Chapter III. Cell-Free Assays

A. Biodot Assay

A quantitative binding assay for p9 HS binding to IGF-I and IGFBP-3 binding to IGF-I was established using the Bio-Dot Microfiltration Apparatus and Zeta-Probe membranes (Bio-Rad, Hercules, CA). The Bio-Dot apparatus has a 96 well template that allows for various combinations of IGF-I, IGFBP-3 and p9 HS to be investigated simultaneously.

The Zeta-Probe membrane is a quaternary amine derivatized nylon membrane that facilitates retention of anionic molecules, such as p9 HS, The general idea is that positive molecules like IGF-I (Figure 1-10), pI 8.8[64], will not bind. IGFBP-3, with a cationic putative heparin-binding region of amino acids and an overall positive charge is also not likely to be retained[51]. (Figure 1-11)

The Biodot Assay was used to investigate the binding interactions of IGF-I, IGFBP-3 and p9 HS. All possible combinations of the three were examined in various concentration ranges with details of the assay included in Chapter 2, Section F. Results are based on triplicate well studies. Figures 3-7 through 3-15 represent a compilation of many experiments. Each experiment included a control value of ^{125}I-IGF-I, ^{125}I-IGFBP-3 or ^{35}S-p9 HS in the absence of any other binding element. These values were taken as 100% and all additions to ^{125}I-IGF-I, ^{125}I-IGFBP-3 or ^{35}S-p9 HS (for example, ^{125}I-IGF-I + 1.2×10^{-11} M p9 HS, ^{125}I-IGF-I + 2.9×10^{-11} M p9 HS, etc.) were analyzed as a percentage of the control value. This allowed for merging of data from several experiments into one figure and a clearer presentation of data. An increase in retention on the membrane of the labeled molecule was indicative of a binding affinity between the two molecules being studied.

Establishing Protocol

A series of experiments was performed to optimize the protocol based on a previous protocol by Forsten and co-workers[66]. p9 HS was expected to be fully retained

on the membrane due to charge attraction while IGF-I and IGFBP-3 were expected to have low to no retention on the membrane due to charge repulsion. The incubation time, the numbers of washes following incubation and the concentration of sample, buffer and bovine serum albumin (BSA) were all varied and analyzed to find conditions where essentially all ^{35}S-p9 HS would be retained and background binding of the ^{125}I-IGF-I and ^{125}I-IGFBP-3 would be obtained. BSA was added to decrease non-specific binding between the molecules themselves and the apparatus. Increasing the concentration of BSA from 1 mg / ml to 2 mg / ml raised the retention of IGF-I on the membrane slightly, (Figure 3-1).

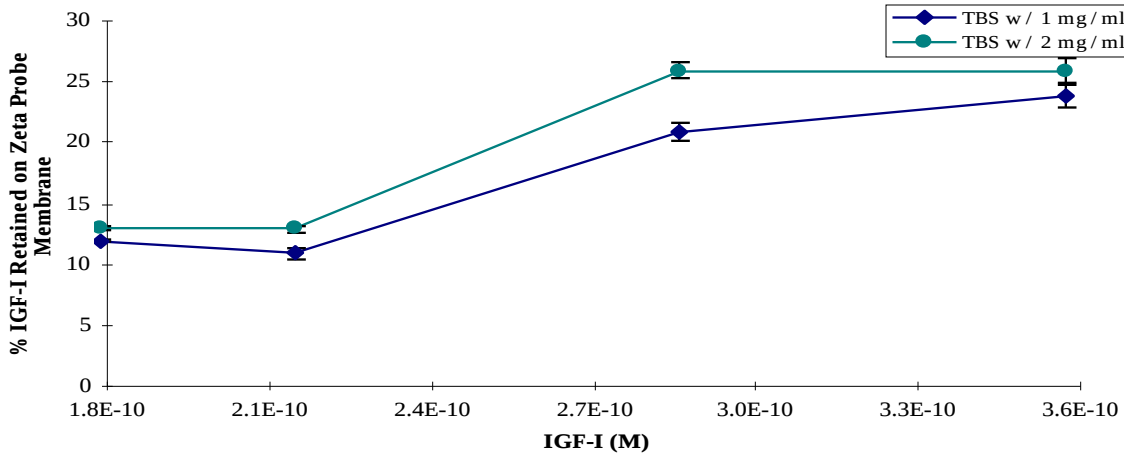

Figure 3-1 The effect of increasing the concentration of Bovine Serum Albumin (BSA) in TBS on ^{125}I-IGF-I retention on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and bar is the standard error of the mean.

Retention on the zeta probe membrane reached the lowest point at 1.8 x 10^{-10} M ^{125}I-IGF-I with 3 washes of TBS / 1mg / ml BSA after a one hour incubation, (Figure 3-2). Increasing the numbers of washes after a one hour incubation slightly decreased the retention of ^{125}I-IGF-I and ^{125}I-IGFBP-3 but did not affect the high retention of ^{35}S-p9 HS, (Figure 3-3). No significant increase ($P \leq 0.05$) of ^{125}I-IGF-I retention to the membrane occured when incubation time was lengthened, (Figure 3-4). Therefore, the final protocol used for the biodot assay was a one hour incubation in TBS, 1 mg / ml BSA with 3 washes after the sample was pulled through the membrane.

Even though BSA was added to decrease non-specific binding between the molecules themselves and the apparatus, a small amount of each molecule clung to the apparatus: ^{125}I-IGF-I, 10%, ^{125}I-IGFBP-3 23%, ^{35}S-p9 HS, 2%. This was determined by

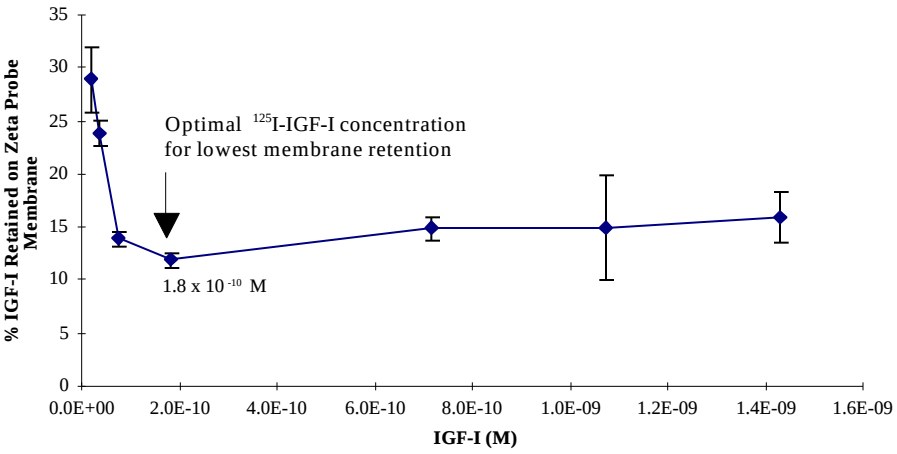

Figure 3-2 The optimal ^{125}I-IGF-I concentration for lowest ^{125}I-IGF-I retention on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

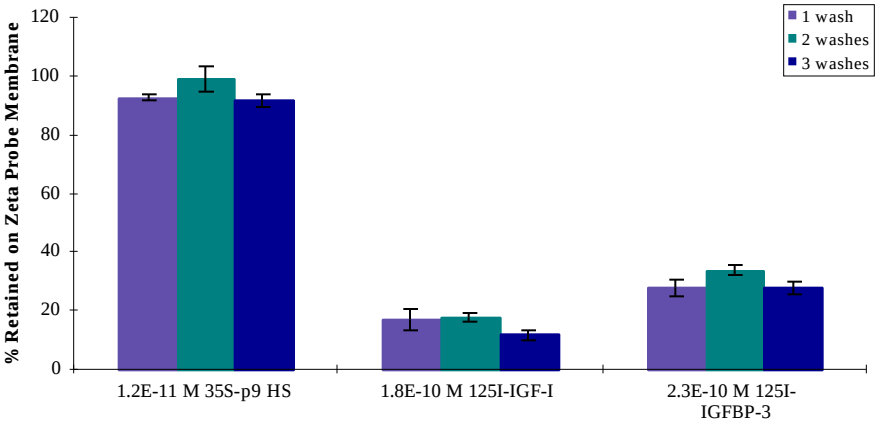

Figure 3-3 The effect of increasing TBS/BSA washes after incubation with labeled p9 HS, IGF-I, IGFBP-3 retention on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

