Journal of Chromatography B, 893–894 (2012) 182–186

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

PEGylation, detection and chromatographic purification of site-specific PEGylated CD133-Biotin antibody in route to stem cell separation

Mirna González-González, Karla Mayolo-Deloisa, Marco Rito-Palomares*

Centro de Biotecnología-FEMSA, Tecnológico de Monterrey, Campus Monterrey, Ave. Eugenio Garza Sada 2501 Sur, Monterrey, NL 64849, Mexico

ARTICLE INFO

Article history:
Received 9 November 2011
Accepted 2 March 2012
Available online 7 March 2012

Keywords:
CD133
Antibody PEGylation
Stem/progenitor cells separation
Streptavidin–biotin conjugation

ABSTRACT

Recovery and purification of stem cells are determining steps in order to obtain the purity and viability required for transplantation. In this context, immunochemical techniques have been widely preferred due to their high selectivity. CD133, a glycoprotein expressed by stem cells, is a well-used marker for isolation of neural stem cells. Transplantation of neural stem cells into patients can promote neural growth and improve neuronal functions. In this study, a new method for site-specific PEGylation of CD133-Biotin antibody is performed through streptavidin–biotin conjugation. Purification was carried out by ion-exchange chromatography. The characterization of the single PEGylated CD133-Biotin antibody was confirmed using electrophoresis with silver staining and I_2–$BaCl_2$ for PEG detection. Moreover, online PEG quantification directly after the chromatographic step was conducted (in each fraction) to detect exact elution times of PEG. In conclusion, the novel CD133-Biotin antibody PEGylation strategy conducted in this study could be used as a process step in route to neural stem cell recovery and purification via the modification of existing techniques such as aqueous two phase systems, PEGylated affinity columns or fluidized chromatography.

1. Introduction

Stem cell transplantation has emerged as a novel therapeutic alternative for several incurable diseases. In this sense, the recovery and purification of stem cells are crucial steps in order to supply the number of cells required and in the degree of purity needed. In other words, the success of a transplant procedure is to a large extent dependent on the effectiveness of the purification and recovery strategies. A desirable recovery method for stem cells has to guarantee high purity and should be sensitive, rapid, quantitative, scalable, non-or minimally invasive to preserve viability and differentiation capacity of the purified cells [1–3]. Currently, there are a wide range of methodologies for stem cell isolation. Nevertheless, there is not a golden standard method that accomplishes all requirements [3–5].

One of the most exploited techniques for stem cell isolation has been the immunochemical, owing the high specificity conferred by the surface marker (cluster of differentiation CD) employed as molecular tagging. For example, one of the most recently used CD for identification of stem cells is the novel CD133 (also known as prominin-1). CD133 is a five-transmembrane glycoprotein [6] that appears to be a reliable marker for the isolation of neural stem cells [7] and has the ability to promote neural growth [8].

Antibody PEGylation has been used in the past decades to improve therapy treatments due to the benefits that the covalent attachment of poly(ethylene glycol) (PEG) to the antibody confers. These advantages include: increase circulating half-lives of antibodies; reduce antigenicity, immunogenicity and toxicity; improve solubility and bioavailability; and enhance proteolytic resistance [9]. Nevertheless, this technique has not been exploited for the recovery and purification of stem cells. In this manner, the conjugation of PEG to a specific antibody (i.e. CD133-Biotin antibody) could confer valuable properties that could be exploited afterwards in downstream process. For example, the recovery and purification of stem cells for clinical applications could be achieved through modified aqueous two phase systems [10], PEGylated affinity columns or fluidized chromatography with PEGylated matrixes. In this sense, a site specific conjugation should be used to preserve the affinity of the interaction between the antibody and antigen. Streptavidin–biotin conjugation is an alternative for a site-specific reaction which can be conducted by employing derivatized PEGs with biotin and biotinylated antibodies (i.e. CD133-Biotin). In this way, streptavidin (with four binding sites for biotin) can bridge two biotinylated reagents and form a site-specific PEGylated antibody. This reaction is highly specific and results in only one product.

