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**Effect of Lysine Modification on the Stability and Cellular Binding of Human  
Amyloidogenic Light Chains**

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**Running Title:** Cellular binding of light chains

**ABSTRACT**

AL amyloidosis is characterized by the pathologic deposition as fibrils of monoclonal light chains (i.e., Bence Jones proteins [BJPs]) in particular organs and tissues. This phenomenon has been attributed to the presence in amyloidogenic proteins of particular amino acids that cause these molecules to become unstable, as well as post-translational modifications and, in regard to the latter, we have investigated the effect of biotinylation of lysyl residues on cell binding. We utilized an experimental system designed to test if BJPs obtained from patients with AL amyloidosis or, as a control, multiple myeloma (MM), bound human fibroblasts and renal epithelial cells. As documented by fluorescent microscopy and ELISA, the amyloidogenic BJPs, as compared with MM components, bound preferentially and this reactivity increased significantly after chemical modification of their lysyl residues with sulfo-NHS-biotin. Further, based on tryptophan fluorescence and circular dichroism data, it was apparent that their conformation was altered, which we hypothesize exposed a binding site not accessible on the native protein. The results of our studies indicate that post-translational structural modifications of pathologic light chains can enhance their capacity for cellular interaction and thus may contribute to the pathogenesis of AL amyloidosis and multiple myeloma.

## 1. INTRODUCTION

Plasma cell dyscrasias, e.g., multiple myeloma, light chain deposition disease, and AL amyloidosis, are associated with the overexpression of monoclonal light chains (LCs), i.e., Bence Jones proteins (BJPs), that deposit, respectively, as renal tubular casts, membrane deposits, and amyloid fibrils [1, 2]. These sequelae have been attributed to primary structural differences in the pathologic protein that induce formation of amorphous, punctuate, or fibrillar tissue aggregates [3, 4]. In the case of AL amyloidosis, other factors such as post-translational modification, including glycosylation and cysteinylolation, have been implicated in LC fibrillogenesis [5-8]. Further, lipid peroxidation and advanced glycation end products have been immunolocalized with AL deposits suggesting these non-enzymatic alterations may be involved in this process [9]. Although it has been shown that adduction of side-chain amino acids can result in aggregation of certain amyloidogenic precursor proteins (i.e.,  $\alpha$ -synuclein and A $\beta$  peptides), heretofore, this effect on LCs has not been studied [10-12].

Surface contact is yet another factor thought to be important in LC fibrillogenesis, based on the fact that amyloid localizes preferentially along the basement membranes of endothelial and epithelial cells and additionally, the presence of raft lipids in isolates of systemic amyloid deposits [13, 14]. In this regard, interaction of LCs with artificial surfaces, such as electronegatively charged mica or membrane lipids, has been shown to promote fibril formation [15, 16] and we have evidenced that their adhesion to plastic surfaces results in a conformational change that renders these molecules reactive with an anti-fibril specific mAb [17]. Notably, these phenomena could lead to an increase in the

local concentration of denatured LCs at cell surfaces, promoting protein aggregation and fibril formation and then recruitment of unmodified proteins into the pathologic deposits.

In this study, we have investigated the effect of lysine modification of monoclonal LC primary amines (through biotinylation) to evaluate the stability of AL amyloid- versus non-amyloid-associated BJPs and to test their capacities to interact with human fibroblast and epithelial cells. We have found that this alteration affected the AL components predominantly, resulting in reduced stability and enhanced cellular binding. On this basis, we suggest that these events can contribute to the pathogenesis of AL amyloidosis.

## 2. MATERIALS AND METHODS

*2.1. Proteins and chemical modification.*  $\lambda$ -type BJPs were isolated from the urine of 2 AL patients in whom there was extensive organ involvement (FRE, IRHE) and 2 with multiple myeloma (MM) who had renal tubular cast nephropathy, but no evidence of amyloid at postmortem examination (JRHE and HUD). The proteins were reconstituted in PBS, pH 7.4, at a concentration of 1-2 mg/ml and were incubated in the dark with a 6 to 20-fold molar excess of sulfo-NHS-biotin (Pierce, Rockford, IL) for 2 h at room temperature; free biotin was removed by size exclusion gel filtration using a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden). Protein concentrations were determined using a micro BCA assay kit (Pierce).

*2.2. Mass spectrometry.* Solutions of native and modified BJPs (0.1-1 mg/ml) were loaded into a 20  $\mu$ l injection loop of an Applied Biosystems (Foster City, CA) 173 capillary HPLC and chromatographed over 90 min using a reverse phase Aquapore 300 C<sub>8</sub> (150  $\times$  0.5 mm) column with a gradient of 15-70% acetonitrile modified with 0.02% trifluoroacetic acid; the eluent was directed into the ion-spray of a PE-Sciex (Applied Biosystems) type 150 EX single quadrupole mass spectrometer and masses were determined using the Biomultiview software provided by the manufacturer.

