UDC 541.64:546 doi: https://doi.org/10.15407/ubj90.02.008

SIMPLE TWO-STEP COVALENT PROTEIN CONJUGATION TO PEG-COATED NANOCRYSTALS

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Covering of nanocrystals (NC) with a polyethylene glycol (PEG) envelop is a common way to increase their hydrophilicity, and compatibility with bio-systems, including increased retention time in the body. Colloidal semiconductor NC, also known as quantum dots (QD), particularly benefit from covering with PEG due to passivation of the inorganic core, while maintaining physical properties of the core. Despite many advantages of covering the surface with PEG, the covalent attachment of protein to hydroxyls of PEG is complicated. Here we propose a simple two-step approach for modification of PEG residues with subsequent covalent attachment of proteins. We were able to achieve specific NC targeting by means of attached protein as well as preserve their optical parameters (fluorescence intensity) in chemical reaction conditions. In the optimized protocol, ensuring removal of chemical byproducts by dialysis, we were able to omit the need for centrifugation (usually a limiting step due to particle size). The obtained NC-protein conjugate solutions contained 0.25x of initial unmodified NC amount, ensuring a low dilution of the sample. During all reactions the pH range was optimized to be between 6 to 8. The proposed approach can be easily modified for covalent targeting of different PEG-covered nanocomposites with proteins.

K e y w o r d s: PEGylated nanocomposites, quantum dots, covalent protein conjugation.

Nanocrystals (NC), particularly quantum dots (QD), are usually poorly compatible with bio-systems, being either toxic, hydrophobic, or unstable upon contact with components of living systems. Covering the NC with polyethylene glycol (PEG) usually overcomes these limitations, making PEGylation a popular modification approach. QD are a type of colloidal semiconductor NC that comprise a promising class of materials for labeling biological systems. Their nanoscale size is compatible with the majority of metabolic and internalization processes observed in cells [1, 2]. Compared to organic fluorophores, QD show exceptional resistance to photodegradation and photobleaching, narrower photoluminescence with high quantum yield, broader absorption bands, larger effective Stokes shifts and higher absorption coefficients. In order to target QD to a specific molecule (e.g. proteins, peptides, organic and inorganic polymers, DNA, carbohydrates) that is present in the biological system under study, their surface must be chemically modified with organic substances. This procedure is called functionalization of nanoparticles and the resulting system may be considered a hybrid organic-inorganic nanocomposite [3], which possesses combined properties of both basic elements: the optical properties of the QD and the biological functionalities of organic molecules. These nanocomposites may be used as biolabels in monitoring biological processes at the molecular level.

Encapsulation of NC by a polymer, for example PEG, increases their water solubility and colloidal stability and reduces the cytotoxic effects. PEG is a polyether compound which is biologically inert, consisting of repeating ethylene ether units with linear or branched polyether (HO-(CH_2CH_2O)-OH) and is

highly water soluble [4]. Despite many advantages of PEGylation, covalent attachment of protein to the hydroxyls of PEG is complicated.

There are many reports about covalent modification of PEG (for example by using carboxyl-PEG-carboxyl) [5] or using approaches to develop rapid PEG-based kits for covalent protein modification (for example, using bis-2-hydroxyethylglycinamide) [6].

The aim of the current work was to develop an effective and simple protocol for covalent and stable conjugation of protein(s) to PEG-coated NC.

Materials and Methods

Fluorescent microscopy was performed with a Carl Zeiss AxioImager A1 DIC/fluorescent microscope (Oberkochen, Germany) using a 1.3 NA 100× oil immersion objective. Fluorescent images were taken by a Zeiss AxioCam MRm III cooled digital CCD camera under constant exposure. Fluorescence of NC was evaluated at red channel (610 nm emission was obtained at 532 nm excitation). Image analysis was performed using Fiji software (National Institutes of Health [NIH], Bethesda, MD, USA).