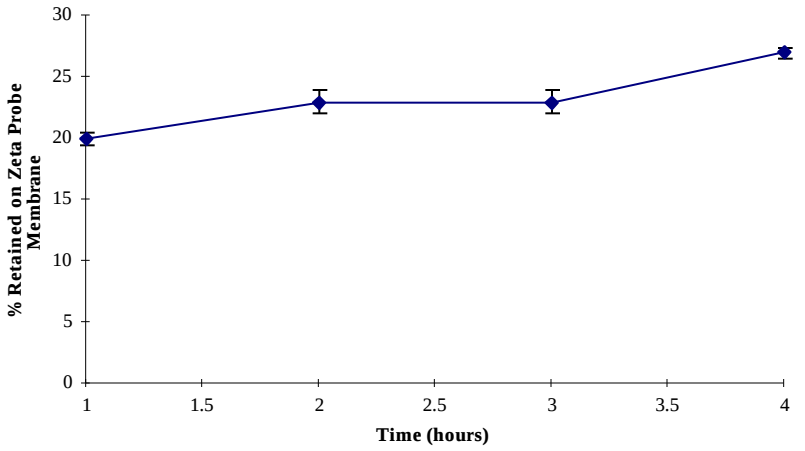

Figure 3-4 The effect of increasing incubation time on ^{125}I-IGF-I retention on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

following normal protocol for two sets of triplicate wells. At the end of the1 hour incubation period, solution buffer was removed and counted for one set; the remaining set was treated as normal. The percentage of counts that stuck to the apparatus for each molecule was obtained by subtracting the percentage of counts present in the solution buffer from the percentage of counts retained on the membrane.

p9 HS Retention on Zeta Probe Membrane

Eighty to one hundred percent retention of ^{35}S-p9 HS on the zeta probe membrane was seen with 1.2 x 10^{-11} M -1.5 x 10^{-10} M p9 HS. Saturation began to occur at values exceeding 1.5 x 10^{-10} M, therefore studies with high concentrations were beneficial in qualitative examination but excluded from quantitative evaluation, (Figure 3-5).

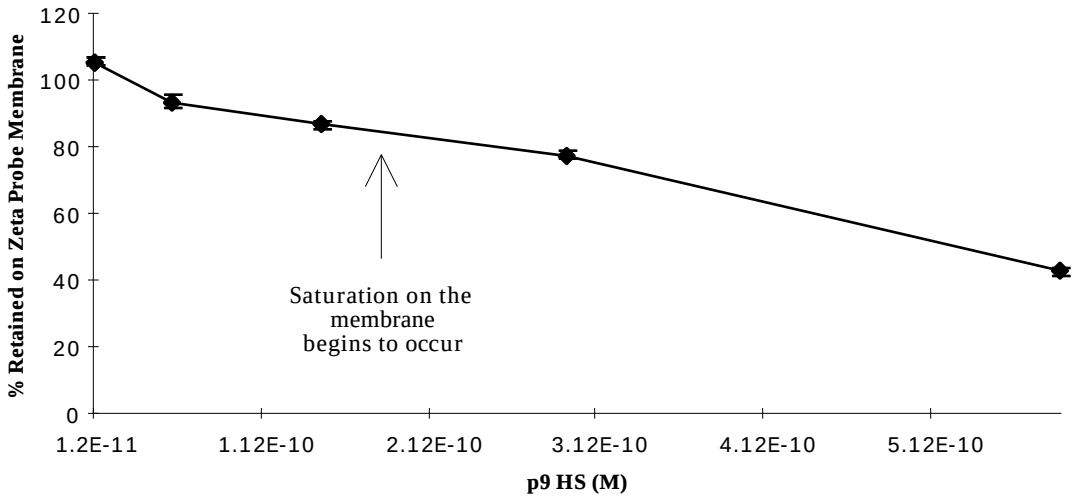

Figure 3-5 Retention of ^{35}S-p9 HS on zeta probe membrane. Saturation occurs at concentrations exceeding1.5 x 10^{-10} M. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

IGFBP-3 Retention on Zeta Probe Membrane

The retention of ^{125}I-IGFBP-3 (1.2 x10^{-10} M - 2.3 x 10^{-9} M) on the zeta probe membrane is less than 50%. ^{125}I-IGFBP-3 retention starts to decline rapidly at molar concentrations exceeding 1.2 x 10^{-9} M, (Figure 3-6). This may be due to saturation on the

membrane. As the concentration of IGFBP-3 increases, binding sites on the membrane are occupied rapidly. At high concentrations, all binding sites may be occupied, thus not allowing all the material put in the system to bind to the membrane. Due to the variable retention of ^{125}I-IGFBP-3 over a range of values, it is difficult to use IGFBP-3 data for quantitative purposes.

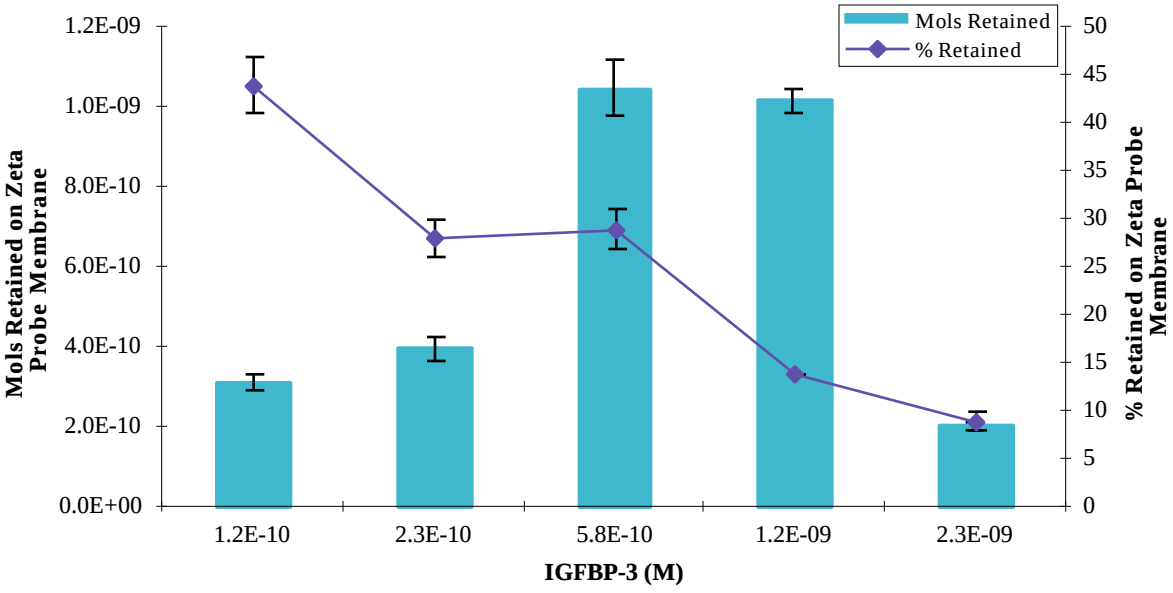

Figure 3-6 Retention of ^{125}I-IGFBP-3 on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean .

IGFBP-3 Retention on the Zeta Probe Membrane in the Presence of p9 HS

p9 HS decreased IGFBP-3 retention on the membrane. To determine the effect of p9 HS on IGFBP-3 retention on the membrane, 2.3 x 10^{-10} M ^{125}I-IGFBP-3 was incubated with 5.9 x 10^{-11} M p9 HS. p9 HS did not have a positive effect on the retention of IGFBP-3; p9 HS drove IGFBP-3 away from the membrane, (Figure 3-7). One possibility is that IGFBP-3 and p9 HS do not bind. Alternatively, the high background level of IGFBP-3 retention may prevent assessment of binding.

The effect of p9 HS on ^{125}I-IGFBP-3 retention on the membrane did not change when exposed to a higher temperature and lengthier incubation time. To determine the effect of p9 HS on IGFBP-3 retention on the membrane, ^{125}I-IGFBP-3 was incubated with

p9 HS (0 - 1.5 x 10-10 M) for 3 hours at 37°C and for 1 hour at room temperature. There was no change in ^{125}I-IGFBP-3 retention on the membrane at 37°C for 3 hours as compared to 1 hour at room temperature, (Figure 3-8).

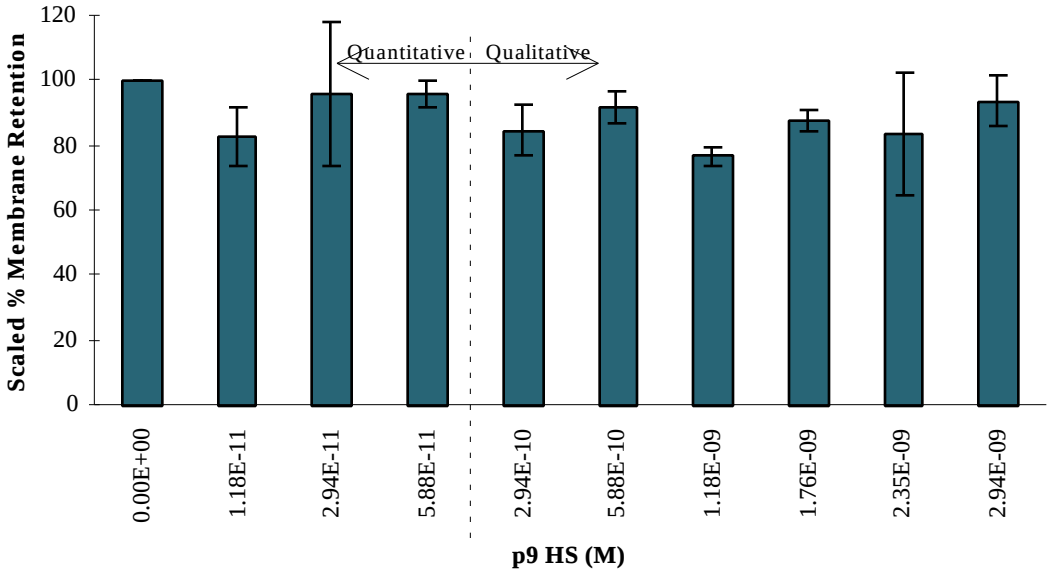

Figure 3-7 The effect of p9 HS on 2.3 x 10^{-10} M ^{125}I-IGFBP-3 retention on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean. Results are scaled as discussed.