*2.3. Immunofixation electrophoresis.* Immunofixation electrophoresis was performed according to the manufacturer's instructions (Paragon, Beckman Coulter, Ireland). In brief, 3-6  $\mu$ l of 0.4 mg/ml solutions of native and biotinylated BJPs were applied to the membrane and immunostained with the anti- $\lambda$  LC antibody. The reaction was visualized with Paragon blue stain.

*2.4. Cells and culture conditions.* Human foreskin fibroblasts were obtained from the American Type Tissue Culture Collection and cultured in EMEM containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, and 0.1 mM non-essential amino acids, supplemented with 10% FCS and penicillin (50 U/ml)/streptomycin (50 µg/ml). Experiments were performed with cells < 41 population doublings. Human renal epithelial cells (HRECs) were supplied by Lonza (Walkersville, MD) and were cultured in basal REC media, supplemented with RE growth medium Bulletkit® that contained hydrocortisone, hEGF, insulin, triiodothyronine, transferrin, gentamicin, amphotericin-B, and 0.5% FBS. The cells used were from passages 4 to 6 and were negative for mycoplasma (Lonza Mycoalert kit).

*2.5. Microscopic analysis of LC-cell interaction.* Cells were grown to confluence on sterile glass coverslips placed in 6-well tissue culture dishes and incubated under 5% CO<sub>2</sub> for 3 h at 37 °C with and without native or biotinylated BJPs (100 µg/0.5 ml). The cells were washed gently × 3 with growth media to remove unbound BJP. Native light chains were further incubated with a biotinylated anti-λ (Fλ-G9) antibody for 1 h at 37 °C and washed to remove unbound antibody [18]. To stain F-actin, Alexa Fluor®-488 phalloidin (Invitrogen, Molecular Probes, Eugene, OR) in cytofix/ cytoperm® reagent (BD Biosciences, Pharmingen, San Diego CA) was added to a final concentration of 30 µg/ml. The cells were incubated for 20 min at 23 °C on a rocking platform, and then for 15 min in a 1:10 dilution of Perm-wash buffer (BD Biosciences, Pharmingen, San Diego CA). Nuclei were stained for 30 min at 37 °C with Hoechst 33342 trihydrochloride-trihydrate/PBS (Invitrogen). Biotinylated LCs were visualized by addition of streptavidin-Alexa Fluor®-594 (Invitrogen) for 1 h at 23 °C. After final PBS and then



water washes, cells were coverslipped using fluorescent aqueous mounting medium (Dakocytomation, Carpinteria, CA) and examined within 72 h with an epifluorescent microscope. Images were recorded with a 3-chip color CCD camera (Diagnostic Spot RT 2.2.0).

*2.6. Quantitative analysis of LC-cell interaction.* Cells were grown to confluence in 96-well plates and incubated under 5% CO<sub>2</sub> for 3 h at 37 °C, with and without native or biotinylated BJPs (50-200 µg/ml in 100 µl volumes). Each well was washed repeatedly with 200 µl of growth media to remove unbound protein and bound BJP was detected using an HRP-labeled anti-human λ LC antibody (Biosource, Camarillo, CA). The wells were washed × 3 and the HRP signal detected using an ABTS solution (KPL, Gaithersburg, MD); the presence of cells was confirmed by microscopy. Absorbance was measured at 405 nm using a BIO-TEK Synergy HT multi-detection microplate reader. To control for non-specific binding of the secondary antibody, absorbance values for wells containing cells, but no BJP, were subtracted from those of test wells. All assays were done in triplicate.

*2.7. Thermal denaturation of LCs.* BJPs (25 µg/ml) were suspended in 2.5 ml PBS in a 3-ml quartz cuvette with stirring. A temperature probe was inserted and the sample heated from 22 to 65 °C at a rate of 2 °C per min. Tryptophan (Trp) fluorescence was measured using an SLM Thermospectronic Aminco Bowman (Series II) spectrofluorometer (excitation, 290 nm; emission, 410 nm). Slit widths of 4 and 8 nm were used and the photomultiplier tube voltage was set at 850 V.

2.8. *Calculation of free energy of unfolding.* The free energy of unfolding for each protein was determined using thermal denaturation data as described previously [19] A correction was made for the contribution of quenched fluorescent Trp residues according to the following transformation:

$$y_{\text{corr}} = (y - c) - mx$$

where  $y$ ,  $m$ , and  $c$  represent, respectively, the original value, the gradient, and the intercept at  $x = 0$ , of the linear quenching of the folded molecule. The  $T_m$  and  $\Delta H_m$  were calculated by nonlinear least-squares analysis of the corrected thermal denaturation data using an equation adapted from Eftink [20]. The value of  $\Delta G$  at room temperature ( $\Delta G_{25}^{\circ}\text{C}$ ) was determined as follows:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln(T/T_m)]$$

where  $T = 298^{\circ}\text{K}$  and  $\Delta C_p$  represented the change in heat capacity associated with unfolding, as calculated theoretically according to the method of Milardi [21]. All parameters were determined using the nonlinear, least-squares fitting program ULTRAFIT (Biosoft, U.K.).

