removed by size-selective precipitation of phi29 DNA-gp3. Phage assembly with such purified DNA-gp3 showed no plaque forming units in pRNA-free negative controls (Fig. 3(B)). His-tagged gp16 that was purified by affinity chromatography on immobilised Ni^{2+} and kept soluble in a PEG-buffer[53] was found to be pure and RNase-free. With all components of the *in vitro* DNA packaging system tested to be RNase-free, the biological activity of pRNA-conjugates in *in vitro* phage assembly could be investigated. pRNA, pRNA-3′-ext., pRNA-biotin, pRNA-streptavidin, and pRNA-ferritin were all found to give rise to infectious phage. However, plaque yield correlated with the molecular size of the attachment to pRNA and ranged from 4×10^8 pfu/ml with pRNA to 6×10^6 pfu/ml with pRNA-ferritin. In order to check for possible direct effects of ferritin on the DNA-packaging reaction, a sample with biotinylated ferritin was prepared like the pRNA-ferritin sample, except that pRNA instead of pRNA-streptavidin was used so that no binding could occur. This sample gave rise to the same plaque yield as pRNA without biotinylated ferritin. When phi29 procapsid was used for DNA-packaging without the reported additional purification, the plaque yield for pRNA and pRNA-conjugates was the same, about 5×10^7 pfu/ml (Fig. 3(B)).

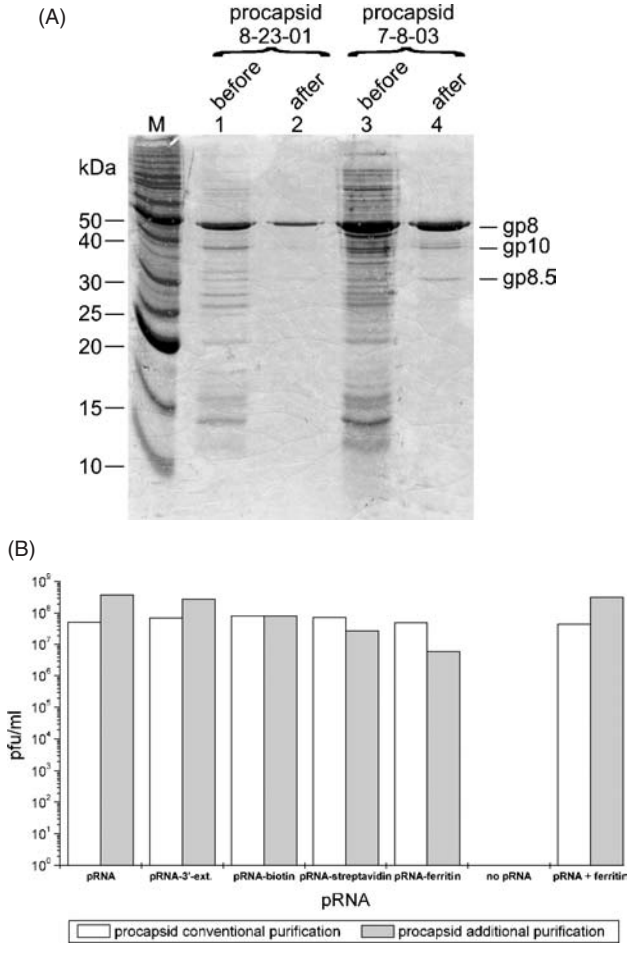

Fig. 3. Comparison of phi29 procapsid preparations after conventional and after additional purification. (A) Coomassie-stained SDS 15% polyacrylamide gel showing two batches of phi29 procapsid preparations (8-23-01 and 7-8-03) before and after the additional purification by sedimentation described in the Materials and Methods section. One μl per sample was loaded; M, molecular mass marker. (B) Phage assembly assay with procapsid (batch 7-8-03) after conventional and after additional purification. Note that plaque forming units are given on a logarithmic scale covering 9 orders of magnitude. With the conventional procapsid preparation, which was found not to be completely free of RNase activity, all pRNAs and labelled pRNAs gave the same plaque yield. After additional purification, plaque yield varied with the size of the label attached to pRNA. pRNA + ferritin stands for a sample that contained biotinylated ferritin and pRNA in the same molar concentrations as in the pRNA-ferritin sample, but not linked by streptavidin. A repeat of this experiment showed the same trend, but because overall plaque yield was lower, the data shown is from a single experiment.