In the present study, the site-specific PEGylation of CD133-Biotin antibody is performed through the streptavidin–biotin

Abbreviations: CD, cluster of differentiation; IEC, ion exchange chromatography; PBS, phosphate buffered saline; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulphate polyacrylamide; UV, ultraviolet.

* Corresponding author. Tel.: +52 81 8328 4132; fax: +52 81 8328 4136.
E-mail address: mrito@itesm.mx (M. Rito-Palomares).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2012.03.002

conjugation. Furthermore, its purification and characterization is achieved, as a first step, in route to neural stem cell recovery and purification for clinical applications.

2. Experimental

2.1. Materials

Poly(ethylene glycol) (molecular weight of 10 kDa) carrying an activated amine group at one end and biotin at the other (NH_2-PEG-Biotin) was purchased from Nanocs Inc. (NY, USA). Immunopure streptavidin was obtained from Thermo Scientific (IL, USA), CD133-Biotin antibody (CD133/2 (293C3)-Biotin, catalogue number 130-090-852) from Miltenyi Biotec (Bergisch-Gladbach, Germany), Tris electrophoresis purity reagent and Precision Plus Protein Kaleidoscope molecular weight standard from Bio-Rad (CA, USA), and Tween 20 from Agdia Inc. (IN, USA). The rest of the reagents were acquired from Sigma–Aldrich Co. (MO, USA).

2.2. CD133-Biotin antibody PEGylation

PEGylation of CD133-Biotin antibody was achieved exploiting the streptavidin–biotin conjugation with a NH_2-PEG-Biotin to CD133-Biotin molar ratio of 850:1 and a streptavidin to CD133-Biotin molar ratio of 32:1. For this, 0.1 mg of streptavidin where dissolved in 100 μL of buffer reaction containing phosphate buffered saline 1× (PBS) (Sigma–Aldrich Co., MO, USA) with 0.05% Tween 20 pH 7.4 and 100 μL of CD133-Biotin. This solution was incubated for 1 h at room temperature and then concentrated to 15 μL using 0.5 mL Amicon Centrifugal filter units of 100 kDa (Millipore, USA). Buffer reaction (485 μL) was added to the 15 μL concentrate and mixed with 500 μL of a solution 1 mg/mL of NH_2-PEG-Biotin 10 kDa (Nanocs, NY, USA). The resulting solution was incubated during 1 h at room temperature. The PEGylated CD133-Biotin antibody was stored at 4 °C.

2.3. Purification of PEGylated CD133-Biotin antibody

The purification of the PEGylated CD133-Biotin antibody was carried out with an Äkta Explorer 100 system (GE Healthcare, Uppsala, Sweden) with a 5 mL ion-exchange column (HiTrap Q-Sepharose Fast Flow, GE Healthcare) pre-equilibrated with buffer A, Tris–HCl 20 mM (pH 8.3). After injection of 0.1 mL of syringe filtered (0.45 μm) sample, the column was eluted with a 10 volume column linear gradient buffer (0–2 M NaCl in buffer A) at a flow rate of 2.5 mL/min. The 5 mL fractions were monitored at 280 nm, collected for online PEG quantification and then concentrated to 1.0 mL using a Centricon 3 kDa (Millipore, MA, USA) for the SDS–PAGE analysis.

2.4. SDS–PAGE analysis

For this, 12.5% (w/v) mini acrylamide gels (10 cm × 7.5 cm × 0.75 cm) were prepared as described by Laemmli [11]. The gels were first visualized for PEG using the barium–iodine method from Skoog [12] eliminating the fixation step. In brief, gels were incubated for 5 min while shaking at room temperature with 5 mL of 5% barium chloride and 2 mL of 0.1 M iodine solution. Subsequently, the gels were washed with sufficient double-distilled water for 15 min while shaking at room temperature. After the PEG staining step, the protocol for the silver staining developed by Blum [13] was carried out as follows: gels were fixed for 40 min with 60 mL of a solution containing 50% methanol, 12% acetic acid and 0.5 mL of 37% formaldehyde per litre. The fixed gels were washed during 30 min with 120 mL of 50% ethanol and treated afterwards with 0.02% sodium thiosulphate for 1 min. Excess thiosulphate was removed from the gel surface by rinsing three times with double-distilled water for 20 s. Silver nitrate impregnation was carried out in 60 mL of 0.2% silver nitrate solution containing 45 μL of 37% formaldehyde for 20 min. Gels were washed two times with double-distilled water for 20 s and developed with 6% sodium carbonate solution containing 0.5 mL of 37% formaldehyde and 4 mg sodium thiosulphate per litre. The reaction was stopped by adding 30 mL of a solution containing 50% methanol and 12% acetic acid and the gels were stored in 50% methanol.