2.9. *Emission spectra of modified and native LCs.* Sample (25  $\mu\text{g/ml}$ ) volumes of 0.5 or 2.5 ml in PBS, pH 7.4, were placed in quartz cuvettes, stirred, and fluorescence measured (excitation, 280 and 295 nm; slit width, 4 nm and emission, 300-450 nm; slit width, 8 nm). Data from 3 repetitions were collected using a scan rate of 1 nm per sec and then averaged.

*2.10. Circular dichroism.* CD measurements were performed with a Jasco 810 spectropolarimeter equipped with a Peltier temperature control unit. Proteins were dissolved in 25 mM Na-phosphate buffer, pH 7.0, at concentrations between 0.8-1.0 mg/ml. The spectra were recorded in a 0.1 cm path-length cuvette (190 - 350 nm) at 25 °C and 37 °C and the data expressed as mean residue ellipticity ( $[\theta]$ ). Thermal denaturation experiments were performed in a 0.1 cm path-length cuvette and spectra recorded in the range of 195 - 350 nm between 25 - 80 °C at 2 °C intervals at a rate of 2 °C/min. Single wavelength kinetics were reported at 205 nm.

*2.11. Statistical analysis.* Unpaired ANOVA, followed by a Tukey multiple comparison post test, was used (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)).

*2.12. LC sequence multiple alignment.* Sequence alignment of the 4 LCs was performed using the CLUSTAL 2.0 Multiple alignment software [22]



### 3. RESULTS

*3.1. Biotin – LC binding.* Quantitative assessment of the number of biotin molecules that bound to the 4  $\lambda$ -type BJPs was determined using liquid chromatography–mass spectrometry (LC-MS) (supplemental data Figure 1) and provided information that was correlated with their pathogenicity and interaction with cultured cells. Electrophoretic analysis (SDS-PAGE) indicated that all had the expected Mr of a disulphide-linked covalent LC dimer (Supplemental data Figure 2).

*3.2. Modification of LC lysines alters cellular interactions.* The 2 amyloidogenic BJPs (FRE and IRHE) preferentially adhered to cultured human cells after attachment of biotin to their lysyl residues. As shown in Figure 1, they interacted more strongly (as evidenced by the red fluorescence) with human foreskin fibroblasts than did the biotinylated non-amyloid counterparts (HUD and JRHE). Notably, there was little or no binding by any of the 4 in their native state. These data were confirmed by a quantitative immunoassay where significant increases were seen, at all concentrations tested, in the binding of biotinylated versus native FRE and IRHE ( $p < 0.001$ ) (Figure 2a and b). This effect also was observed in the case of JRHE ( $p < 0.01$ ) when this protein was tested at higher concentrations (Figure 2c). Notably, lysyl modification of non-amyloidogenic HUD did not significantly change its interaction with HRECs (Figure 2d).

In their native state the 2 AL proteins FRE and IRHE adhered to HRECs significantly more than native JRHE at 100  $\mu\text{g/ml}$  (FRE vs JRHE  $p < 0.001$  and JRHE vs IRHE  $p < 0.01$ ) and 200  $\mu\text{g/ml}$  ( $p < 0.001$  comparing both AL proteins to JRHE). Native HUD exhibited increased adhesion to HREC relative to native JRHE at all concentrations

tested ( $p < 0.05$  at 50  $\mu\text{g/ml}$  and  $p < 0.001$  at 100 and 200  $\mu\text{g/ml}$ ). However, HUD did not differ in its basal adhesion to HRECs when compared with the 2 AL-LCs.

*3.3. Effect of lysine modification on LC thermodynamic stability.* The change in Trp fluorescence intensity of native and biotinylated BJPs, resulting from increasing the temperature from 22 to 60  $^{\circ}\text{C}$ , was monitored and the midpoint transitional temperature ( $T_m$ ) determined. In the case of native FRE, this value was 53.7  $^{\circ}\text{C}$ , which decreased to 47  $^{\circ}\text{C}$  after addition of 12 biotins per dimer. Similarly, native HUD and JRHE had  $T_m$  values of 55  $^{\circ}\text{C}$  and 55.6  $^{\circ}\text{C}$ , respectively, which went down to 48.8  $^{\circ}\text{C}$  (HUD) and 49  $^{\circ}\text{C}$  (JRHE) after maximal biotinylation (Figure 3a). In the case of IRHE, the  $T_m$  was 51.5  $^{\circ}\text{C}$ , but dropped to 48.5  $^{\circ}\text{C}$  after addition of 8 biotins to the dimer. Comparable results were obtained by CD analysis of thermally denatured FRE; namely, there was reduced stability after lysine modification (Figure 3b). Calculation of the Gibbs Free Energy ( $\Delta G$ ) for all 4 BJPs (Table 1) revealed that this value changed dramatically after biotinylation of FRE and HUD (2.68 and 4.4, respectively) as compared to the native proteins (5.21 and 6.19, respectively). The amyloidogenic BJP IRHE was already quite unstable with a  $\Delta G$  of 3.64, which fell to 3.37 after lysine modification, while the value for the MM-associated BJP JRHE increased from 5.55 to 6.27.