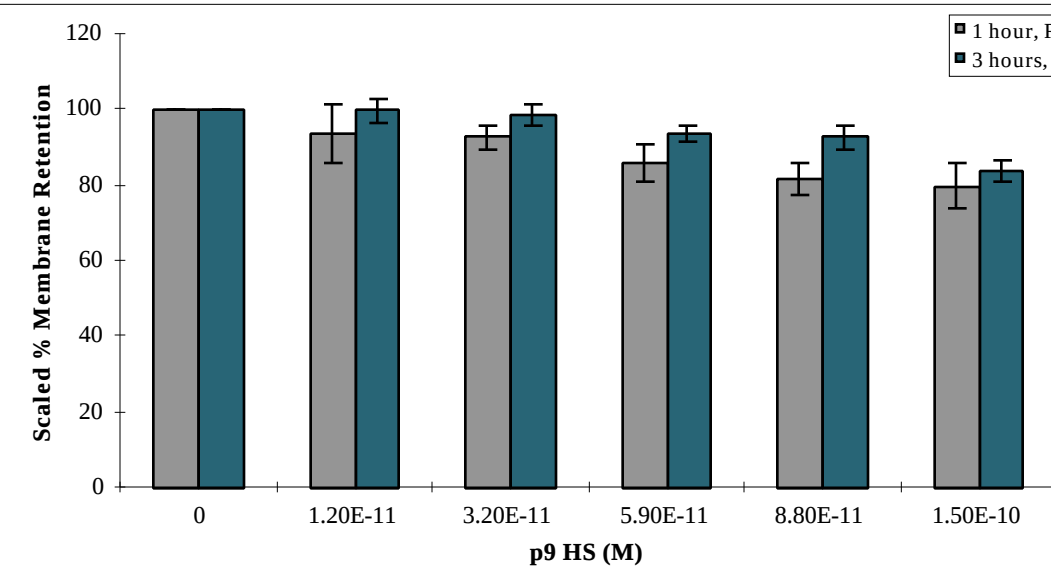

Figure 3-8 The effect of increasing time and temperature on ^{125}I-IGFBP-3 retention on zeta probe membrane in presence of p9 HS. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

IGF-I Retention on Zeta Probe Membrane

Eleven to thirty percent background retention of ^{125}I-IGF-I to the zeta probe membrane was seen with 1.8 x 10^{-11} M - 1.4 x 10^{-9} M IGF-I. The lowest retention occurred at 1.8 x 10^{-10} M IGF-I, (Figure 3-2).

Retention of IGF-I in the Presence of p9 HS

p9 HS increased IGF-I retention on the membrane slightly in the unsaturated area of interest (0 - 5.9 x 10^{-11} M) and largely increased IGF-I retention on the membrane at higher p9 HS molar concentrations (2.9 x 10^{-10} M - 2.9 x 10^{-9} M). To establish the effect of p9 HS on ^{125}I-IGF-I retention on the zeta probe membrane, 1.8 x 10^{-10} M ^{125}I-IGF-I was incubated with p9 HS (0 - 2.9 x 10^{-9} M). p9 HS did not have a large effect on IGF-I retention on the membrane in the lower concentration range investigated with the maximum increase of 25% over normal IGF-I retention seen at 5.9 x 10^{-11} M p9 HS . As the p9 HS concentration increased above the saturation point, IGF-I retention on the membrane increased profoundly, (Figure 3-9). It is difficult to quantitate how much of the IGF-I / p9 HS complex is actually retained on the membrane after the saturation point, therefore this data was only used for illustration of the binding capability. p9 HS increased IGF-I retention on the membrane and therefore does bind to IGF-I.

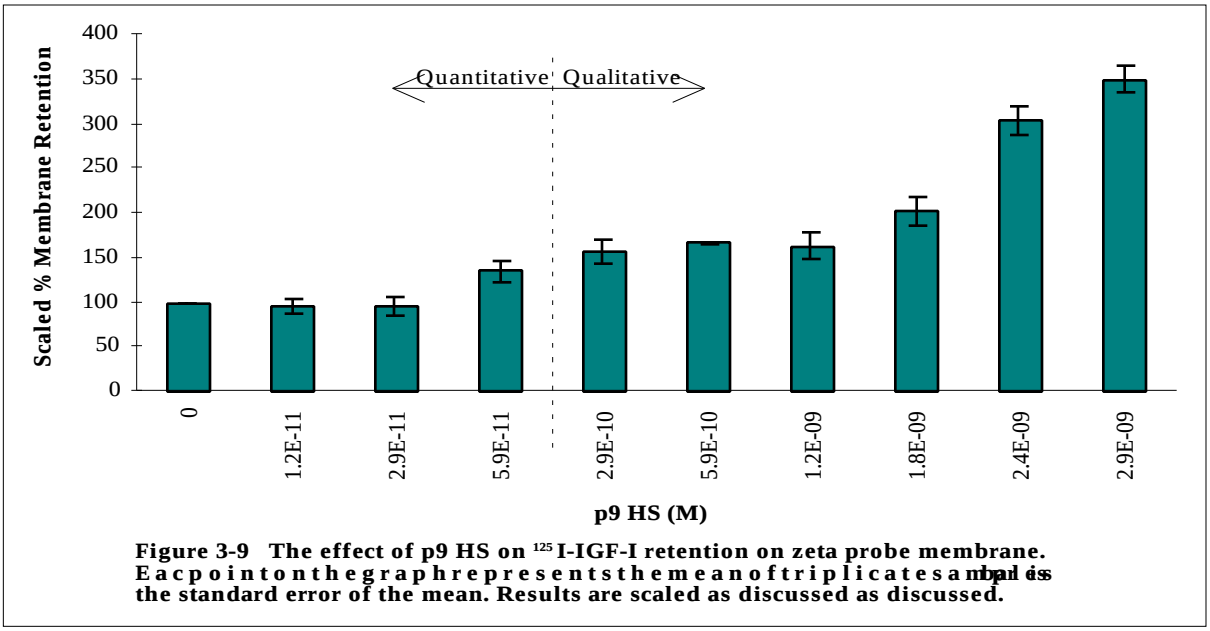

Figure 3-9 The effect of p9 HS on ^{125}I-IGF-I retention on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean. Results are scaled as discussed as discussed.

The effect of p9 HS on ^{125}I-IGF-I retention on the membrane did not change when exposed to a higher temperature and lengthier incubation time. To determine the effect of p9 HS on IGF-I retention on the membrane, 3.6 x 10^{-10} M ^{125}I-IGF-I was incubated with p9 HS (0 - 1.5 x 10^{-10} M) for 3 hours at 37°C and for 1 hour at room temperature. There was no change in ^{125}I-IGF-I retention on the membrane at 37°C for 3 hours as compared to 1 hour at room temperature, (Figure 3-10). Therefore, increasing the temperature and incubation time did not affect p9 HS binding to IGF-I. There was no significant increase ($P \leq 0.05$) seen at the higher IGF-I concentration and p9 HS concentrations (0 - 1.5 x 10-10 M) than the small increase in ^{125}I-IGF-I retention seen with 1.8 x 10^{-10} M ^{125}I-IGF-I and p9 HS (0 - 5.9 x 10-11 M). It is important to note that this higher concentration was not used in quantifying the binding affinity (K_D) of p9 HS and IGF-I.