3.3. Isolation and Analysis of Procapsid/pRNA-Conjugate Complexes

After binding pRNA or pRNA-conjugates to phi29 procapsids, samples were loaded on top of sucrose gradients and separated by rate zonal ultracentrifugation. pRNA-gold conjugates migrated and formed a broad peak, but all other described pRNA-conjugates including pRNA-ferritin stayed on top of the gradient and could be separated from procapsid/pRNA-conjugate complexes. Scintillation counting of tritium labelled pRNA showed that all these pRNA-conjugates bound to procapsid, and the proportions of procapsid-bound to free molecules was very similar for pRNA and the pRNA conjugates (Fig. 4). As the next step, we tested whether pRNA conjugates bound to procapsids in a functionally correct, i.e., in a DNA-packaging competent way. Procapsid/pRNA-conjugate complexes from the peak fractions of the ultracentrifugation experiment (Fig. 4) were subjected to DNA packaging and phage

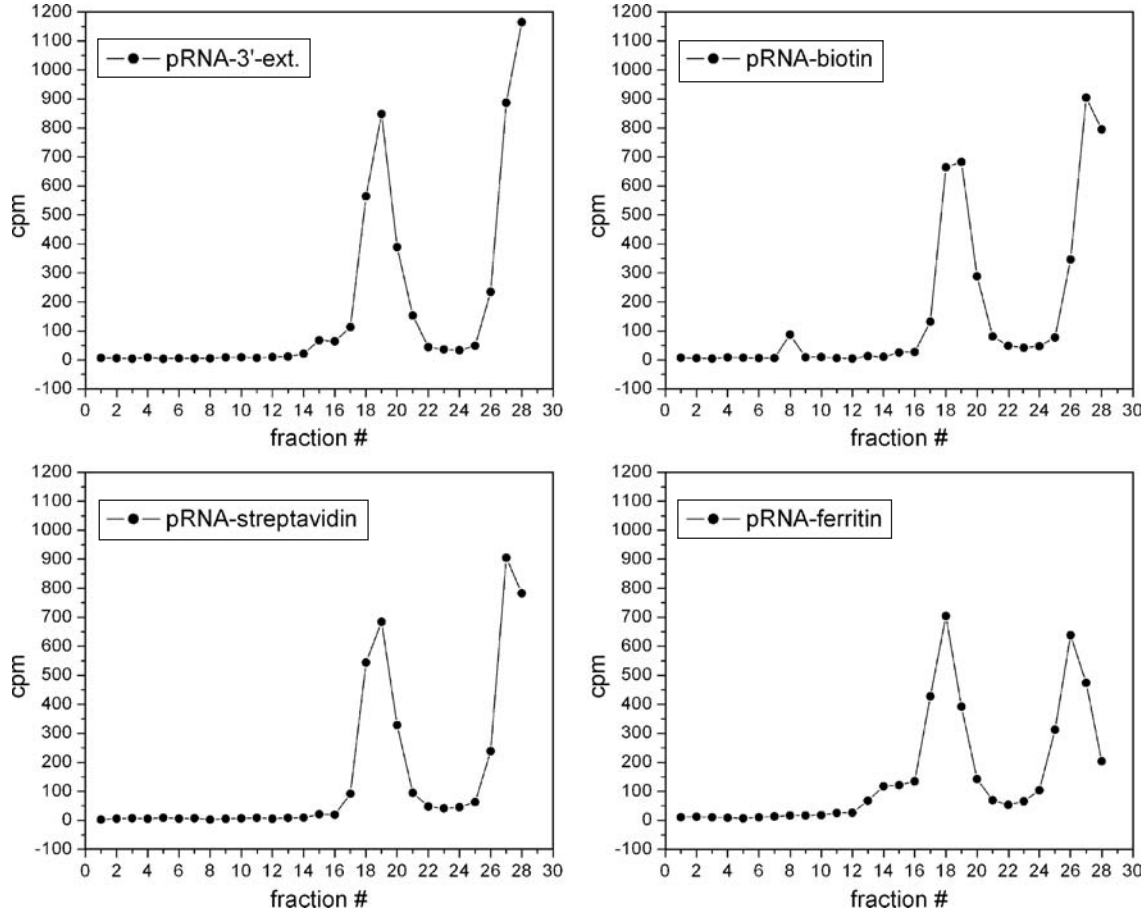

Fig. 4. Sedimentation by sucrose gradient ultracentrifugation of modified pRNAs bound to phi29 procapsids. The tritium label was on pRNA, and sedimentation is from right to left. The peaks centered around fraction 18 (pRNA-ferritin) or 19 (others) represent procapsid-pRNA complexes. Excess modified pRNA or modified pRNA in the absence of procapsid (not shown) stayed on top of the gradient. All modified pRNAs bound to procapsid with similar efficiency. Cpm, counts per minute.

assembly with and without RNase H treatment. RNase H cleaves RNA specifically in an RNA/DNA hybrid region. Therefore, RNase H cleaved pRNA conjugates so that the modification was removed from core pRNA (Fig. 1). This cleavage was confirmed by gel electrophoresis (Fig. 2). Without RNase H cleavage, the plaque yield of procapsids with pRNA-conjugates was dependent on the molecular mass of the pRNA modification. However, after the modification was cleaved off with RNase H, all samples gave rise to very similar plaque forming units per milliliter (Fig. 5). This experiment was repeated with a different purified procapsid preparation (prepared from batch 8-23-01, Fig. 3(A)), and the results were reproducible (not shown).

3.4. Transmission Electron Microscopy of Procapsid/pRNA-Ferritin Samples