Human cervical adenocarcinoma HeLa cells were obtained from the American Type Culture Collection and cultured in RPMI-1640 and Eagle's minimum essential medium (EMEM) (Sigma-Aldrich Co., USA). The media were supplemented with 4 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin (PAA, Pasching, Germany), and fetal calf serum (Gibco-BRL, Eggenstein, Germany) at 10%. Cultures were maintained under 5% CO_2 at 37 °C. Cell necrosis was induced by incubation of cells, grown on slides, at 56 °C for 30 min. ConA lectin was a kind gift of Prof. V. Antonyuk, Lectinotest laboratory, Ukraine. Incubation of cells with NC was done by addition of NC-PEG-CO-NH-ConA suspension to cells (final concentration 1% v/v), incubating for 10 min at 37 °C and imaging.

Results and Discussion

Reaction of a covalent protein attachment using zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is widely used in conjugation chemistry [7]. We also effectively used this approach to covalently attach proteins, particularly lectins, to inorganic rare-earth NC with subsequent tissue targeting via intravenous administration in animals [8-10]. Besides, the reaction produced no toxic byproducts, since EDC was transformed into a non-toxic urea derivative. Thus, we choose this approach for PEG modification. Both amino and carboxyl groups are available in protein molecules, allowing flexible options for conjugation. To convert the hydroxyl of the PEG residue into a carboxyl group to be further functionalized we used the reaction with monochloroacetic acid (MCAA), as previously described [7, 11]. MCAA is a small molecule (MW 94,5), and the unreacted compound is effectively removed from reaction media by dialysis. The obtained carboxyl residue on PEG was conjugated to amine groups of proteins using EDC as a zero-length cross-linker, as described below.

As model NC we have selected core/shell CdSe/CdS QD with a 5.2 nm diameter and a maximum of the first absorption band at λ = 570 nm. These NC showed a maximum in fluorescence at 581 nm with 33 nm a full width at half maximum (FWHM), and were easily traceable under TexasRed filter using microscopy and 254/360 nm transilluminator during chemical modifications. As a model protein for conjugation we selected lectin ConA from *Canavalia ensiformis*, possessing specificity to glucosylated oligomannose residues found inside the cell [12], and also able to bind some surface glycans on specific immune cell subpopulations. HeLa cells used for testing do not express surface targets of ConA, thereby allowing a convenient testing of lectin-nanocomposite specificity.

Modification of NC-PEG with monochloroacetic acid (MCAA). The following protocol has been chosen as optimal for the modification of NC-PEG with MCAA (schematically shown on Fig. 1, *A*):

1. We prepared reaction buffer (RB), containing 1 M MCAA in 3 M NaOH (important: use glass vials).

2. The conjugation buffer is quite reactive , thus we tested a few variations of conjugation with NC-PEG, by mixing 1 volume of NC-PEG with:

• 1⁄4 volume of RB, providing 0.25 M MCAA and pH ~ 12.

• 1⁄10 volume of RB, providing 0.1 M MCAA and pH ~10.

• 1⁄100 volume of RB, providing 0.01 M MCAA and pH ~8.

3. The obtained mixtures were incubated for 70 min at room temperature with intensive stirring.

4. The reaction was stopped by adding solid NaH_2PO_4 (4 mg/ml) to neutralize the pH to <6, controlled by measuring pH.

5. Excess reactants were removed by dialysis against dH_2O overnight. Then the resulting volume

was measured. The obtained product was NC-PEG-COOH, that could be used for further conjugation.

The influence of 3 different pH environments on NC fluorescence, along with untreated NC and RB alone is shown on Fig. 1, *B* and normalized fluorescence (accounting for NC dilution) is shown on Fig. 1, *C*.

Available protocols propose using basic pH (>12) for conjugation, however this condition can degrade the used NC (and is potentially harmful for many other NC). However, at pH 8 we observed almost complete preservation of fluorescence (Fig. 1, *B* and *C*).