2.5. Online PEG quantification

Online PEG quantification for the detection of the exact elution times of PEG was carried out directly after the chromatographic step. In brief, 176 μL of each collected fraction was treated with 44 μL of 5% barium chloride and 22 μL of 0.1 M iodine solution [14]. Next, they were incubated for 15 min before analysis at 535 nm.

3. Results and discussion

Antibody PEGylation has been widely used, however, to our best knowledge, no reports are found in literature concerning PEGylation of CD133 antibody. This conjugation was achieved through the site-specific reaction streptavidin–biotin (Fig. S1). Streptavidin poses four binding sites with an extraordinarily high affinity for biotin that can bridge two biotinylated reagents (PEG and CD133-Biotin antibody, in this case). The streptavidin–biotin complex has a great resistance to organic solvents, denaturants, detergents, proteolytic enzymes, and extremes of temperature and pH making this conjugation very stable and one of the strongest non-covalent interactions known in nature.

The main advantage of utilizing this site-specific reaction over the amine-reactive chemistry (most common way of attaching PEG to protein) [9] is the preservation of the affinity interaction between the antibody and its antigen. Other positive effects that are embedded with the PEGylation include: increase circulation half-lives, greater environmental and proteolytic stability [9].

The purification of PEGylated CD133-Biotin antibody was performed with a Q-Sepharose resin. Fig. 1 illustrates the chromatogram profile of the PEGylated CD133-Biotin antibody reaction and reaction's reagents. The native negatively charged CD133-Biotin antibody is shown in green (Fig. 1). It is clearly observed that practically a minor amount is present in the reaction (red line at approximately 40 mL). This proves the high affinity of streptavidin with biotin. The first red peak of the PEGylated reaction (at about 6 mL) is positively charged. Therefore is not retained on the anionic adsorbent. Presumably, this peak represents the unbound NH_2-PEG-Biotin that did not react with the conjugated CD133-Biotin-Streptavidin. At this same time (around minute 2), a small amount of the standard of streptavidin and PEG are visualized, implying they are not retained on the column. Normally, the PEG standard will not give a signal at 280 nm. But in this case, a modified PEG is used and the UV absorption may come from traces of intermediate (tosylate) left during the synthesis, as explained by the supplier.

From the chromatogram in Fig. 1, the elution of the negatively charged compounds is achieved with a considerably low conductivity. Hence, a concentrated salt gradient is not required to displace these weak anionic complexes. The obtained elution profile was the following: NH_2-PEG-Biotin standard followed by the PEGylated CD133-Biotin antibody, then the streptavidin standard and the last peak represents the native CD133-Biotin antibody. The resolution obtained allowed the separation of each standard from the PEGylated CD133-Biotin antibody (third red peak), supporting the use of anion exchange purification strategy. With this evidence, it can be stated that the PEGylated CD133-Biotin antibody acquires a weaker