Fractions 17, 18, and 19 of the procapsid/pRNA-ferritin ultracentrifugation shown in Figure 4 were pooled and studied by transmission electron microscopy of negatively stained preparations (Fig. 6). About one third of the procapsids had ferritin bound to them. In most procapsids the unique vertex that is the site of DNA translocation and harbors the connector could be identified. This vertex is known to be the binding site for pRNA,[55] and all procapsid-bound ferritin particles appeared to be tethered to the side of the procapsid with the connector. The number of ferritin particles per procapsid varied. In order to be able to give an impression of the number of particles per procapsid, one section of an electron microscopy grid was chosen at random for counting. Of 111 procapsids with attached ferritin in this section there were 14 procapsids with one ferritin particle attached, 15 with two, 39 with three, 30 with four, 10 with five, 2 with six, and 1 with seven particles. Sometimes procapsids with even more ferritin particles were observed.

3.5. Binding of pRNA-5 nm and pRNA-10 nm Gold Conjugates to phi29 Procapsids

Five nanometer colloidal gold particles and pRNA-5 nm gold conjugates sedimented during ultracentrifugation in

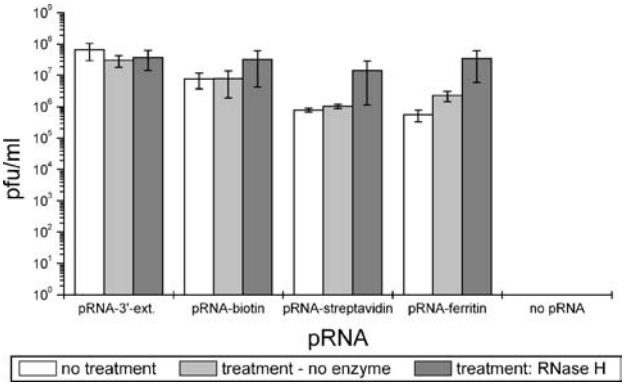

Fig. 5. Plaque yield of infectious phage assembled with pRNA-conjugates. Procapsid-pRNA complexes were isolated from the sucrose gradient ultracentrifugation shown in Figure 4, so that no excess, unbound pRNA-conjugates were present to form new procapsid/pRNA complexes in the course of the experiment. The samples were subjected to *in vitro* DNA packaging and phage assembly either directly, after treatment with RNase H to remove the labels, or after treatment with RNase H buffer and incubation but no enzyme, as a control. After stripping off the labels by RNase H treatment, all samples gave the same plaque yield, indicating that labelled pRNAs had assembled to form functionally correct rings. With the labels still attached, plaque yield varied with the size of the label. Note that plaque forming units were plotted on a logarithmic scale. Means and standard deviations are from three independent repeats of the experiment.