Protein conjugation to NC-PEG-COOH.

6. An equal volume of 0.1 M MES (2-(N-Morpholino)ethanesulfonic acid hydrate) buffer, pH 5 was added to NC-PEG-COOH obtained in step 5.

7. Lectin ConA was dissolved in 0.1 M MES buffer, pH 5 (the conjugation rate: 2 mg of protein/1 ml of NC-PEG-COOH, the concentration of NC: 0.02 mg/ml).

8. Lectin ConA was mixed with NC-PEG-COOH and then dry EDC, MW 191.7, (Sigma-Aldrich) was added to obtain a 0.25 M solution.

9. The reaction lasted 2 h at room temperature, with mixing.

10. Dialysis against 10 mM Tris, pH 6.8 was performed. The final volume of NC-PEG-CO-NH-protein was 4x the initial NC-PEG volume, (that is, i.e., fluorescence and concentration of NC in the final sample was 0.25x the initial concentration of NC-PEG used in the reaction at step 2). If needed, preservative compounds of choice can be added at this step.

Application of conjugated NC. The obtained NC-PEG-CO-NH-ConA conjugates were tested for specific interaction with living and necrotic human HeLa cells. Conjugation with protein resulted in a slight increase in size of NC-PEG-CO-NH-protein complexes (Fig. 2, *B*) vs unconjugated NC-PEG (Fig. 2, *A*); those complexes represented a small percentage of NC manifested aggregation. Still both NC-PEG and NC-PEG-protein demonstrated a prominent Brownian motion, and were detected due to bright point spread function of initial point objects using a cooled Zeiss CCD camera; this did not make possible the direct measurement of the size of NC-PEG-protein, but we estimated it as 0.1 to 0.2 μm for NC-PEG-CO-NH-ConA basing on the behavior of particles with known sizes. Incubation of NC-PEG-CO-NH-ConA with viable cells demonstrated no staining (in fact, there was negative contrasting of viable cells with intact membrane vs positive background due to unbound NC-PEG-CO-NH-ConA fluorescence), as shown in Fig. 2, *C* (differential interference contrast [DIC] microscopy) and Fig. 2, *D* (fluorescence). At the same time, treatment of necrotic HeLa cells allowed effective staining on intracellular compartments with NC-PEG-CO-NH-ConA, as shown in Fig. 2, *E* (DIC) and Fig. 2, *F* (fluorescence), clearly demonstrating accumulation of conjugates on the surface and inside dying cells.

Conclusions. Here we propose the optimized protocol for a covalent coupling of proteins to NC-PEG. The method ensures removal of chemical by-products by dialysis, and it results in a minimal final dilution of NC-protein conjugates (4x dilution). The proposed approach omits the need for centrifugation of small NC, which is usually a limiting step due to the needed high accelerations of > 20 000 g. Besides, it allows the pH of the reaction mixture to be maintained in the range between 6 to 8, preserving the properties of both protein and NC. The proposed approach can be easily modified for covalent targeting of different PEG-bearing nanocomposites.

Acknowledgements

This work was supported by Grants from the Ministry of Healthcare of Ukraine (project 0116U000759). We thank are thankful to Cedars-Sinai Medical Center's International Research and Innovation in Medicine Program, and the Association for Regional Cooperation in the Fields of Health, Science and Technology (RECOOP HST Association) for their support. MB acknowledges the National Science Centre for its financial support within the Sonata 8 project no. UMO-2014/15/D/ST5/02744.