*3.4. Effect of lysine modification on LC charge, secondary structure, and fluorescence spectra.* As found electrophoretically, the addition of biotin to the 4 BJPs effectively increased their pI, rendering them more electronegative (Figure 4). Native IRHE, FRE,

and JRHE had similar migration patterns, while unmodified HUD was slightly more anionic.

The Trp emission spectra (excitation, 295nm) of the BJPs at 37 °C decreased after biotin labeling (Figure 5). This effect was greater for the IRHE and HUD proteins, as compared to FRE and JRHE. Similar results were observed when Trp/Tyr fluorescence was measured (excitation, 280nm).

The CD spectra of native and biotinylated proteins revealed that they were composed primarily of  $\beta$ -sheets with minimal amounts of  $\alpha$ -helix present (Figure 6). The spectra of both forms were similar, evidencing that their shape was not altered by lysine modification. However, biotinylation of the LCs did result in an increase in the negative maxima at 217 nm, indicative of enhanced  $\beta$ -sheet formation [23]. This alteration was most apparent for FRE and JRHE.

### *3.5 Multiple alignment comparison of LC sequences*

The sequence alignment data indicates that there is a significant amount of homology between the 4  $\lambda$  LCs but particularly between the 3  $\lambda$ 3a LCs (Table 2). All of the lysyl residues are located within the framework regions of the LC variable domains except Lys29 in the CDR1 of FRE (Figure 7). Neither IRHE nor HUD contains a Lys in FR1 whereas both FRE and JRHE have a Lys at position 15 in this region. HUD and IRHE both contain one Lys in FR2. This is the only lysyl residue within the variable region of IRHE. HUD however has a second Lys within FR3. None of the  $\lambda$ 3a LCs has a Lys

within this region. The position of the Lysyl residues within the constant domains are similar for all LCs with the exception of an extra Lys192 in HUD and Lys190 in JRHE (Figure 7).

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#### 4. DISCUSSION / CONCLUSION

Lysine modification has been reported to alter protein conformation and function and has been implicated in the conversion of amyloidogenic precursor proteins, i.e.,  $\beta$ -microglobulin,  $\alpha$ -synuclein and A $\beta$  peptide, into fibrils [10-12, 24]. Similarly, glycation of albumin via lysyl side chain modifications can lead to the formation of fibrils or amorphous aggregates [25]. The effects of lysyl adduction on pathogenic LCs has not previously been investigated. Although biotinylation is unlikely to occur *in vivo* the effects observed in this study may be mirrored by natural modifications such as glycation or lipoxidation [26]. Our model system has provided evidence that biotinylation of 2 amyloid-forming LCs (FRE and IRHE) and 2 non-amyloid forming LCs (JRHE and HUD) resulted in altered stability and conformation and, notably these corresponded with differences in their propensities to adhere to cultured human fibroblast and epithelial cells. In this regard, although a relationship between LC instability and fibrillogenic potential previously has been established [19], our experimental data suggest that decreased stability of amyloidogenic LCs alone does not account for their cellular avidity. Comparison of the unmodified LCs showed that there was a concentration-dependent increase in adhesion levels observed for the 2 AL LCs (FRE and IRHE) as compared to JRHE. However, no significant difference between HUD ( $\lambda$ 1b) and the 2 AL LCs ( $\lambda$ 3a) was observed. JRHE is a cast-forming protein *in vivo* whereas there are no reports of pathologic deposits associated with the HUD LC. The native HUD protein exhibits significantly more avidity for fibroblast and epithelial cells than JRHE. However, modification of the lysine residues on the  $\lambda$ 3a LC JRHE increased its binding to HRECs while it had little or no effect on the  $\lambda$ 1b LC HUD.



Lysyl modification of the 2 AL-associated LCs did not lead to any significant alteration in their secondary structure, in-as-much as the  $\beta$ -sheet conformation was maintained. While the CD spectra of both the AL- and MM- associated BJPs were similar to those reported for other LCs [27] and the biotinylated BJPs did not differ in their CD profiles, their  $\beta$ -sheet content was increased relative to their native forms, as has been observed for other molecules [10, 12, 25]. Additionally, charge had no apparent effect on their capacity to bind cells in that the increased pIs of the biotinylated proteins did not correlate with their capacity to bind cells or form amyloid *in vivo*. The Trp emission spectra of the 4 BJPs after biotinylation showed a decrease in fluorescence intensity, which may reflect a conformation change in the microenvironment around one or more of the 4 Trp residues found in all LCs [28]. This effect also has been shown in the case of human serum albumin, where a subtly altered structure and change in function has been observed [29, 30]. Based on our data, we hypothesize that lysine modification led to conformational changes in the amyloidogenic LCs that exposed a binding site not accessible on the native proteins. In this regard, it has been shown that partial denaturation of LCs increased their binding to the heat shock protein BiP via specific peptide sequences [31]. A number of these sequences are located close to or include Trp 35 and are buried within the core of the LC domain including one high affinity peptide [31]. The Trp 35 within IRHE, FRE and JRHE has a Lys residue that is 4 amino acids distant from it; however, HUD has a Lys that is 10 amino acids removed. Therefore, a conformational change induced by lysine modification in the vicinity of this Trp may effect the 3  $\lambda$ 3a LCs similarly, i.e. resulting in increased adhesion to epithelial cells, but not the  $\lambda$ 1b LC HUD. Modification of IgG lysyl residues also has been found to