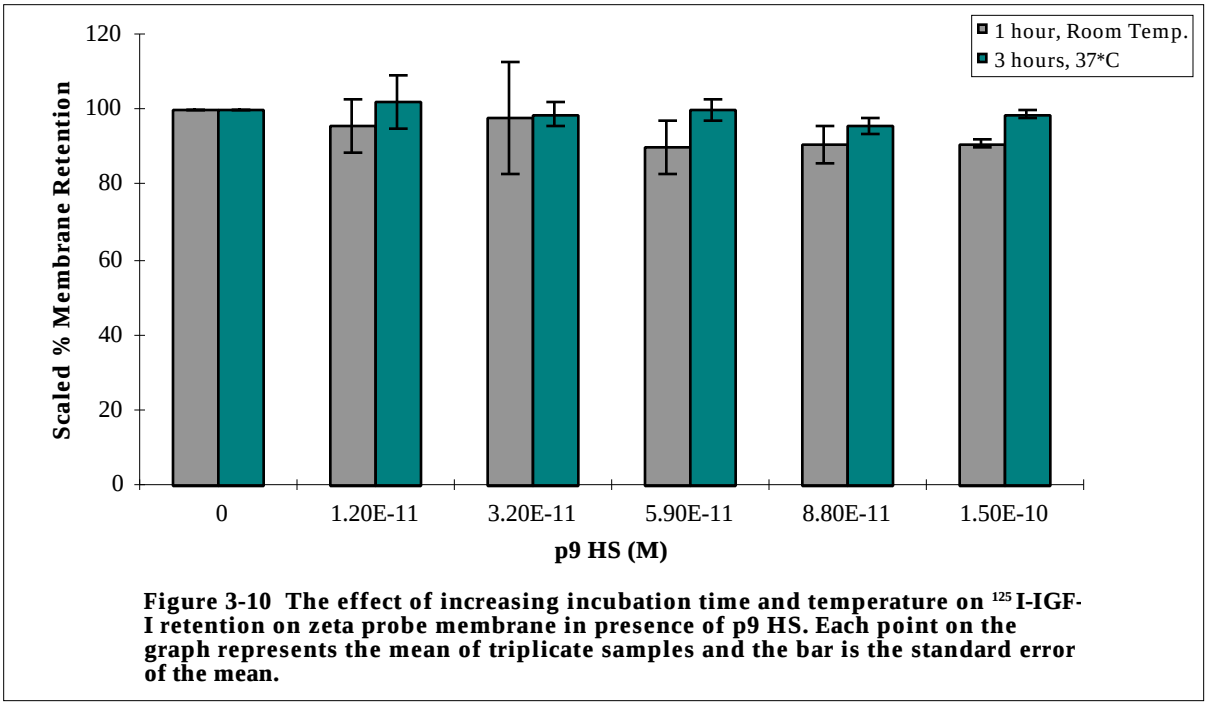

Figure 3-10 The effect of increasing incubation time and temperature on ^{125}I-IGF-I retention on zeta probe membrane in presence of p9 HS. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

Retention of IGF-I in the Presence of IGFBP-3

IGFBP-3 increased IGF-I retention on the membrane over the IGFBP-3 concentration range examined with the maximum increase at 2.3 x 10^{-9} M. To ascertain the effect of IGFBP-3 on ^{125}I-IGF-I retention on the zeta probe membrane, 1.8 x 10^{-10} M ^{125}I-IGF-I was incubated with IGFBP-3 (0 - 1.2 x 10^{-7} M). IGFBP-3 had a half maximal affect on IGF-I retention at 5.8 x 10^{-10} M. The maximum increase was seen at 2.3 x 10^{-9} M.

Leveling off occurred at molar concentrations exceeding 2 .3 x 10^{-9} M, (Figure 3-11). Leveling off could be due to saturation of IGF-I with IGFBP-3 due to the high molar concentration of IGFBP-3 (2.3 x 10^{-9} M) added compared to IGF-I (1.8 x 10^{-10} M). Assuming a one site model, molar concentrations above 2.3 x 10^{-9} M IGFBP-3 well exceed the 1.8 x 10^{-10} M IGF-I present in the system. IGFBP-3 did increase the retention of IGF-I on the membrane and therefore does bind IGF-I. However, only a fraction of IGFBP-3 was retained on the membrane and it is unclear how IGF-I impacts that retention. It is not known if all the complexes were retained or a fraction equivalent to the fraction of IGFBP-3 retained. Thus, the binding affinity (K_D) of IGFBP-3 and IGF-I was quantified with this data for comparison to other cell-free assays, but not used for model purposes.

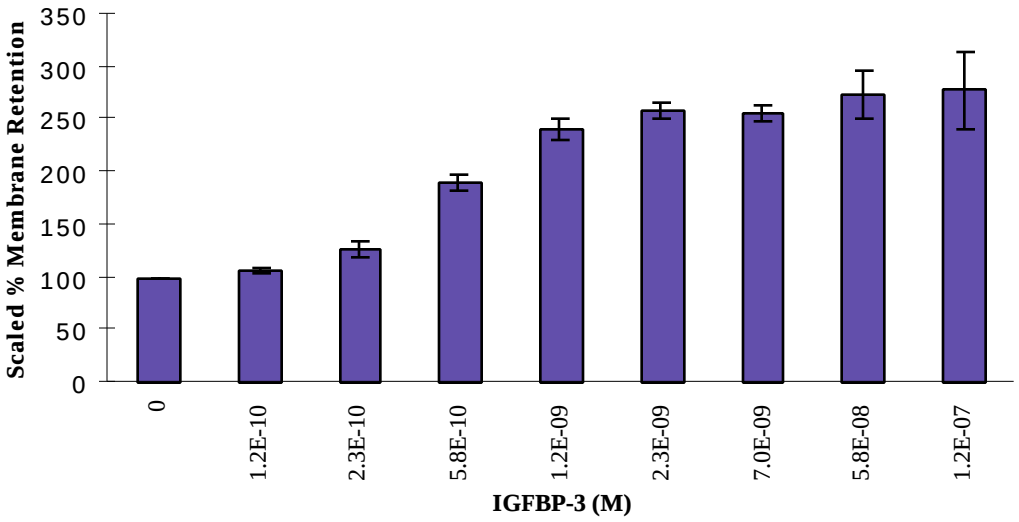

Figure 3-11 The effect of IGFBP-3 on ^{125}I-IGF-I retention on zeta probe membrane. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean. Results are scaled as discussed.

The effect of IGFBP-3 on ^{125}I-IGF-I retention on the membrane did change when exposed to a higher temperature and lengthier incubation time. To determine the effect of IGFBP-3 on IGF-I retention on the membrane, 3.6 x 10^{-10} M ^{125}I-IGF-I was incubated with IGFBP-3 (0 - 2.3 x 10^{-9} M) for 3 hours at 37°C and, for comparison,1 hour at room temperature. There was no specific trend with ^{125}I-IGF-I retention on the membrane at 37°C for 3 hours, (Figure 3-12). However, there was a substantial decrease of ^{125}I-IGF-I retention at IGFBP-3 concentrations above 5.8 x 10^{-10} M at 37°C and 3 hours than with

^{125}I-IGF-I retention at room temperature for 1 hour. Therefore, increasing the temperature and incubation time does affect IGFBP-3 binding to IGF-I. This may be due to dissociation of IGFBP-3 from IGF-I during the longer incubation time or an affect of the temperature on IGFBP-3 retention on the membrane. No further studies were done to elucidate what was occuring, but it may be worth following up on.

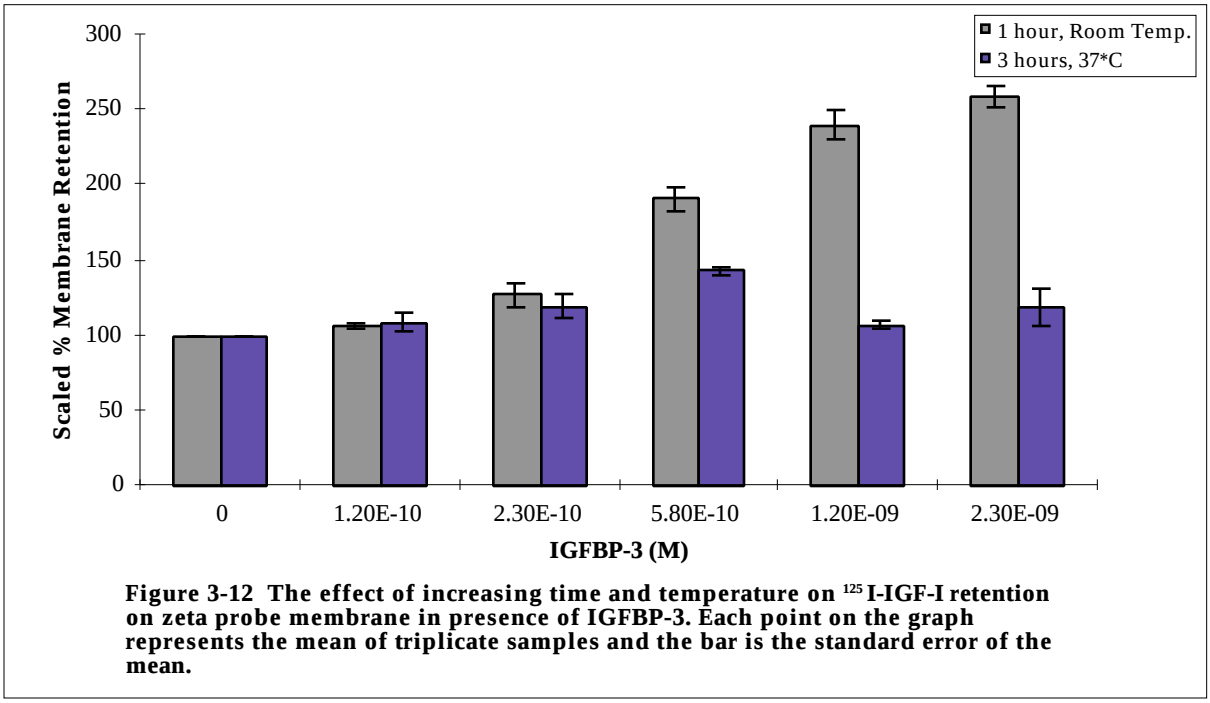

Figure 3-12 The effect of increasing time and temperature on ^{125}I-IGF-I retention on zeta probe membrane in presence of IGFBP-3. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