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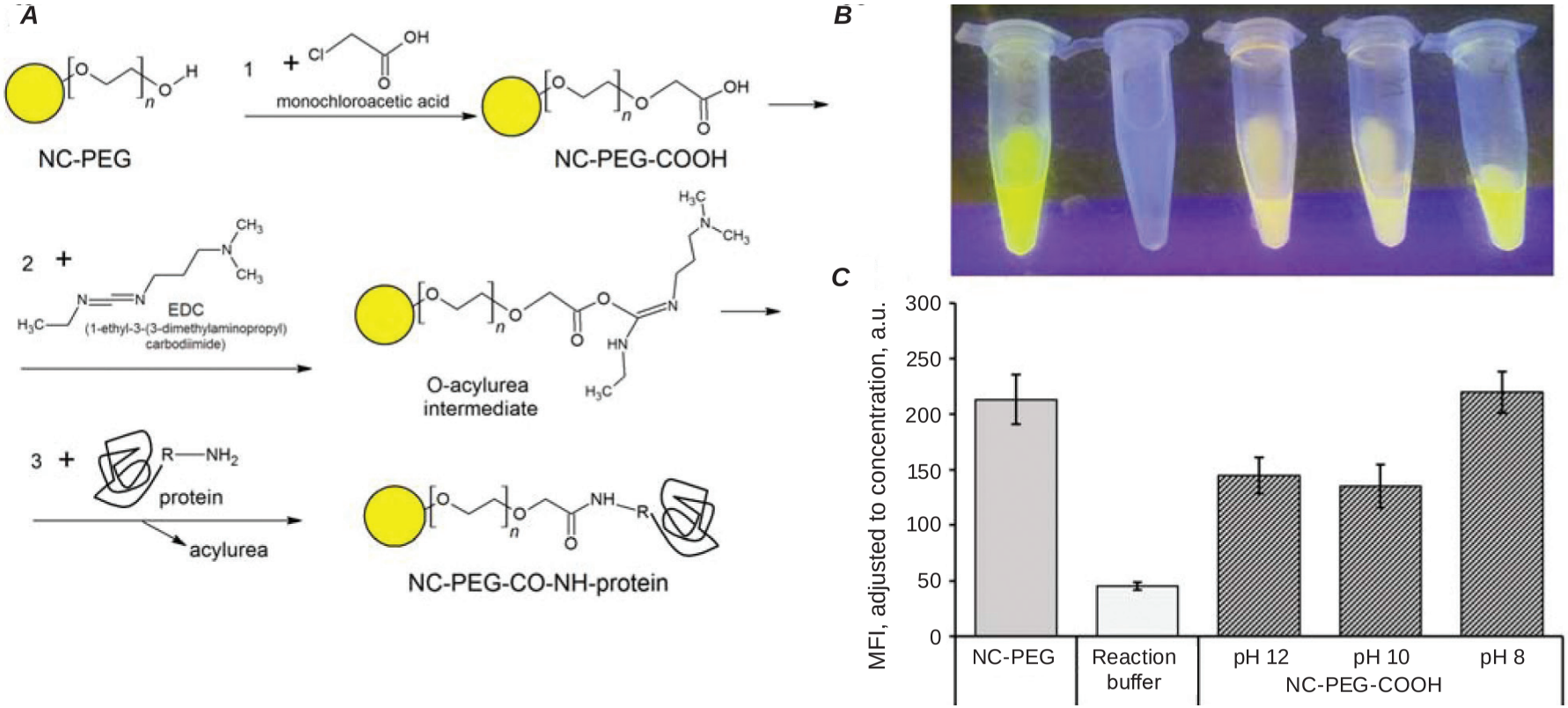

Fig. 1. ***A*** *– Scheme of specific conjugation of NC to proteins. Step 1 – NC-PEG is reacted with MCAA to produce NC-PEG-COOH, while unreacted MCAA is removed by dialysis. Step 2 – EDC is added to provide O-acylurea intermediate, the latter reacts with the amine group of protein (in Step 3), while unreacted acylurea is removed by dialysis; the resulting product is NC-PEG-CO-NH-protein.* ***B*** *and* ***C*** *– Effects of different pH environments used in Step 2 on photoluminescence properties of NC-PEG-COOH.* ***B*** *– Photoluminescence of NC-PEG-COOH (solutions in the tubes are those given below bars in part* ***C****),* ***C*** *– Corresponding MFI values, normalized to NC concentration. Photoluminescence is almost completely preserved at pH 8. NC, nanocrystals; PEG, polyethylene glycol; MCAA, monochloroacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; MFI, mean fluorescence intensity*