a sucrose gradient and formed a broad peak, which overlapped the peak formed by phi29 procapsid. However, when pRNA-5 nm was allowed to bind to phi29 procapsid before ultracentrifugation, formation of procapsid/pRNA-5nm complexes was detected by a change in sedimentation rate (Fig. 8(A)). When individual fractions were used for *in vitro* DNA-packaging and phage assembly, they gave rise to infectious phage (Fig. 8(B)). Fractions of procapsid/pRNA-5 nm and procapsid/pRNA-10 nm ultracentrifugations were also pooled, concentrated by ultrafiltration, and analysed by transmission electron microscopy (Fig. 9). The gold particles were attached to the side of procapsids with the connector, indicating that binding was mediated by the specific procapsid/pRNA interaction. Procapsids had up to six gold particles attached, and the number of particles per procapsid was distributed roughly like in the procapsid/pRNA-ferritin samples.

4. DISCUSSION

Our experiments showed that pRNA could be conjugated to gold and ferritin particles, and that these conjugates were competent in pRNA-mediated binding to phi29 procapsids. Previously, reports on conjugates between nanometer-sized particles and nucleic acids focussed on DNA. For example, DNA oligonucleotide-nanoparticle conjugates were described as a substrate for the rational formation of macroscopic nanoparticle aggregates or defined supramolecular assemblies of gold nanocrystals with possible applications in biosensing, nanostructuring,

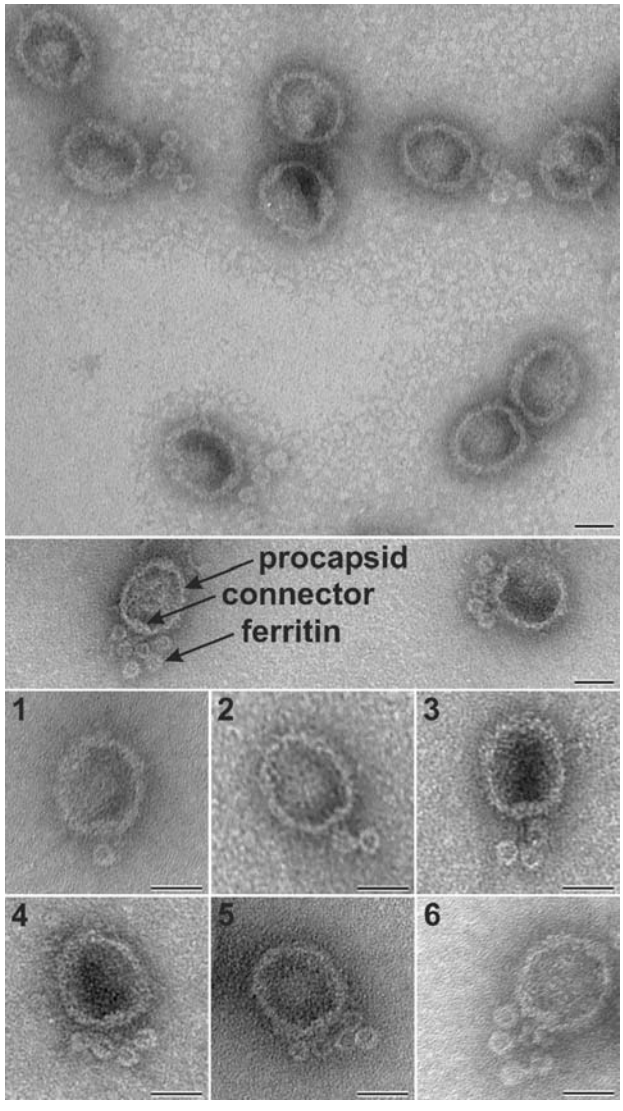

Fig. 6. Transmission electron micrographs (negative stain with uranyl acetate) of phi29 procapsids with bound pRNA-ferritin. The sample was taken from the peak (pool of fractions 17, 18, and 19) of the sucrose gradient ultracentrifugation shown in Figure 4. Note that the connector at the portal vertex can be identified in most of the procapsids. The connector at this unique vertex is the binding site for pRNA[55] and, as shown here, was also the binding site for the pRNA-ferritin conjugates. Bars: 25 nm.