Retention of IGF-I in the Presence of p9 HS and IGFBP-3

IGFBP-3 increased the retention of IGF-I on the membrane in the presence of p9 HS . To determine the effect of IGFBP-3 on IGF-I retention in the presence of p9 HS, 1.8 x 10^{-10} M ^{125}I-IGF-I was incubated with p9 HS(0 - 6.5 x 10^{-11} M) and IGFBP-3 (0 - 8.7 x 10^{-10} M) There was a 10% increase of IGF-I retention when p9 HS (0 - 3.2 x 10^{-11} M) was added without IGFBP-3 and a 25% increase of IGF-I retention with 6.5 x 10^{-11} M p9 HS. The addition of IGFBP-3 (0 - 8.7 x 10^{-10} M) on ^{125}I-IGF-I (1.8 x 10^{-10} M) retention on the membrane in the presence of p9 HS (0 - 6.5 x 10^{-11} M) resulted in a larger increase than IGF-I and p9 HS in the absence of IGFBP-3. The higher concentration of IGFBP-3 (8.7 x 10^{-10} M), in the presence and absence of p9 HS, increased IGF-I retention by approximately 125% whereas the lower concentrations of IGFBP-3 (1.7 x 10^{-10} M) increased IGF-I retention by 50%, (Figure 3-13).

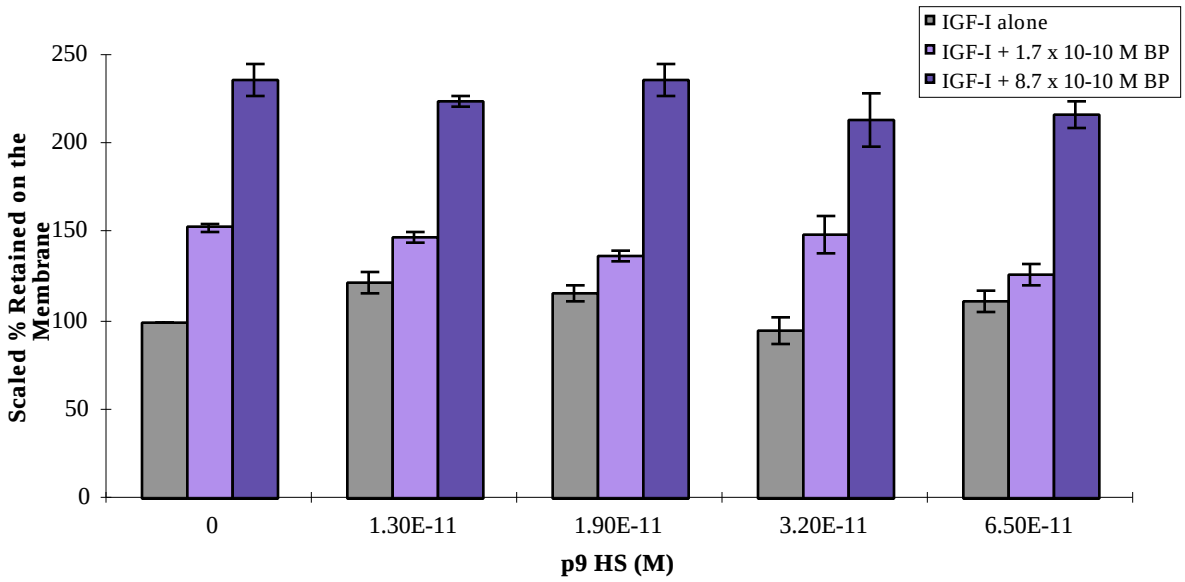

Figure 3-13 The effect of IGFBP-3 on ^{125}I-IGF-I retention on zeta probe membrane in the presence of p9 HS. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean. Results are scaled as discussed.

Figure 3-14 demonstrates the same examination of p9 HS, IGFBP-3 and IGF-I interactions as those shown in Figure 3-13, however, higher molar concentrations of IGFBP-3 and p9 HS were utilized. There was a 10% increase of IGF-I retention when 5.9 x 10^{-12} M p9 HS was added without IGFBP-3 and a 50% increase when 1.5 x 10^{-10} M p9 HS was added without IGFBP-3 . The addition of IGFBP-3 (0 - 2.9 x 10^{-9} M) to ^{125}I-IGF-I and p9 HS (0 - 1.5 x 10^{-10} M) increased IGF-I retention as compared with IGF-I retention when only p9 HS was present. There was a 60% increase in IGF-I retention with the addition of 1.2 x 10^{-10} M IGFBP-3 in the presence of p9 HS and a 90% increase in IGF-I retention with the addition of 1.2 x 10^{-9} M and 2.9 x 10^{-9} M IGFBP-3 in the presence of p9 HS. The molar concentrations of p9 HS examined (0 - 6.5 x 10^{-11} M p9 HS) may not have been high enough to produce a notable affect on IGF-I retention with the IGFBP-3 amount (0 - 8.7 x 10^{-10} M) present.

To further explore the effect of p9 HS on ^{125}I-IGF-I retention on the membrane in an acceptable range of p9 HS, 3.6 x 10^{-10} M ^{125}I-IGF-I was incubated with IGFBP-3 (0 - 2.3 x 10^{-9} M) and 5.9 x 10^{-11} M p9 HS. As seen previously, IGFBP-3 without p9 HS increased IGF-I retention on the membrane. The addition of p9 HS to ^{125}I-IGF-I and IGFBP-3 decreased IGF-I retention slightly over the range examined, (Figure 3-15).

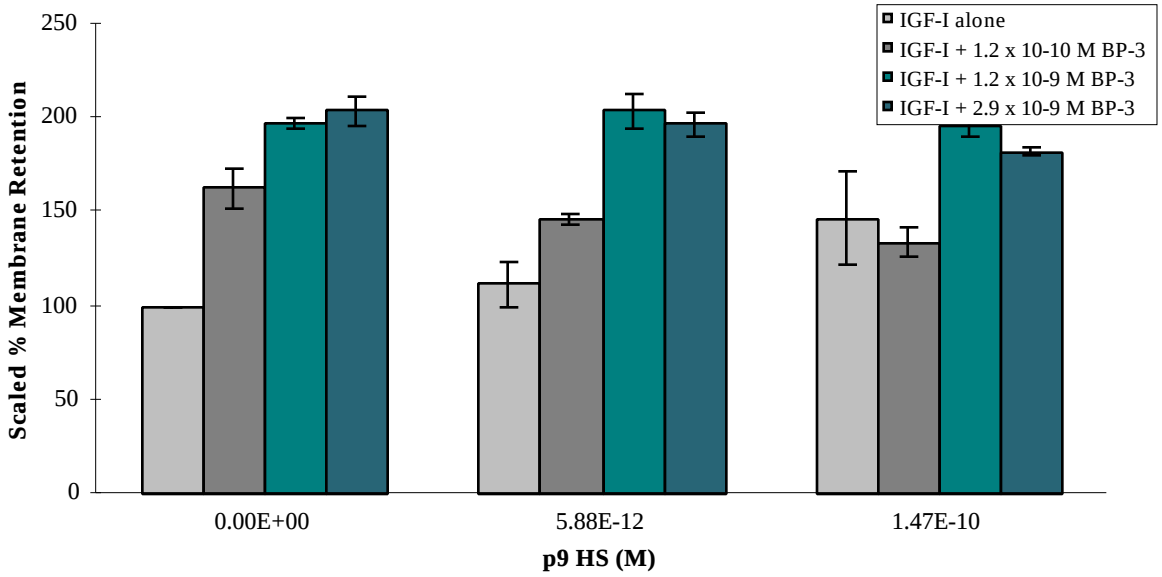

Figure 3-14 The effect of p9 HS on ^{125}I-IGF-I retention on zeta probe membrane in presence of IGFBP-3. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean. Results are scaled as discussed.