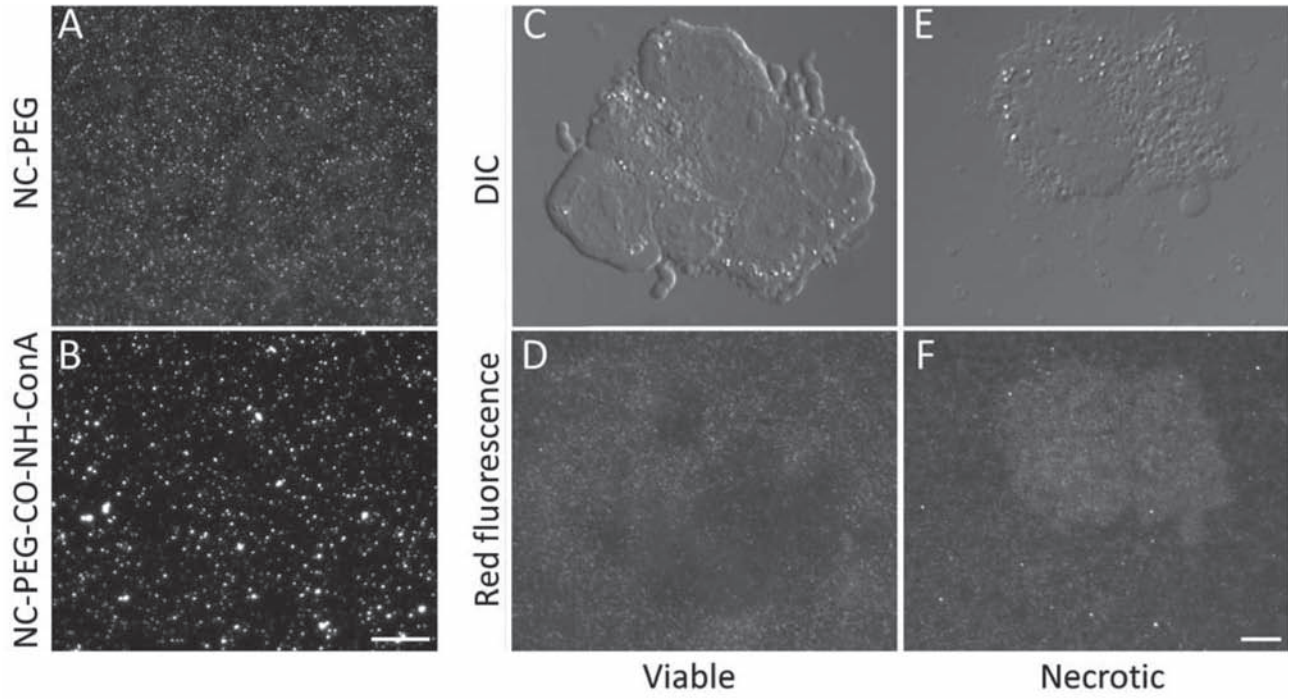

*Fig. 2. Targeting intracellular glycans of HeLa cells with created NC-PEG-CO-NH-ConA. **A** – NC-PEG (before protein conjugation step) and **B** – NC-PEG-CO-NH-ConA (after protein conjugation step). NC-PEG-CO-NH-ConA complexes were incubated with viable (**C**, **D**) or necrotic (**E**, **F**) HeLa cells. Fluorescent (**A**, **B**, **D**, **F**) and DIC (**C**, **E**) microscopy. Scale bar equals 5 μm. NC, nanocrystals; PEG, polyethylene glycol; DIC, differential interference contrast*

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Received 21.03.2018