and molecular electronics.[34, 56] Of such described conjugates, the functionality for biosensing e.g., Refs. [57, 58] or nanotechnology depended on the sequence of DNA oligonucleotides and Watson-Crick basepairing. However, compared with DNA, RNA molecules which adopt a defined fold have much more versatile abilities for biomolecular interactions and can also function as biocatalysts. Moreover, RNA aptamers with novel binding affinities can be generated *in vitro*.[59, 60] Therefore, RNA-nanoparticle conjugates like the ones we described may be a desirable contribution to the development of RNA-based nanostructuring[28, 61, 62] or biosensing.[63] In applications where the inherent susceptibility of RNA to

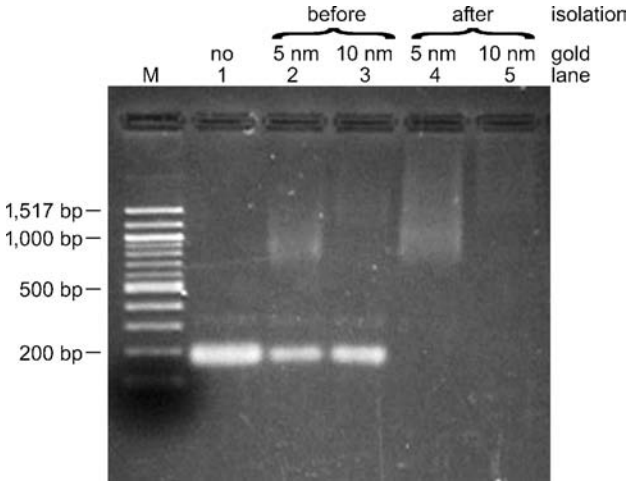

Fig. 7. Agarose gel (2%; ethidium bromide stained) with pRNA-gold conjugates. pRNA-thiol was coupled to 5 nm and 10 nm colloidal gold ("before" isolation), and the conjugates were isolated by sedimentation in the ultracentrifuge ("after" isolation). Lane 1: pRNA-thiol without gold; M, molecular mass marker: 100 bp DNA Ladder, New England Biolabs.