significantly impair antigen recognition, presumably as a result of altered conformation [32]. Further, reduced immune function in patients with diabetes has been attributed to lack of antigen recognition induced by adduction of glucose to lysine residues on IgG variable regions [33].

Although it is possible that the observed LC adherence to human cells was caused by reduction in overall surface charge density, we postulate that side chain modifications (such as those involving lysine) led to subtle conformational changes that exposed a peptide sequence(s) reactive with cell surface receptors. A number of such receptors for human LCs have been identified in the kidney and include Tamm-Horsfall protein, as well as the glycoproteins cubilin and megalin [34, 35]. Further, AL LCs have been shown to adhere to an, as yet, unidentified receptor on mesangial cells [36] and a relationship between the receptor for advanced glycation end products (RAGE) and amyloid deposition has been noted [37-41]. Our study, which shows that modification of  $\lambda$ 3a LCs by biotinylation of lysyl residues augments their binding to cultured cells, suggests that this interaction is receptor-mediated and may occur *in vivo*. We posit that post-translational alteration of LCs is an important physiological process that can influence their stability and conformation, as well as cell binding capability, resulting in fibrillar or other types of pathologic deposits such as amorphous aggregates.

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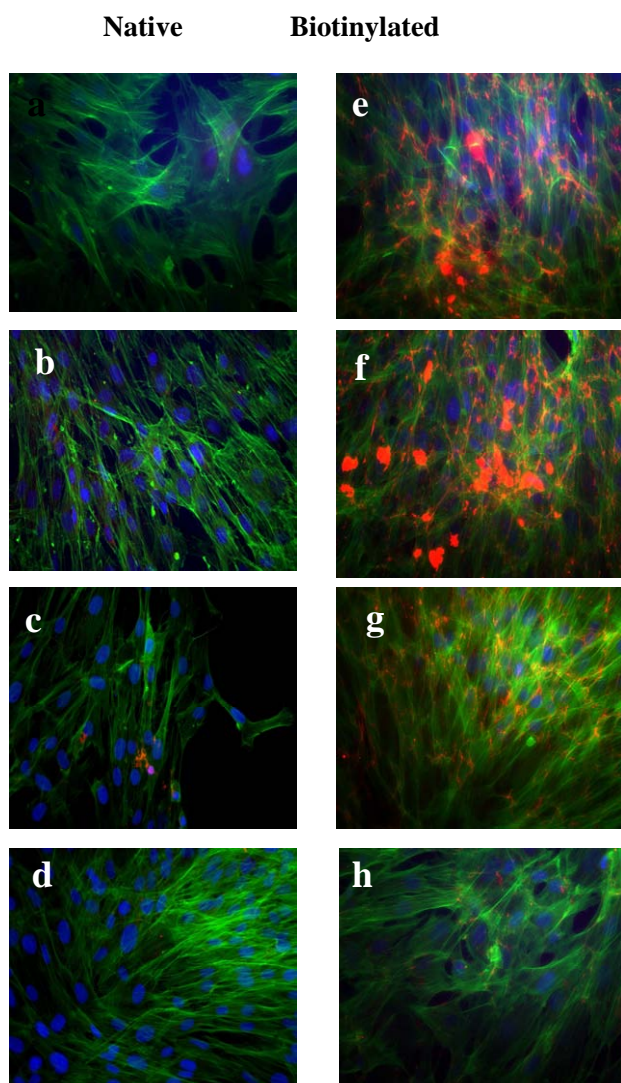
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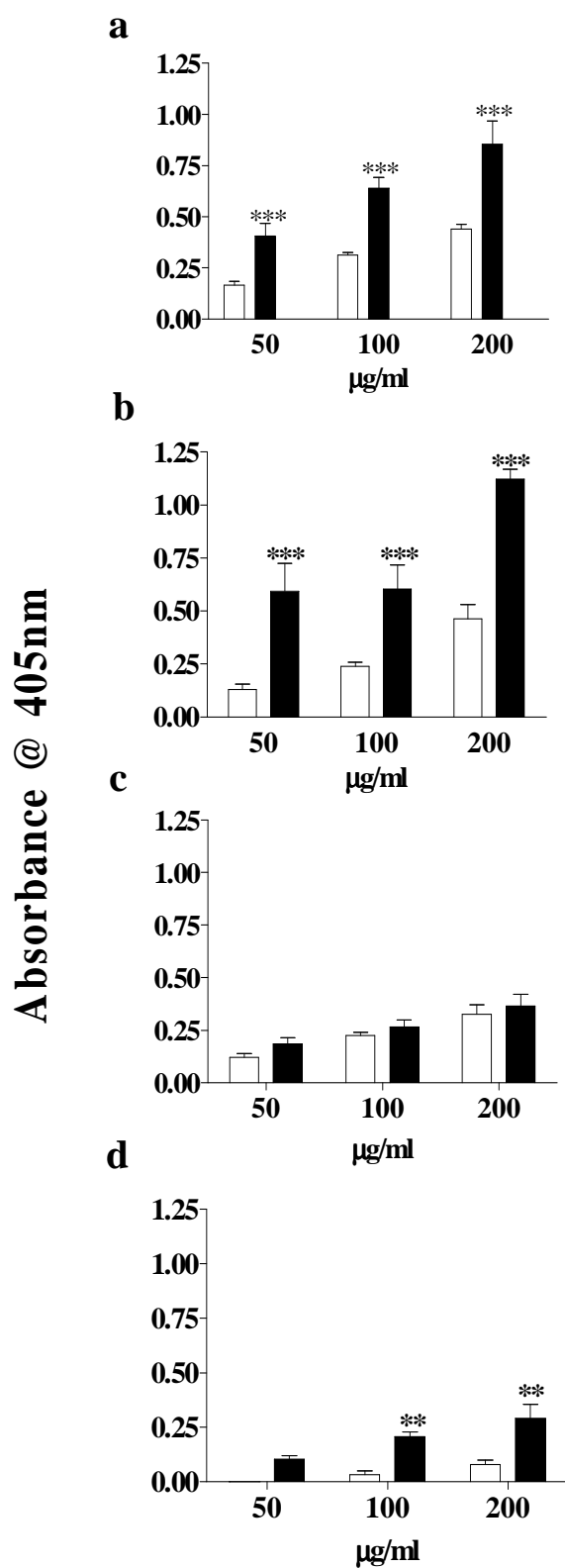
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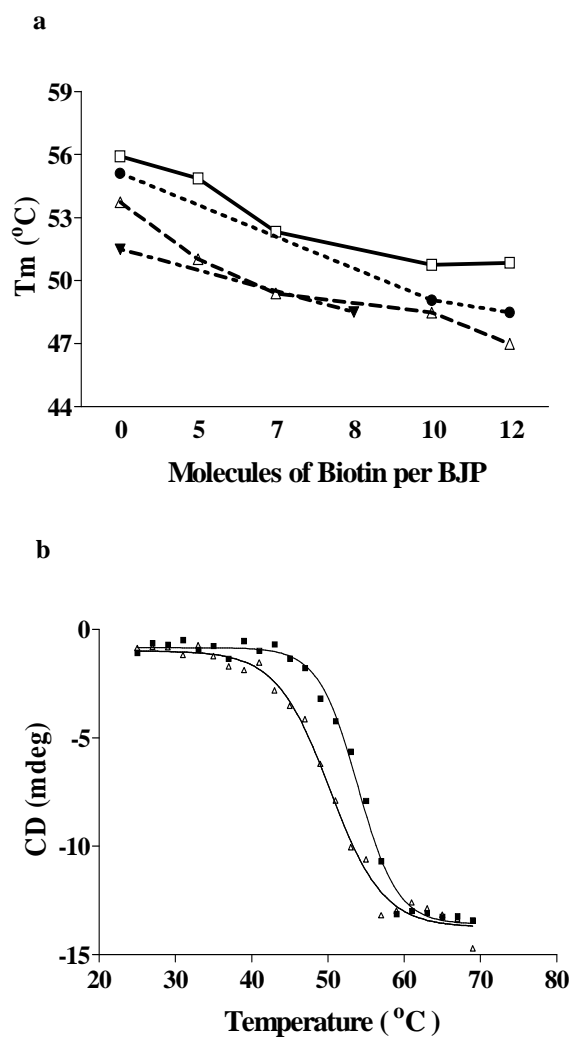


**Figure 1:** Immunocytochemical demonstration of the binding LCs to human foreskin fibroblasts. Cells were exposed to native or biotinylated AL-associated BJPs FRE (Panels a and e) and IRHE (Panels b and f) and myeloma-associated BJPs HUD (Panels c and g) and JRHE (Panels d and h). Red, green and blue fluorescence indicates the presence of LCs, F-actin, and cell nuclei, respectively (magnification X 400).