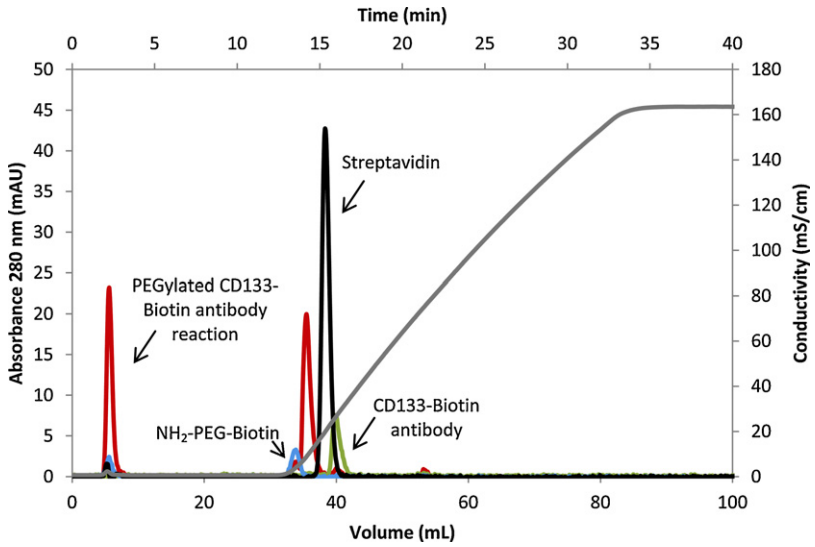

Fig. 1. Chromatographic profiles using ion exchange chromatography of the PEGylated CD133-Biotin antibody reaction and reaction's reagents. Column: HiTrap Q-Sepharose Fast Flow, buffer A: Tris–HCl 20 mM pH 8.3, buffer B: buffer A + 2 M NaCl, Flow: 2.5 mL/min, loop: 100 μL. Volume column: 5 mL. Red line: PEGylated CD133-Biotin antibody reaction; blue line: NH_2-PEG-Biotin reagent; black line: streptavidin reagent and green line: CD133-Biotin antibody reagent. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

negative charge through the process reaction in comparison to its native antibody standard.

The SDS–PAGE gels were run to present evidence of the PEGylation of CD133-Biotin antibody (Figs. 2 and 3). First, the gels were stained with I_2–$BaCl_2$ (Figs. 2A and 3A) to determine the presence of PEG. Then they were silver stained (Figs. 2B and 3B) to visualize proteins. NH_2-PEG-Biotin standard is seen with the I_2–$BaCl_2$ staining at 50 and 20 kDa (Fig. 2A lane 1). Meanwhile, the PEGylated CD133-Biotin antibody reaction displays two bands of PEG, one greater than 100 kDa and other at 20 kDa (Fig. 2A lane 7). With this evidence, it can be stated that the PEGylated CD133-Biotin antibody is the one with a higher molecular weight. While the excess PEG that did not reacted conserves the same weight of 20 kDa, as its standard. To this respect, the I_2–$BaCl_2$ method detects the presence of PEG in a fast, simple, economical, and non-toxic qualitative way based on the formation of a barium iodide complex with PEG [14]. But it is important to mention that the molecular weight of the bands of PEG read with respect to the Precision Plus Protein Kaleidoscope will not correspond to the real weight of the PEG.

The silver staining method (Fig. 2B lane 7) confirms the presence of PEGylated CD133-Biotin antibody at about 100 kDa. In this manner, the visualization of PEG (Fig. 2A lane 7) and protein (Fig. 2B lane 7, dotted square) at the same spot of the gel concludes that CD133-Biotin antibody is indeed PEGylated. The reaction of the PEGylated CD133-Biotin antibody also contains streptavidin that did not react at approximately 15 kDa (Fig. 2B). This coincides with the band in Fig. 2B lane 5 which contains the streptavidin standard (circulated region). Lane 9 shows the CD133-Biotin antibody standard, which contains two bands of approximately 25 kDa and 50–60 kDa. The heaviest band of 50–60 kDa is still visible on lane 7 (PEGylated CD133-Biotin antibody). But the band of 25 kDa is apparently the PEGylated and thus increases its molecular weight, as it is not visualized on lane 7 (PEGylated CD133-Biotin antibody).

Online PEG quantification of the collected fractions after ion exchange chromatography (IEC) was also conducted with the I_2–$BaCl_2$ method employing a calibration curve. The concentration of PEG in each fraction of the PEGylated CD133-Biotin antibody reaction (Fig. 4, green line) and the PEG standard (Fig. 4, blue line) were calculated with this novel procedure of online PEG quantification. An overlapping of these data with the chromatographic profile of the PEGylated CD133-Biotin antibody reaction (Fig. 4, red dotted line) was performed. The presence of PEG