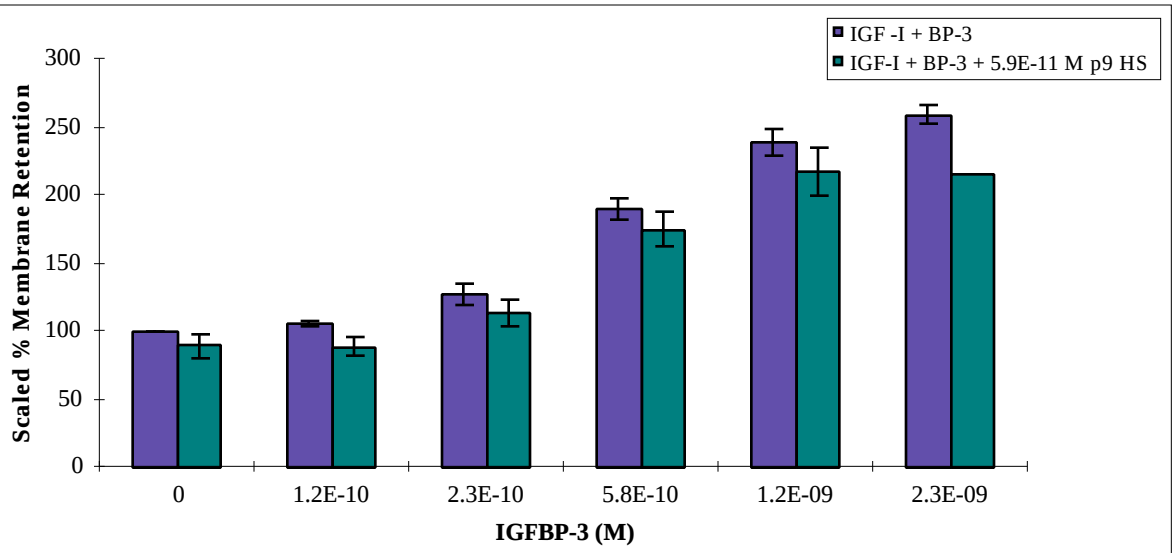

Figure 3-15 The effect of IGFBP-3 on ^{125}I-IGF-I retention on zeta probe membrane in absence and presence of 5.9 x 10^{-11} M p9 HS. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

The addition of IGFBP-3 to IGF-I and p9 HS resulted in a large increase of IGF-I retention. There was a decrease in IGF-I retention with the addition of p9 HS to IGF-I and IGFBP-3. These results suggest: 1) IGF-I and IGFBP-3 can bind to the same site on

p9 HS; 2) p9 HS drives IGFBP-3 off the membrane; 3) p9 HS prevents IGF-I binding to IGFBP-3 or releases IGF-I from IGF-I / IGFBP-3 complex. Further analysis would be necessary to distinguish between these potential mechanisms.

The biodot assay was not particulaly valuable to this study quantitatively. However, qualitative conclusions can be made. IGF-I and IGFBP-3 have a binding affinity for each other. A binding affinity, K_D ($K_D = k_{on} / k_{off}$), was determined for IGF-I and IGFBP-3 and will be discussed in Chapter 5. However, the parameter value will not be used due to the lack of quantitative data on IGFBP-3 retention on the membrane. IGF-I and p9 HS have a binding affinity for each other. The binding affinity, K_D ($K_D = k_{on} / k_{off}$), was determined for IGF-I and p9 HS from biodot data and will be discussed in Chapter 5. The parameter value can be used for modeling purposes due to the quantitatable amount of p9 HS retained on the zeta probe membrane. In comparison, IGFBP-3 seems to have a higher binding affinity for IGF-I than p9 HS has for IGF-I as seen from IGF-I retention levels. IGFBP-3 and p9 HS do not have a discernible binding affinity for each other from the biodot assay and may compete for binding sites either on the zeta probe membrane or IGF-I. This was also seen with the slight decrease in IGF-I retention seen when p9 HS was added to IGF-I and IGFBP-3.

B. Charcoal Assay

A quantitative binding assay for p9 HS and IGFBP-3 binding to IGF-I was established modifying a procedure described by Booth and co-workers[62]. Briefly, IGF-I and p9 HS or IGFBP-3 were incubated at room temperature for one hour at which point 5% charcoal (2% BSA) was added to bind free ^{125}I-IGF-I, centrifugation separates free ^{125}I-IGF-I from bound ^{125}I-IGF-I. The charcoal pellet (free ^{125}I-IGF-I) and the supernatant (bound ^{125}I-IGF-I complex) were separated and counted in a gamma counter (Beckman Instruments, Irvine, CA).

Establishing Protocol

Several experiments were performed to evaluate any potential masking effects of 5% charcoal on the ^{125}I signal when using a scintillation counter. The scintillation counter is one of the most sensitive techniques used for detecting and quantifying

radioactivity. The measurement technique is applicable to all forms of nuclear decay emissions (gamma ray emitting nucleotides, alpha and beta particles and electron capture). A radiolabeled sample is mixed with a liquid chemical medium containing a fluorescent compound that emits a flash of light when it absorbs the energy of the gamma rays released during decay of the radioisotope[6]. Setting a plastic vial holding the radiolabeled material with the scintillation fluid into a dark enclosure allows the photon intensity to be surveyed. The pertinent photon intensity is dependent on many factors: the type of radiolabeled material, the type of scintillation fluid, maximum energy of emission and other solutes dissolved along with the radiolabeled material. While the scintillation counter has the same efficiency of a gamma counter for counting gamma rays (78%), chemical or color quenching with charcoal can affect recognized counts with the scintillation counter, but not with the gamma counter. Chemical quenching induces energy losses in the transfer from solvent to solute and is dependent on the amount of the chemical and the characteristics of the sample. Color quenching depends on the color of the interfering chemical and the path length over which the photon must travel. Figure 3-16 demonstrates the decrease in energy that results from chemical or color quenching.

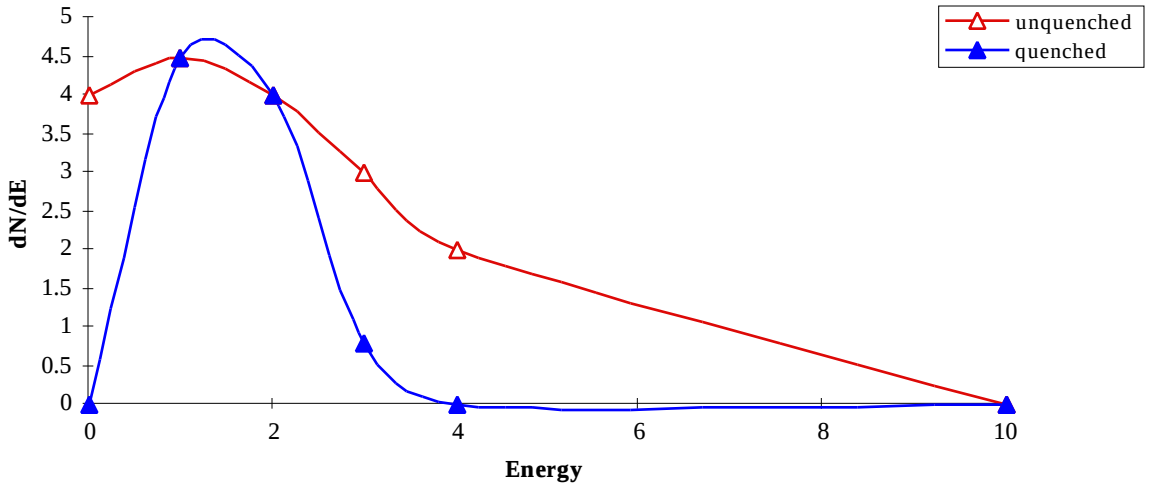

Figure 3-16 The effect of color or chemical quenching on energy emission of radiolabeled particle which is related to liquid scintillation counter recognized CPMs. (Packard 2100TR Scintillation Counter Instruction Manual)

To evaluate the effect of 5% charcoal on ^{125}I-IGF-I counts per minute (CPM), ^{125}I-IGF-I (0.4 ng), ^{125}I-IGF-I with TBS (0.1 and 0.2 ml) and ^{125}I-IGF-I with charcoal and TBS (0.1 and 0.2 ml) were added to scintillation vials and counted. Charcoal decreased the recognized CPMs by 74% and 85% with 0.1 ml and 0.2 ml of charcoal, respectively. The masking effect of the charcoal was decreased when less charcoal was present, (Figure 3-17). To evaluate the effect of charcoal masking on a range of IGF-I concentrations, samples of ^{125}I-IGF-I (7.1×10^{-11} M - 3.6×10^{-10} M) and 5% charcoal (.1 and .2 ml) was counted, (Figure 3-18). There was no change in the masking effect over the range of concentrations examined. As seen in Figure 3-17, 0.1 ml 5% charcoal had less of a depletion effect than did 0.2 ml 5% charcoal, however, the highest percentage of CPMs that were recognized by the scintillation counter in the presence of 0.1 ml charcoal was still only 30% of the actual CPMs present.

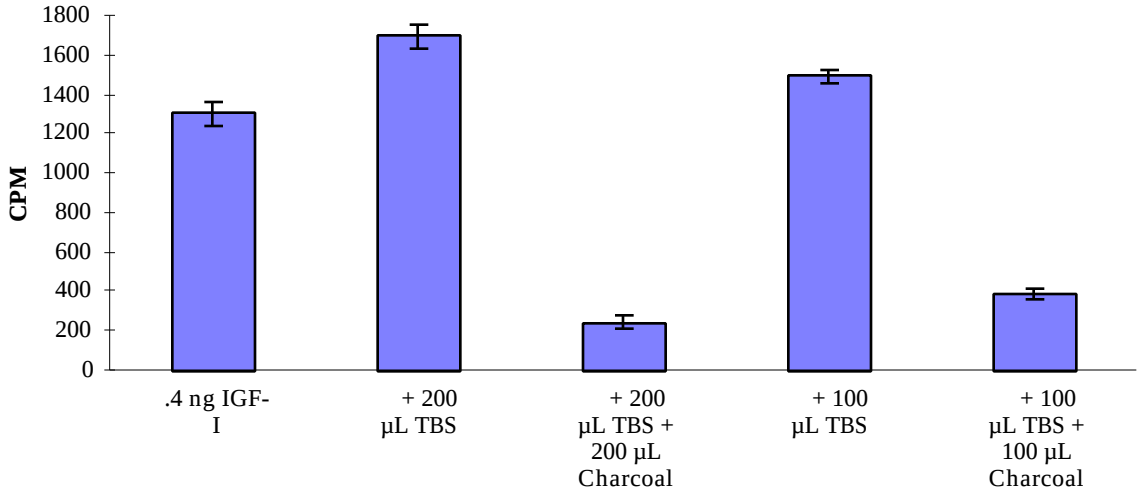

Figure 3-17 The effect of 5% Charcoal Masking on ^{125}I-IGF-I CPMs when using a liquid scintillation counter. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

The scintillation counter is clearly not the optimum means of measuring the charcoal assay results. Charcoal does not influence the recognized ^{125}I-CPMs when counted on a gamma counter making it the superior choice for assay quantitation.