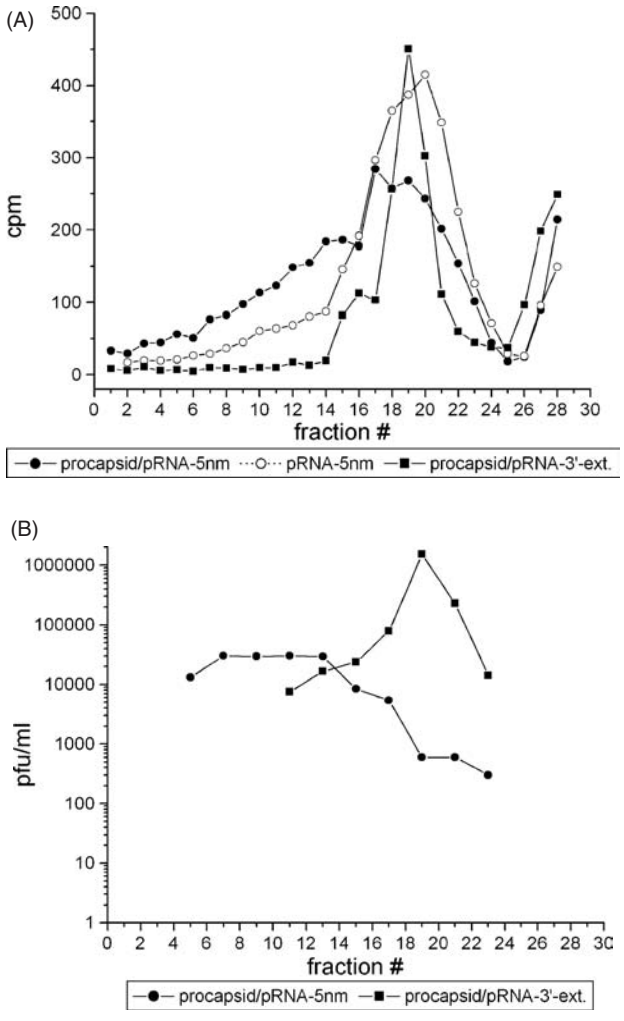

Fig. 8. Sedimentation (from right to left) of pRNA-5 nm gold conjugates, alone or after incubation with phi29 procapsids, by sucrose gradient ultracentrifugation. pRNA was tritium-labelled. (A) The amount of pRNA in each fraction was determined by liquid scintillation counting. cpm, counts per minute. (B) Fractions of the sucrose gradient ultracentrifugations shown in (A) were used for *in vitro* phage assembly. pfu/ml, plaque forming units/ml.

enzymatic degradation is a problem, the use of RNase inhibitors or chemical modifications at the 2′ position of ribose for increased nuclease resistance may be feasible. Another possible application of RNA conjugated to electron dense labels might be in the study of location and transport of specific RNA molecules inside cells by electron microscopy. Such investigations are desirable in light of the recognition that RNA-based mechanisms can play a major role in gene regulation, and that these mechanisms may be exploited for therapeutic applications. Previously reported RNA-nanoparticle conjugates were mostly limited to the formation of complexes between RNA and colloidal gold by adsorption with no site-specificity,[64, 65] to the application in arrays for gene expression analysis,[66, 67] or to labelling with the small 1.4 nm nanogold.[68]

Our finding that conventional phi29 procapsid preparations had a low level of RNase contamination, and that partial pRNA degradation had an influence on the *in vitro* DNA packaging and phage assembly assay with pRNA conjugates, showed that results from this assay need to be considered with caution. The RNA sequences following the nucleotide 117 are sensitive to RNase activity. The higher plaque yield with high molecular mass pRNA conjugates can be explained with partial RNA degradation when conventional rather than specially purified phi29 procapsid preparations were used in the *in vitro* assay: Loss of the label in a fraction of the pRNA conjugates would cause an increase in plaque yield.

Ultracentrifugation showed that when modified pRNAs were incubated with procapsid, all pRNA modifications gave rise to similar ratios of bound to free pRNA (Fig. 4), and the phage assembly assay (Fig. 5) suggested that pRNA to procapsid binding was biologically correct. After the labels were cleaved off from procapsid-bound pRNA with RNase H, full DNA packaging activity was restored. The phage assembly assay of procapsid/pRNA-conjugate complexes before RNase H treatment showed that modifications to pRNA decreased DNA packaging efficiency with dependence on the molecular mass of the label. This result supports the conclusion from previous work,[4, 69–71] that the region of pRNA where the labels were attached plays an essential role in the DNA packaging reaction.

The observation by electron microscopy that particles were attached to procapsids at the connector was direct evidence that pRNA mediated supramolecular assembly of procapsid-particle complexes. The expected arrangement of particles in rings, based on the ring of pRNA molecules around the connector at the packaging vertex of the procapsid, could not be observed because the linker between pRNA and nanoparticle was too long and flex-