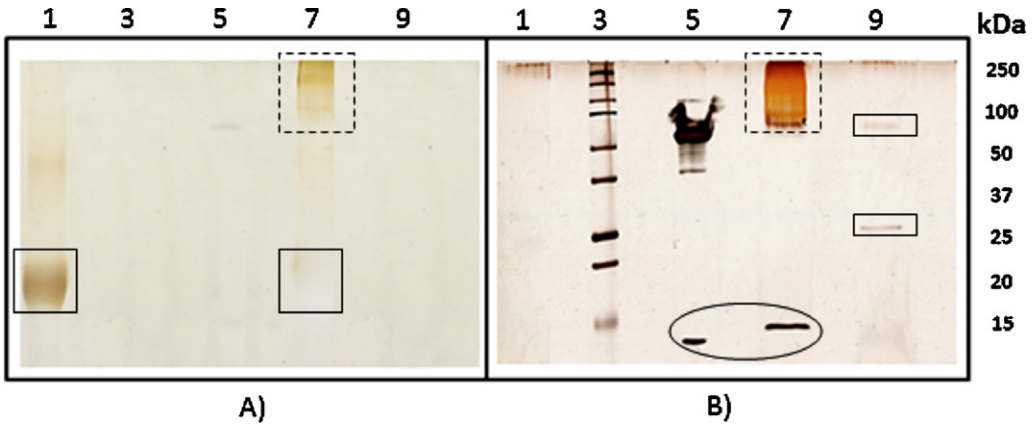

Fig. 2. SDS–PAGE analysis of PEGylated CD133-Biotin antibody reaction and reaction's reagents with I_2–$BaCl_2$ staining for PEG detection (A) and silver staining for protein detection (B). The samples are: NH_2-PEG-Biotin 5 μg (lane 1), molecular weight marker Precision Plus Protein Kaleidoscope 1.5 μg (lane 3), streptavidin 0.83 μg (lane 5), PEGylated CD133-Biotin antibody (lane 7), and CD133-Biotin antibody 0.5 μg (lane 9).