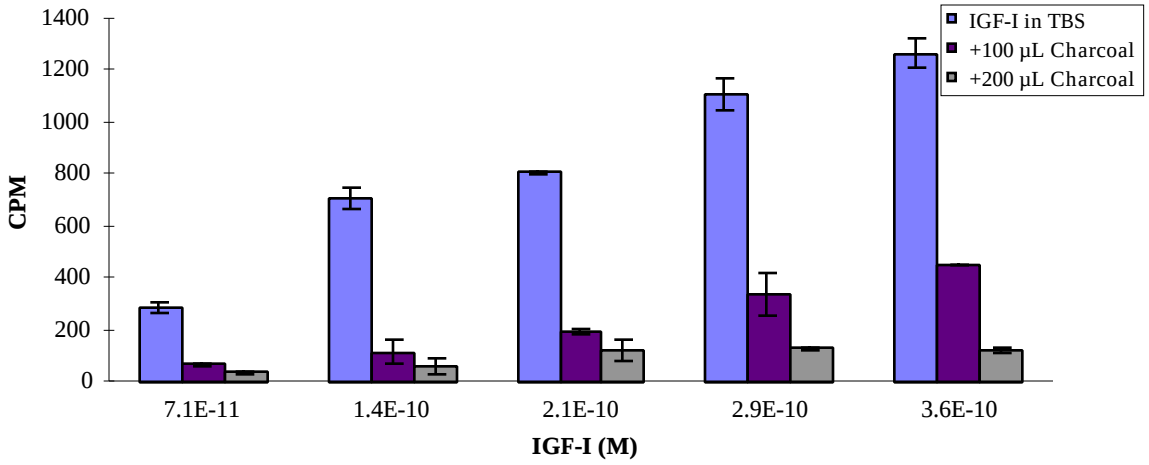

Figure 3-18 The effect of charcoal on a range of ^{125}I-IGF-I CPMs. The maximum CPMs recognized by the scintillation counter was 30% of the actual counts present when charcoal was added.
Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

IGF-I and IGFBP-3 Binding

Using the charcoal assay it was shown that increasing molar concentrations of IGFBP-3 increased the amount of IGF-I bound as a complex with IGFBP-3, (Figure 3-19). To determine the binding affinity of IGFBP-3 for IGF-I, 3.6 x 10^{-10} M ^{125}I-IGF-I was incubated with IGFBP-3 (0 - 2.3 x 10^{-9} M) with charcoal acting as the separation agent for free from bound ^{125}I-IGF-I. IGFBP-3 reduced the amount of free ^{125}I-IGF-I found in the charcoal pellet. Increasing the concentration of IGFBP-3 increased the amount of bound IGF-I (% non-precipitable ^{125}I-IGF-I by the charcoal). The specific binding of ^{125}I-IGF-I and IGFBP-3 is shown in Figure 3-20. Non-specific binding was determined by the amount of ^{125}I-IGF-I found in the supernatant when no IGFBP-3 was added. The binding affinity, K_D ($K_D = k_{on} / k_{off}$), was determined for IGF-I and IGFBP-3 from charcoal assay data and will be discussed in Chapter 5. In contrast with the biodot assay, the parameter value from the charcoal assay can be used for modeling purposes. The charcoal assay was a closed system and therefore, all IGFBP-3 added was confined to the system and could be accounted for.

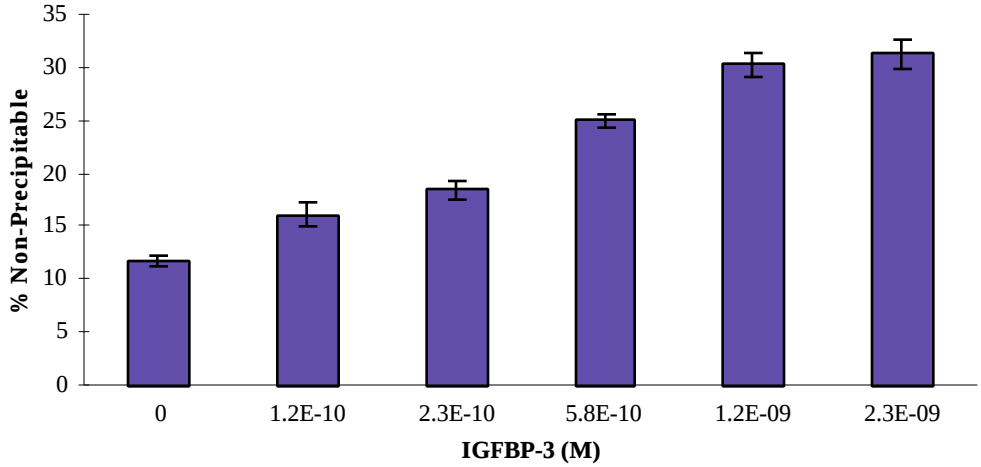

Figure 3-19 Charcoal Assay: The effect of IGFBP-3 on non-precipitable ^{125}I-IGF-I. Each point on the graph represents the mean of duplicate experiments with triplicate sets and the bar is the standard error of the mean.

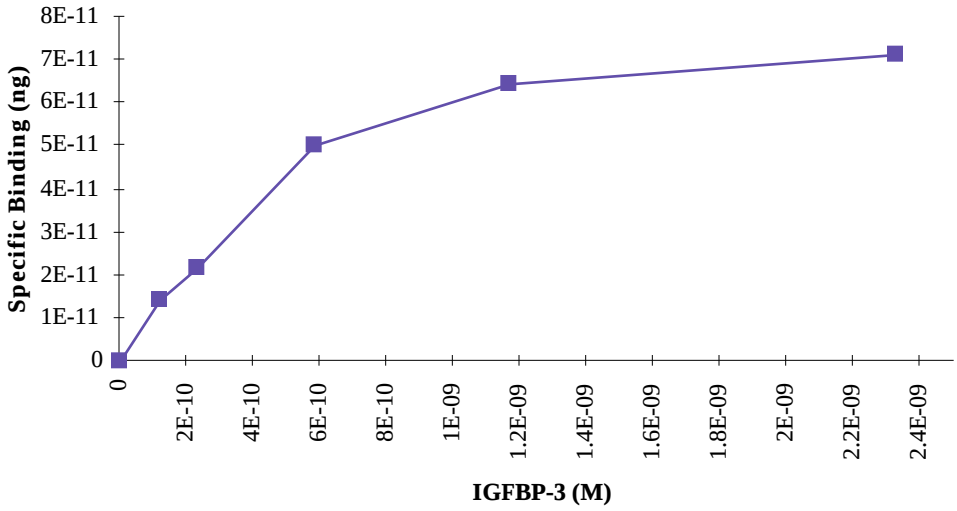

Figure 3-20 Charcoal Assay: Specific binding between IGF-I and IGFBP-3. Each point on the graph represents the mean of duplicate experiments with triplicate sets and the bar is the standard error of the mean..

IGF-I and p9 HS Binding

Using the charcoal assay, it was shown that increasing molar concentrations of p9 HS increased the amount of IGF-I bound as a complex with p9 HS, (Figure 3-21). To determine the binding affinity of p9 HS for IGF-I, 3.6×10^{-10} M ^{125}I-IGF-I was incubated with p9 HS (0 - 2.9×10^{-10} M) with charcoal acting as the separation agent for free from bound ^{125}I-IGF-I. p9 HS slightly decreased the amount of free ^{125}I-IGF-I found in the charcoal pellet. Increasing the concentration of p9 HS increased the amount of bound IGF-I (% non-precipitable ^{125}I-IGF-I by the charcoal).

