Preparation of Peptide-Functionalized Gold Nanoparticles Using One Pot EDC/Sulfo-NHS Coupling

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Supporting Information

**ABSTRACT:** Although carbodiimides and succinimides are broadly employed for the formation of amide bonds (i.e., in amino acid coupling), their use in the coupling of peptides to water-soluble carboxylic-terminated colloidal gold nanoparticles remains challenging. In this article, we present an optimization study for the successful coupling of the KPQPRPLS peptide to spherical and rodlike colloidal gold nanoparticles. We show that the concentration, reaction time, and chemical environment are all critical to achieving the formation of robust, peptide-coated colloidal nanoparticles. Agarose gel electrophoresis was used for the characterization of conjugates.

**INTRODUCTION**

Applications of nanotechnology in biomedical and physical sciences require sophisticated nanoparticles with precisely defined chemical composition, size, shape, and functionality. In particular, understanding the nanoparticle surface chemistry is of critical importance to obtaining stable and functional nanoparticles. A popular type of surface capping ligand, frequently utilized for the preparation of biocompatible gold nanoparticles, is based on thiol-containing oligoethylene glycols with a terminated amine or carboxylic group [i.e., monocarboxy(1-mercaptooundec-11-yl) hexa(ethylene glycol) (OEG)]. The thiol group of OEG binds to the gold surface, the ethylene glycol unit imparts hydrophilic character to the ligand, and the terminal carboxy or amine group serves as a binding site to conjugate functional biomolecules such as peptides and antibodies. The coupling of biomolecules to inorganic colloidal nanoparticles is quite challenging because one needs to consider not only the several chemical parameters that affect the kinetics of the reaction but also the robustness of the final product (biomolecule—nanoparticle).

There are various methods available for cross-linking biomolecules to nanoparticles. Among the most commonly used strategies are so-called click chemistry and EDC/sulfo-NHS coupling. EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] is one of the most commonly used carbodiimides, which catalyzes the formation of amide bonds between carboxy and amine groups. Its popularity is derived from its high solubility in water and the ease of removal of the byproduct. However, sulfo-NHS (N-hydroxy sulosuccinimide) is used to increase the stability of active intermediates in coupling reactions via the formation of active ester functional groups with carboxylates. Several reports demonstrate the successful coupling of biomolecules to water-soluble nanoparticles using EDC or its derivatives as the main coupling reagent. For example, Nie and co-workers showed the coupling of proteins to mercaptoacetic acid-capped quantum dots, and Mason and co-workers showed the successful conjugation of antibodies to carboxy-terminated PEG dithiol-coated gold NPs. However, the employment of EDC to bind biomolecules efficiently to water-soluble colloidal nanoparticles remains a challenge. Quite often, the biomolecules do not bind efficiently to the particles or the particle—biomolecule conjugates are not stable.

In this article, we demonstrate our recent studies in identifying the appropriate chemical conditions for the successful coupling of the KPQPRPLS peptide to carboxy-terminated oligoethylene glycol gold nanoparticles (OEG NPs). The KPQPRPLS—nanoparticle conjugates are of critical importance to the activation of angiogenic genes, as has been shown in earlier studies. A number of experimental parameters are explored, including the variation of reagent concentration, the reaction time, and the type of nanocrystal morphology.

**EXPERIMENTAL SECTION**

**Synthesis of Peptide-OEG NPs.** Spherical gold NPs were prepared according to the well-established citrate reduction method and then stabilized with bis(1-sulfonatophenyl)phenyl phosphine dehydrate dipotassium salt (BSPP). Gold nanorods (NR) were synthesized following the procedure by El-Sayed and co-workers. Both types of gold NPs were functionalized with OEG. In detail, an aqueous solution of OEG (5 mg/mL, 200 μL) was added to a solution of gold spheres (10 mL, 5 nM) or gold nanorods (5 mL, OD 0.5) while stirring. The mixture was incubated for 2 h at room temperature and then overnight at 4 °C. Then, the particles were purified by centrifugation (three times, 16,400 rpm, 15 min) and redispersed in 0.01 M sodium borate buffer at pH 9. Prior to the coupling reactions, the particles were characterized using a nanoeztasizer (Supporting Information).

**EDC/s-NHS Coupling Reactions.** The KPQPRPLS peptide was employed for the coupling experiments. The peptide was designed to be slightly hydrophilic, containing an N-terminus lysine that can be coupled...
to carboxylic acids. In a typical reaction, a solution of the peptide (10 μL, 1 mg/mL, MW = 922.1, in 0.01 M sodium borate buffer, pH 9) was added to OEG NPs (0.5 mL, 1.5 nM for spherical or OD = 0.3 for NRs, in 0.01 M sodium borate buffer, pH 9) and mixed. To this solution, aqueous solutions of coupling reagents EDC (5 μL, 0.2 M) and sulfo-NHS (10 μL, 0.2 M) were introduced simultaneously. Then, the reaction mixture was stirred for 24 h at room temperature. The particles were purified by centrifugation/decantation (three times, 16,400 rpm, 15 min) and redispersed in tris-borate-EDTA buffer (0.5/TBE) at pH 9.