RESEARCH ARTICLE

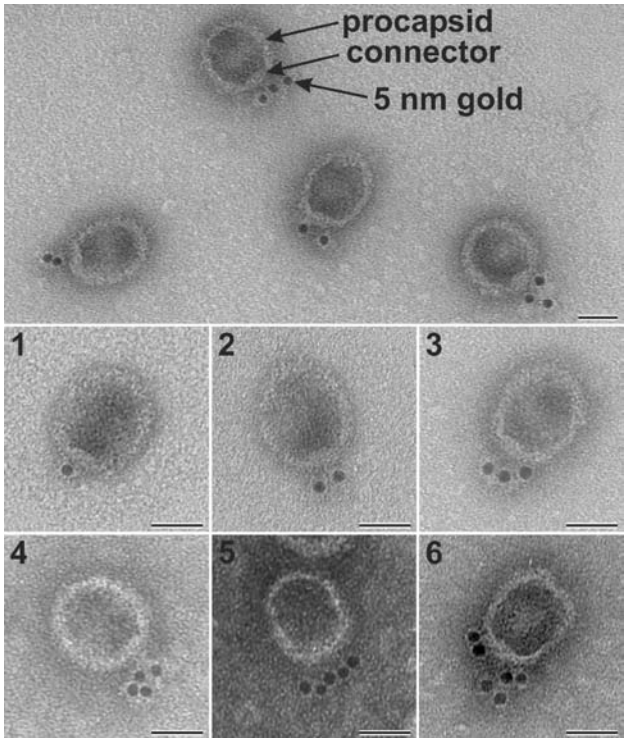

Fig. 9. Transmission electron micrographs (negative stain with uranyl acetate) of phi29 procapsids with bound pRNA-5 nm gold. The procapsid/pRNA-gold complexes were isolated by sucrose gradient ultracentrifugation. Note that the gold particles were attached to procapsids at the portal vertex, which is the known binding site for pRNA. Bars: 25 nm.

ible. Also, procapsids were attached to the carbon film of the electron microscopy grids with their prolate side and therefore appeared in side view. Only particles within about 20 nm from the connector could be considered to be linked specifically through pRNA. This distance is composed of the double-helical domain of pRNA that is not involved in procapsid binding,[69] the 26 bp RNA–DNA hybrid extension of pRNA, the linkers of the chemical moieties, and streptavidin in the case of pRNA-ferritin. Some particles were observed that were further away from the connector and therefore could not have been bound specifically and directly. Especially with ferritin, we observed that sometimes particles bound together, giving rise to procapsids with higher numbers of attached ferritin particles. Non-specific aggregation and binding of more than one biotinylated ferritin particle per pRNA-streptavidin, although sterically unfavorable, cannot be ruled out. Binding of particles to each other may also have been due to the specific dimerization of pRNA in the presence of magnesium ions. Even though we used a molar excess of particles over pRNA, some of the particles would be expected to carry more than one pRNA molecule. Accordingly, pRNA-10 nm gold conjugates with a lower molar ratio of gold to pRNA than pRNA-5 nm gold conjugates showed a higher tendency to form small groups. The ultimate result of our work would be the preparation of self-assembled procapsid-nanoparticle complexes, where almost every procapsid has the same number of linked nanoparticles, equivalent to the number of pRNA molecules in a functional ring. Our observation of a distribution of the number of particles per procapsid may, in addition to some aggregation, be due to assembly intermediates with incomplete pRNA rings or to partial loss of label. A possible approach of increasing the stability of pRNA-nanoparticle conjugates would be to use pRNA variants with covalent modifications at their 5′ end.[4, 33, 42, 72] Decreasing the length and flexibility of the linker between pRNA and nanoparticles may also allow the arrangement and observation of nanoparticle rings. Another desirable experimental improvement would be to ensure that no particles are linked to more than one pRNA molecule.

Since pRNA-nanoparticle conjugates bound to procapsids efficiently and in the biologically correct way, pRNA may be useful to direct assembly of functional nanoparticles. Defined groups of nanoparticles may become desirable elements for molecular electronics, nonlinear, optics, and nanotechnology. Since pRNA rings can be assembled in a pre-programmed way from mixtures of pRNA molecules,[22, 73] which could in theory each be modified with a different particle or nano-component, the formation of defined groups of nanoelements, which together are capable of performing novel functions, may be feasible.

ABBREVIATIONS

pRNA	packaging RNA
gp	gene product
ds	double-stranded
pfu	plaque forming units

Acknowledgments: The authors wish to thank Debby Sherman for access to a transmission electron microscope and her technical and scientific assistance, and Dan Shu and Songchuan Guo for generously providing materials. This work was supported in part by NIH grant R01-EB003730 to P. G., and an Erwin-Schrödinger fellowship from the Austrian Science Fund FWF to W.-D. M.

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Received: 4 April 2007. Revised/Accepted: 16 May 2007.