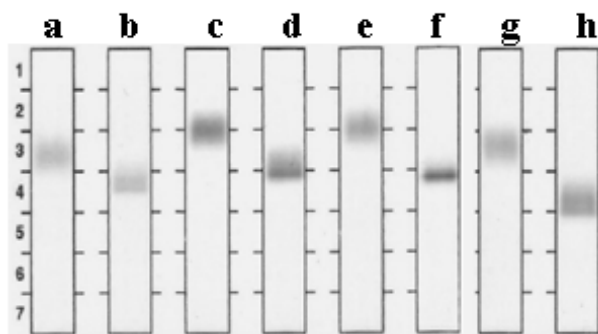


**Figure 2:** Comparison of native and biotinylated LCs interaction with human renal epithelial cells. Amyloid-associated BJPs: FRE (a) and IRHE (b) and myeloma-associated BJPs HUD (c) and JRHE (d). Native (□) and biotinylated (■). The data represent mean  $\pm$  SEM, (n = 3). \*\* p < 0.01, \*\*\* p < 0.001



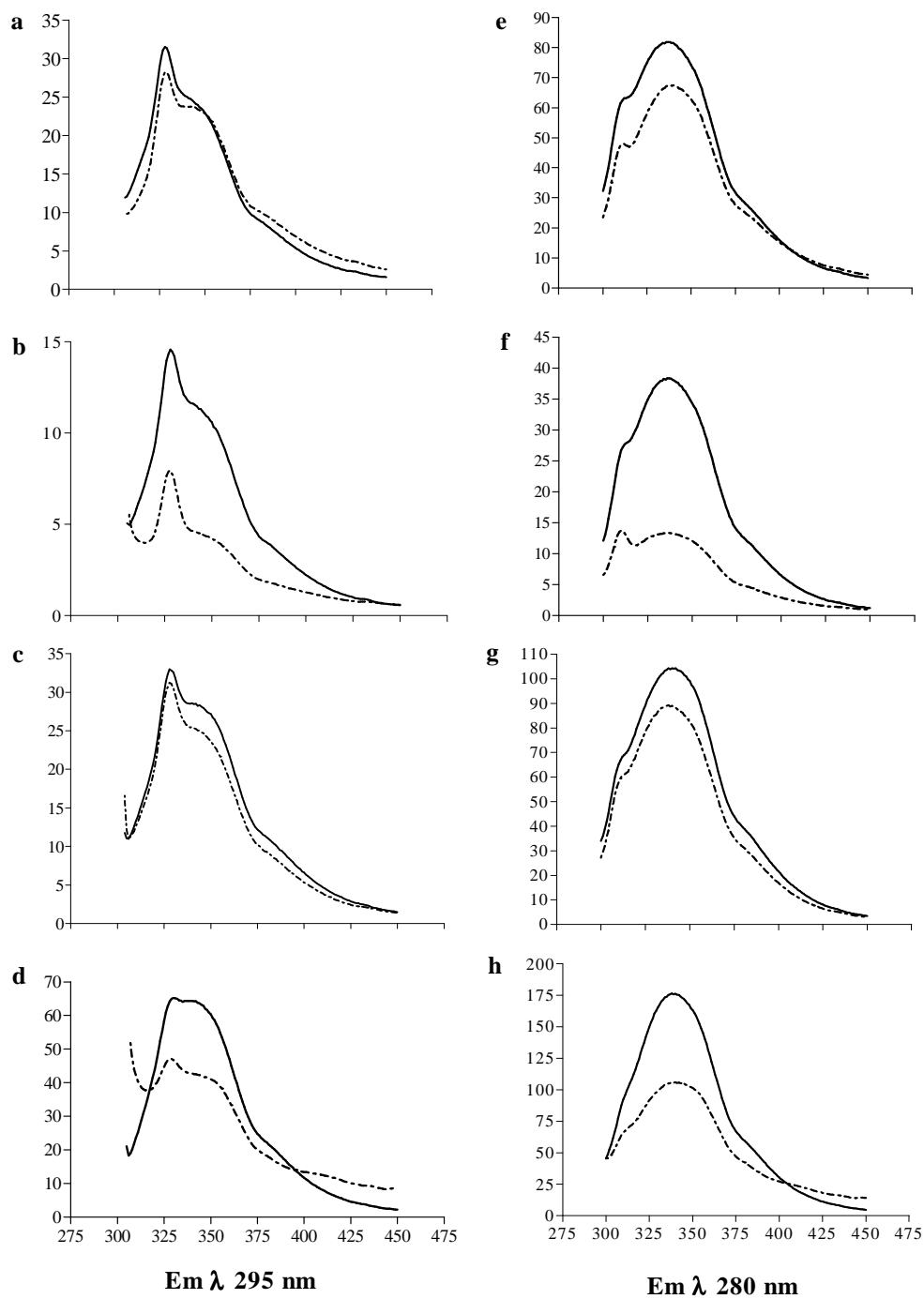
**Figure 3:** Thermal denaturation of native and biotinylated LCs.

(a) Number of biotin molecules on amyloid-associated BJPs, FRE ( $\Delta$ ), and IRHE ( $\blacktriangledown$ ) and myeloma-associated BJPs, HUD ( $\bullet$ ), JRHE ( $\square$ ). (b) Circular dichroism spectra of native ( $\blacksquare$ ) and biotinylated FRE ( $\Delta$ ) containing  $\sim 10$  biotins per BJP.