For the different types of experiments, the following reaction parameters were varied one at a time while keeping the rest of the parameters the same as in the typical reaction.

(a) The amounts of EDC and sulfo-NHS were varied in the reaction mixture while keeping the molar ratio between them constant at 1:2 (EDC/sulfo-NHS). The added volumes were kept the same as in the typical reaction. The minimum used quantity of EDC/sulfo-NHS was 1 μmol/2 μmoles, and the maximum was 10 mmol/20 mmol, respectively.

(b) The amount of peptide added to the reaction mixture was varied while keeping the volume of introduced solution the same as in the typical reaction. The minimum introduced quantity of the peptide was 10.85 nmoles, and the maximum was 10.85 μmoles.

(c) The concentration of sodium borate buffer was varied from 0.1 to 0.001 M, and the concentrations of all other reactants were kept constant, as in the typical reaction.

(d) The reaction time, measured after all reagents were added to the reaction mixture, was varied between 24 and 48 h.

(e) Two shapes of OEG NPs, spherical and rodlike, were used in EDC/sulfo-NHS coupling reactions. This was to investigate if morphologically different NPs can still be successfully conjugated to the peptide using the typical reaction conditions.

**Gel Electrophoresis.** Gel electrophoresis was employed to determine the variations in charge and size as previously reported for gold particles of different functionalities. A horizontal agarose gel system was used in all experiments. The agarose gel (0.75%) was prepared by dissolving agarose (0.45 g, Sigma-Aldrich) in 0.5/TBE (60 mL, pH 8). Liquid agarose was poured into a gel tray (10 × 7 cm², Bio-Rad) fixed within the gel caster (Bio-Rad). Next, a teeth comb (Bio-Rad) was placed in the middle slot, and the gel was left to cool and solidify for 30 min at room temperature. The gel caster was leveled, ensuring the formation of an evenly thick (1 cm) matrix with identical wells (well capacity 41.6 μL). The gel was placed in a mini-sub cell GT base (Bio-Rad) along with TBE buffer (0.5/TBE) for submersion of the gel beneath 5 mm of liquid. The colloids (16 μL, 50 nM spherical or OD = 10 for nanorods, in 0.5/TBE buffer) were mixed with glycerol (4 μL, 30%, in 0.5/TBE buffer) and loaded into the wells. The gel was run for 1 h using 10 V (steady current) per 1 cm of gel. Digital images of gels were taken with a Canon Power Shot A480 digital camera.

**RESULTS AND DISCUSSION**

Water-soluble carboxdiimides, such as EDC, are some of the most popular reagents used in cross-linking reactions. EDC reacts with carboxylic acids and forms reactive α-acylisourea intermediates, which are then attacked by a nucleophile (i.e., a
and the concentration of the peptide is modified when the concentrations of the coupling reagents are kept fixed. Variations in the electrophoretic mobilities of Pep-OEG NPs arise from water, can also act as a nucleophile, cleaving off the intermediate and releasing isourea, thus inactivating EDC. To improve the coupling efficiency, EDC can be used in conjunction with sulfo-NHS. Sulfo-NHS forms an active ester with the carboxylic acid attached to the nanoparticle surface. This type of intermediate ester is very hydrophilic and stable and hydrolyzes relatively slowly in water, offering an extra advantage for coupling reactions. In the presence of amine nucleophiles, the sulfo-NHS ester is rapidly hydrolyzed, allowing the formation of an amide bond.

Although the EDC/sulfo-NHS coupling reaction can be performed in several steps, in our experiments we chose to add all of the reagents in one step. The successful coupling, for the different reaction parameters, was evaluated by gel electrophoresis. All conjugation reactions were performed under alkaline conditions to reduce the possibility of peptide polymerization when the peptide was present in excess. In the first set of experiments, the concentrations of EDC and sulfo-NHS were varied, and the concentration of the peptide was kept steady. As can be seen in Figure 1A, a mobility trend (V shape) is observed for lanes 4A–8A, indicating that different coupling efficiencies depend on the experimental conditions. The electrophoretic mobility of batch 6A is significantly lower than those of the others, implying that a larger number of peptides is conjugated to the nanoparticles. When a higher number of peptides is associated with the nanoparticles, then the particles are delayed in the gel matrix because of their larger hydrodynamic size and their less-negative charge (because the peptide used in our experiments has a slightly positive charge). Figure 1B shows the variations in the electrophoretic mobilities of Pep-OEG NPs when the concentrations of the coupling reagents are kept fixed and the concentration of the peptide is modified. Again, it is evident that the most effective coupling conditions are observed for lane 5B. When a large amount of coupling reagents, the particles have a very low mobility on the gel. This could be due to particle agglomeration caused by peptide cross-linking. In any case, from the results presented in Figure 1, it is clear that the best conditions to achieve the conjugation of the peptide to the particles are for a concentration of 1 mmole of EDC, 2 mmoles of sulfo-NHS, and 1.09 μmoles of peptide.