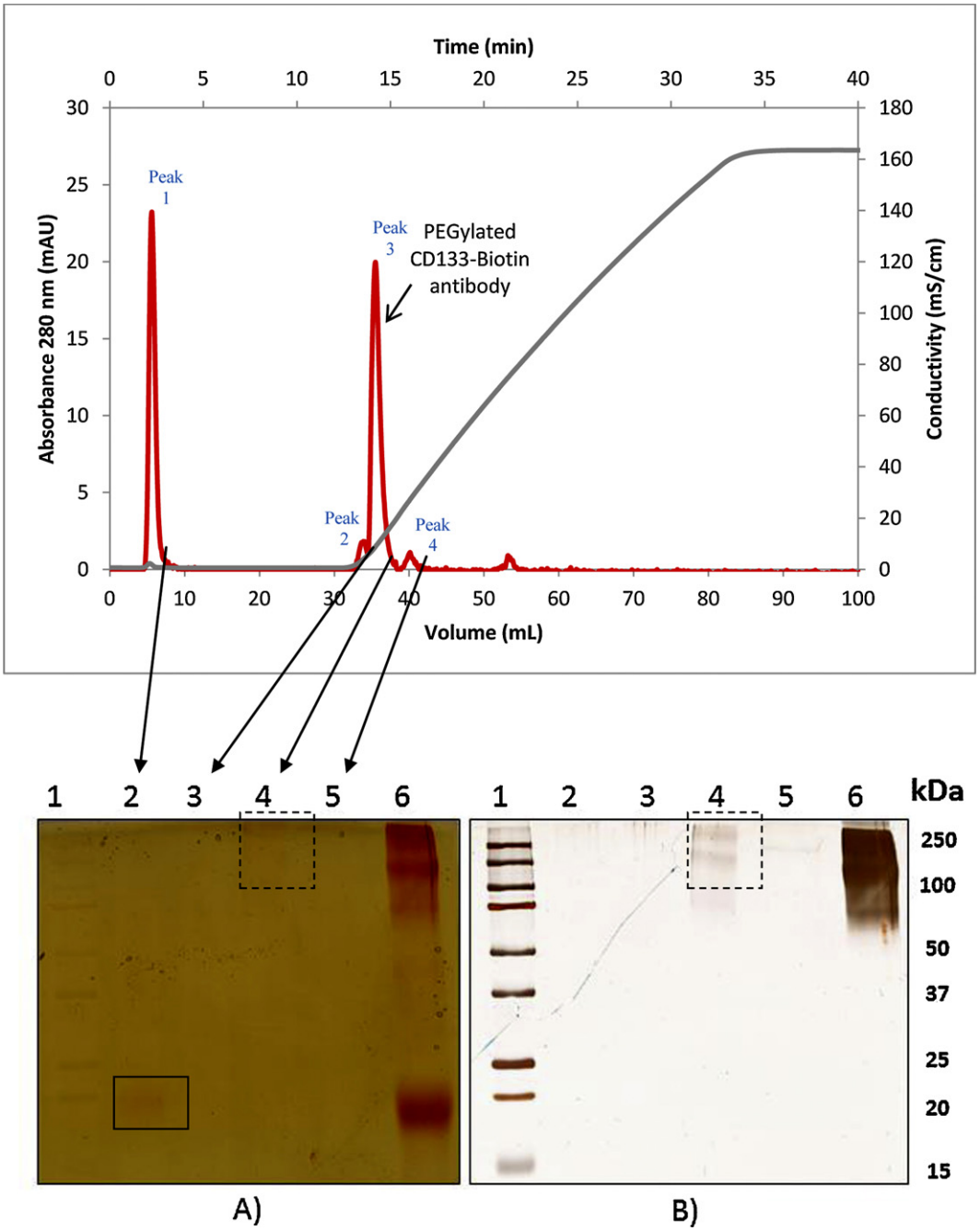

Fig. 3. SDS–PAGE analysis of purified fractions of PEGylated CD133-Biotin antibody after ion exchange chromatography with I_2–$BaCl_2$ staining for PEG detection (A) and silver staining for protein detection (B). The samples are: molecular weight marker Precision Plus Protein Kaleidoscope 1.5 μg (lane 1), peak number one of the purified PEGylated CD133-Biotin antibody (lane 2), peak number two of the purified PEGylated CD133-Biotin antibody (lane 3), peak number three of the purified PEGylated CD133-Biotin antibody (lane 4), peak number four of the purified PEGylated CD133-Biotin antibody (lane 5), and PEGylated CD133-Biotin antibody before ion exchange chromatography (lane 6).

agrees with the previous information presented. The first green peak is the excess PEG that did not reacted. Meanwhile, the second green peak is the PEG that PEGylates CD133-Biotin antibody, which elutes at around 35 mL. Another important aspect of the online PEG quantification method is that the concentration of PEG quantified at the end of the chromatography corresponds to the initial concentration of PEG utilized during the PEGylation reaction. Furthermore, this method, as the PEG staining procedure implemented on SDS–PAGE gels, is fast, easy, non-toxic, economical, but the difference is that this technique is quantitative.

The fractions obtained from the IEC were also analysed with an SDS–PAGE (Fig. 3). PEGylated CD133-Biotin antibody corresponds to the third peak (Fig. 3B lane 4) and the excess PEG that did not react was collected in the first peak (Fig. 3A lane 2). Moreover, there is no evidence of excess PEG in another fraction, confirming the effectiveness of the separation technique capable of separating in a single step the free PEG and other contaminants from the PEGylated product. Additionally, the purification of PEGylated CD133-Biotin antibody also increases the resolution in the SDS–PAGE gel, allowing the clear visualization of 2 bands of approximately 250 kDa and 150 kDa (Fig. 3B lane 4). The results reported here clearly