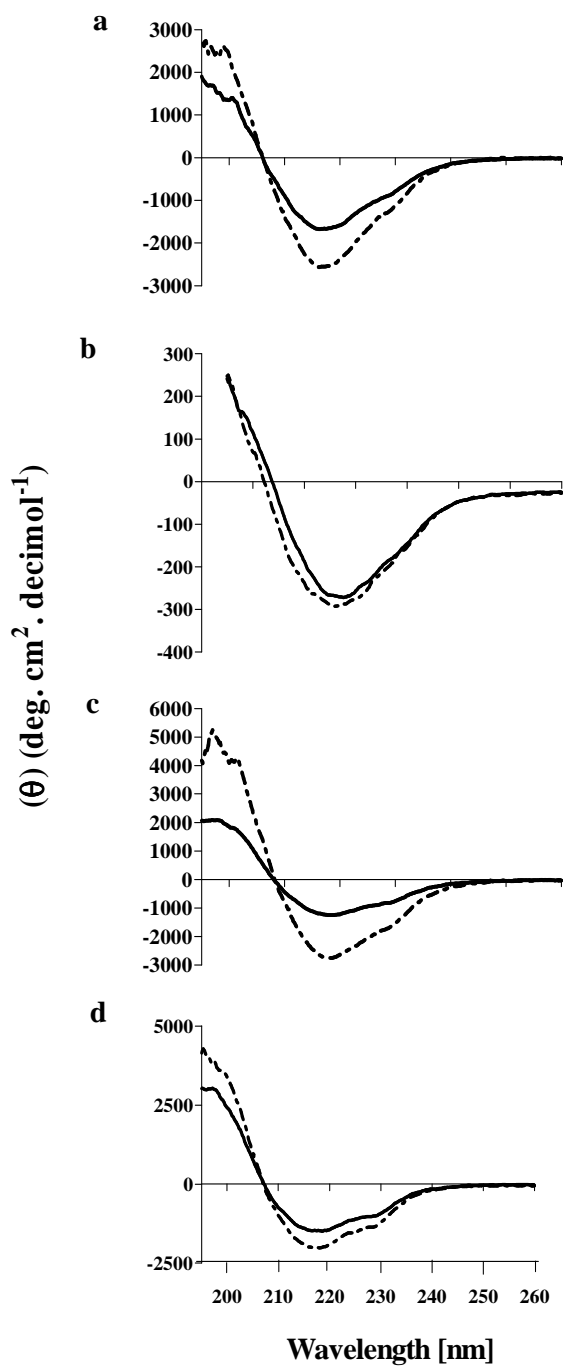
**Figure 4:** Electrophoretic mobility of native and biotinylated LCs.

Immunofixation electrophoresis: of (biotinylated/native) BJPs: amyloid-associated BJPs, IRHE (a/b), FRE (c/d), JRHE (e/f), and HUD (g/h). Proteins were immunostained with an anti- $\lambda$ -chain antibody.



**Figure 5:** Fluorescence emission spectra of native and biotinylated LCs.

Native (—) and biotinylated (---) (25  $\mu\text{g/ml}$  in PBS), amyloid-associated BJPs, FRE (a, e), and IRHE (b, f), and myeloma-associated BJPs JRHE (c, g) and HUD (d, h).



**Figure 6:** Circular dichoric spectra of native and biotinylated LCs.

Native (—) and biotinylated (---), amyloid-associated BJPs, FRE (a) and IRHE (b) and myeloma-associated BJPs, JRHE (c) and HUD (d).



**Figure 7:** LC sequence alignment: Gaps are indicated by a “-” and the lysines in each sequence are highlighted. “\*” denotes residues in that column are identical in all sequences “:” denotes conserved substitutions observed “.” denotes semi-conserved substitutions observed using the CLUSTAL 2.0. multiple alignment program.

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**Table 1:** Thermodynamic parameters of native and biotinylated LCs

Parameter	FRE		IRHE		HUD		JRHE	
	Native	Biotin <sup>a</sup>	Native	Biotin <sup>b</sup>	Native	Biotin <sup>c</sup>	Native	Biotin <sup>d</sup>
<b>T<sub>m</sub></b> (K)	327.03	320.29	324.30	321.30	328.30	323.30	329.22	324.15
<b>ΔH<sub>m</sub></b> (Kcal/mol)	59.37	39.02	45.44	47.12	67.81	56.95	59.14	78.62
<b>C<sub>p</sub>Total</b> (Kcal/Kmol)	3.50	3.50	3.44	3.44	3.40	3.40	3.55	3.55
<b>ΔG(T)</b> (Kcal/mol)	5.21	2.68	3.64	3.37	6.19	4.40	5.55	6.27

a, b, c, d: 12, 8, 12, 10, biotins/dimer

**Table 2:** Pairwise identity of aligned LC sequences

	FRE	IRHE	JRHE	HUD
FRE	**	90	88	79
IRHE	90	**	88	78



JRHE	88	88	**	76
HUD	79	78	76	**

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