Another very interesting observation in Figure 1A is that the degree of coupling seems to correlate in an unexpected way with the concentration of the coupling reagents used in every experiment. If we assume the ideal coupling conditions (lane 6A) to be a reference point, then the coupling yield seems to decrease similarly by increasing or decreasing the concentrations of EDC and sulfo-NHS. One may speculate that the lower the concentration of coupling reagents, the lower the efficiency of coupling (lanes 4A and 5A).

The explanation of the lower coupling efficiency at higher concentrations of EDC and sulfo-NHS (lanes 7A and 8A) does not seem to be very obvious. With more coupling agents, it is expected that more coupling events would take place. Here, it is possible that a competition event takes place. The larger amounts of EDC and sulfo-NHS may promote the polymerization between the highly concentrated active species (peptides), thus reducing the quantity of peptides available for coupling to the nanoparticle surface.

The last observation in Figure 1 is that the individual reagents (peptides, EDC, and sulfo-NHS) do not influence the nanoparticle mobility in the gel (lanes 2A and 3A) unless they are all present in the reaction mixture.

Moreover, similar mobility is observed for samples that contain different amount of coupling reagents (lanes 4B, 6B, and 8B). This means that for the given experimental conditions the nanoparticles remain inert to a large amount of the peptide.

To realize further the optimum coupling conditions, we investigated the influence of the buffer concentration in the
reaction mixture while keeping all other experimental parameters as in the typical coupling reaction. The agarose gel in Figure 2 shows that for all samples the particles are delayed in the matrix, thus indicating a successful coupling (lanes 2C–5C). However, in lower buffer concentrations, a lower nanoparticle mobility is observed in the gel (lanes 6C and 7C), suggesting a more efficient coupling of the peptides to the particles. For a smaller buffer concentration (lane 8C), some particles do not run in the gel, indicating a degree of agglomeration. The variation in particle mobility in lane 8C may be due to the lower capacity of the borate buffer. It is difficult to realize how sudden pH variations, as the reaction proceeds, could affect the degree of coupling. However, there is a strong possibility that sudden charge changes could cause the agglomeration of the colloids.

To evaluate if the incubation time affects the degree of coupling, we performed two independent reactions where all of the experimental conditions were kept constant and only the incubation times were varied to 24 or 48 h. Figure 3 shows in both cases the particle delay on the gel (lanes 3D and 6D), indicating a successful coupling. However, as expected, lanes 1D and 4D show that plain OEG NPs are stable at different reaction times; when the particles are mixed with the coupling reagents but no peptide, the results vary (lanes 2D and 5D). Specifically, when particles and coupling reagents (without peptide) are incubated together for 48 h the sample smears on the gel, indicating a variation in charge and/or the agglomeration of particles. This observation suggests that the coupling reaction takes place in the first 24 h.

In the final experiment, we investigated if the chosen coupling conditions are suitable for the conjugation of the peptides to short aspect ratio gold nanorods. This is an essential experiment to realize if the anisotropy of the particles influences the coupling reaction (physicochemical characteristics of the particles in Supporting Information). Figure 4 shows the gel electrophoresis of spherical and rodlike gold nanoparticles when the typical experimental coupling conditions were applied. It is evident that in both cases the peptide-coated nanoparticle samples are delayed in the gel matrix, thus indicating a successful coupling.

Therefore, it was concluded that the experimental conditions chosen for the coupling of the peptide to 16 nm spherical nanoparticles can be employed for the conjugation of the same peptide to gold nanorods with dimensions of 16 × 47 nm² (nanozetasizing measurements in Supporting Information).
CONCLUSIONS

This work provides insight into the chemical conditions required for the conjugation of the KPQPRPLS peptide to nanoparticles of sphere or rodlike shape using EDC/sulfo-NHS coupling. It was shown how different experimental conditions affect the degree of peptide coupling as well as the nanoparticle stability. Beyond the importance of the KPQPRPLS-nanoparticle conjugates as highlighted in earlier work, we believe that our observations will be the cornerstone for more in-depth studies concerning the functionalization of biomolecules to particles and the optimization of conditions to retain the stability of colloidal nanoparticles.

ASSOCIATED CONTENT

Supporting Information. Physicochemical characteristics of the particles. This material is available free of charge via the Internet at http://pubs.acs.org.

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