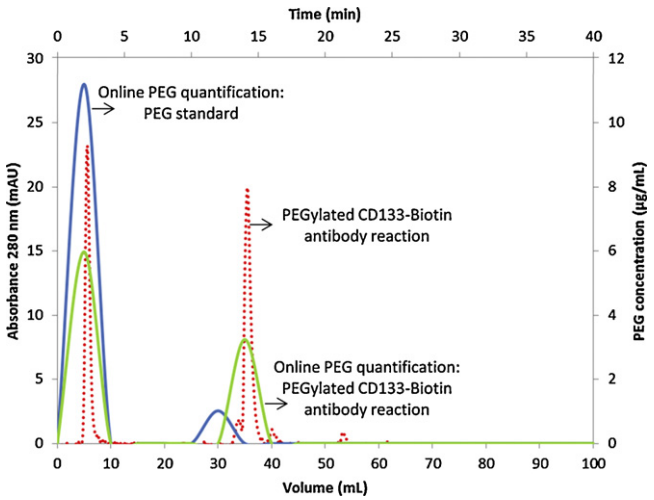

Fig. 4. Overlapping of PEGylated CD133-Biotin antibody reaction chromatogram and online PEG quantitation using I_2–$BaCl_2$. Blue line: online PEG quantification of the PEG standard; green line: online PEG quantification of the PEGylated CD133-Biotin antibody reaction; and red dotted line: chromatogram of the PEGylated CD133-Biotin antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

demonstrated that the PEGylation, purification and characterization of CD133-Biotin antibody was effectively conducted obtaining a site-specific PEGylated CD133-Biotin antibody.

4. Conclusions

This study reports the successful PEGylation of CD133-Biotin antibody via streptavidin–biotin conjugation. This site-specific reaction is highly effective and thus only produces a single product. The online PEG quantification allows a rapid visualization of the fractions which contains the excess PEG as well as the PEG that PEGylates CD133-Biotin antibody. The novel PEGylated CD133-Biotin antibody prepared in this study could be used as a practical strategy in immunoaffinity aqueous two phase systems, PEGylated affinity columns or fluidized chromatography. Hence exploiting the potential advantages conferred by the PEG to the antibody with respect to these same techniques utilizing native antibodies.

Acknowledgements

The authors wish to acknowledge the financial support of Tecnológico de Monterrey, Bioprocess research chair (Grant CAT161), the Zambrano-Hellion Foundation and of the CONACyT for the fellowships of M. González-González, No. 223963 and K. Mayolo-Deloisa, No. 27470.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.03.002.

References

[1] K. Melnik, M. Nakamura, K. Comella, L.C. Lasky, M. Zborowski, J.J. Chalmers, Biotechnol. Prog. 17 (2001) 907.
[2] R. Pethig, A. Menachery, S. Pells, P. De Sousa, J. Biomed. Biotechnol. 2010 (2010) 1.
[3] M. González-González, P. Vázquez-Villegas, C. García-Salinas, M. Rito-Palomares, J. Chem. Technol. Biotechnol. 87 (2012) 2.
[4] J. Seligman, S. Slavin, I. Fabian, Stem Cells Dev. 18 (2009) 1263.
[5] G. Gaipa, M. Tilenni, S. Straino, I. Burba, G. Zaccagnini, D. Belotti, E. Biagi, M. Valentini, P. Perseghin, M. Parma, C.D. Campli, A. Biondi, M.C. Capogrossi, G. Pompilio, M. Pesce, J. Cell. Mol. Med. 14 (2010) 1619.
[6] S. Miraglia, W. Godfrey, A.H. Yin, K. Atkins, R. Warnke, J.T. Holden, R.A. Bray, E.K. Waller, D.W. Buck, Blood 90 (1997) 5013.
[7] Y. Wu, P.Y. Wu, Stem Cells Dev. 18 (2009) 1127.
[8] H.R. Martinez, M.T. Gonzalez-Garza, J.E. Moreno-Cuevas, E. Caro, E. Gutierrez-Jimenez, J.J. Segura, Cytotherapy 11 (2009) 26.
[9] A.P. Chapman, Adv. Drug Deliv. Rev. 54 (2002) 531.
[10] A.F. Sousa, P.Z. Andrade, R.M. Pirzgalska, T.M. Galhoz, A.M. Azevedo, C.L. da Silva, M. Raquel Aires-Barros, J.M.S. Cabral, Biotechnol. Lett. 33 (2011) 2373.
[11] U.K. Laemmli, Nature 227 (1970) 680.
[12] B. Skoog, Vox Sang. 37 (1979) 345.
[13] H. Blum, H. Beier, H.J. Gross, Electrophoresis 8 (1987) 93.
[14] X.W. Gong, D.Z. Wei, M.L. He, Y.C. Xiong, Talanta 71 (2007) 381.