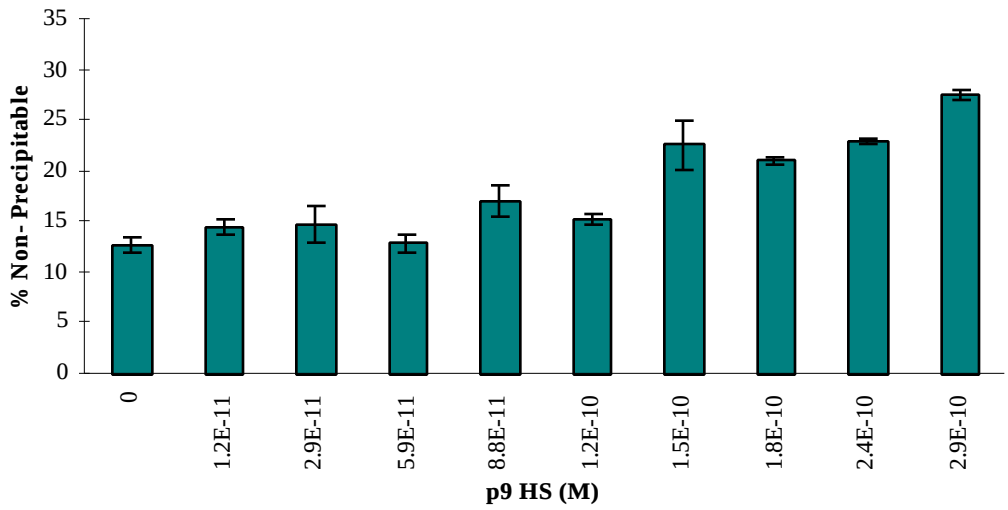

Figure 3-21 Charcoal Assay: The effect of p9 HS on non-precipitable ^{125}I-IGF-I as seen with the charcoal assay. Each point on the graph represents the mean of duplicate experiments with triplicate sets and the bar is the standard error of the mean.

The specific binding of IGF-I and p9 HS is shown in Figure 3-22. Specific binding was determined by the amount of ^{125}I-IGF-I found in the supernatant when no p9 HS was added. There was not a tremendous increase in specific binding when p9 HS was added in low concentrations (0 - 1.2×10^{-10} M). However, a greater increase was seen with higher p9 HS concentrations (1.5×10^{-10} M - 2.9×10^{-10}M). A very small amount of ^{35}S-p9 HS pelleted (16%) which was decreased to 9% by the addition of IGF-I. Due to charcoal masking effects when using the scintillation counter, these results should be interpreted with caution and could not be further investigated. The binding affinity, K_D ($K_D = k_{on} / k_{off}$), was determined for IGF-I and p9 HS from charcoal assay data and will be discussed in Chapter 5. The parameter values found from the biodot assay and the

charcoal assay are in close comparison and provide a check for both assays. It should be noted that the restriction on the p9 HS concentration by membrane saturation during the biodot assay made the charcoal assay the preferable technique for affinity determination.

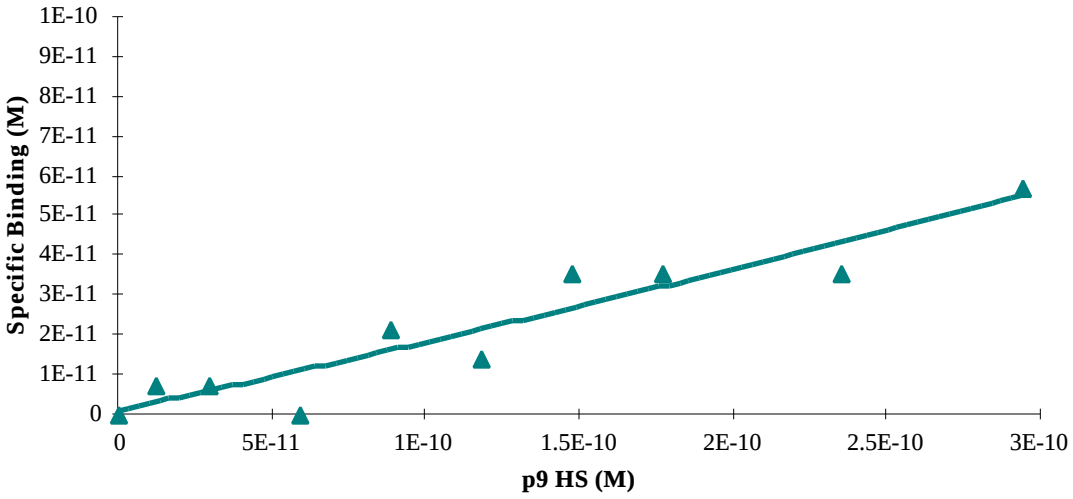

Figure 3-22 Charcoal Assay: Specific binding of IGF-I and p9 HS. Each point on the graph represents the mean of duplicate experiments with triplicate sets and the bar is the standard error of the mean.

C. IGFBP-3 and p9 HS Binding: Gel Chromatography

Gel filtration chromatography was investigated as a possible means of determining whether there is a binding affinity between p9 HS and IGFBP-3. A qualitative binding assay for IGFBP-3 and p9 HS was established using a Sepharose CL-6B gel chromatography column. CL-6B columns separate globular proteins in the range of 10,000 and 4,000,000 MW. IGFBP-3 is included in this range and therefore should elute within the gel. p9 HS is much larger than IGFBP-3 and should elute in the void volume. The CL-6B column will separate by size with the largest molecules leaving the column first followed by the smaller molecules in order of their sizes. If there is an affinity between p9 HS and IGFBP-3, a large peak consisting of p9 HS/^{125}I-IGFBP-3 complex will elute first with a peak consisting of the smaller molecular weight protein molecules (unbound IGFBP-3) following. However, since this is a transient binding

event, there is lag time from the top of the column to the bottom in which dissociation of the complex may occur. This may result in a broadened smear rather than a peak. This makes it difficult to establish a binding affinity between the two molecules.

^{125}I-IGFBP-3 (18,000 CPM / ng) was run over a CL-6B column (1.5 x 50 cm, 1 ml / min flow rate) following a one hour room temperature incubation either alone or with p9 HS (0 - 50 ng). IGFBP-3 was seen as two peaks. The addition of p9 HS caused a very slight shift in the peak sizes, (Figure 3-23). The second peak was smaller in size with the addition of p9 HS while the void peak was larger. This suggests that there was a small binding affinity between p9 HS and IGFBP-3.

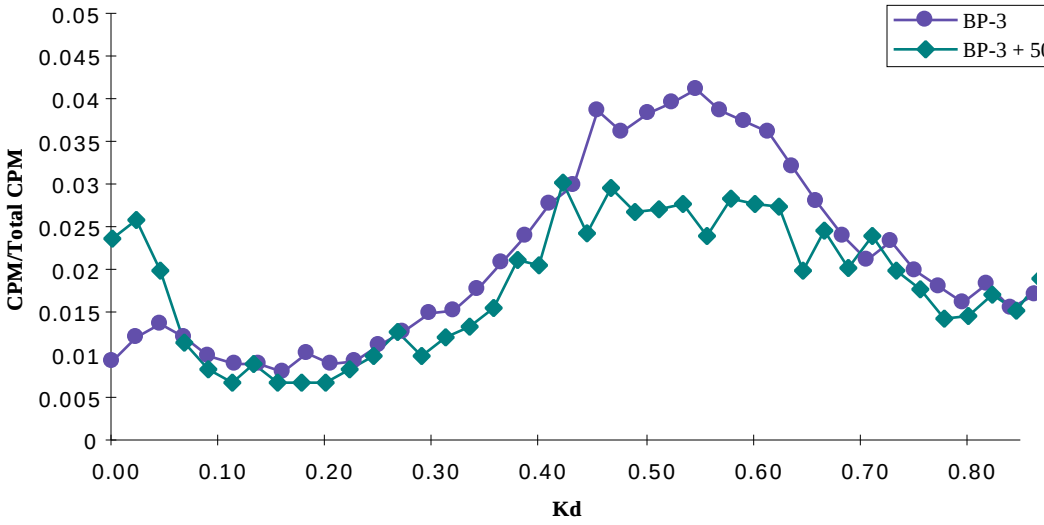

Figure 3-23 The effect of p9 HS on ^{125}I-IGFBP-3 as seen with a CL-6B Column, (1 cm D, 1 ml/min flowrate). Results represent two separate runs and are presented as a fraction of the total CPMs for each Kd value to allow for variations in each run.

D. Capacity Binding Assay

The capacity of ^{35}S-p9 HS to bind IGFBP-3 and ^{125}I-IGFBP-3 to bind p9 HS was explored using a modified method of Baird and co-workers[64]. The assay was aimed at discerning the binding characteristics of IGFBP-3 with p9 HS. Uniform nitrocellulose membrane discs (.5 cm diameter) were subjected to a 27 hour incubation at 37°C with unlabeled IGFBP-3 (0 - 2.2 x 10^{-7} M) or p9 HS (0 - 7.1 x 10^{-9} M). The unlabeled material

was put directly onto the discs to insure that all material would be contained on the discs. A control study was done with 2500 CPM / 5 µL ^{125}I-IGFBP-3 and 30 ng / 5 µL ^{35}S-p9 HS for 27 hours at 37°C to insure that all of the unlabeled IGFBP-3 and p9 HS would be retained on the disc. 95% of ^{125}I-IGFBP-3 and essentially 100% of ^{35}S-p9 HS was retained on the disc, (Figure 3-24).

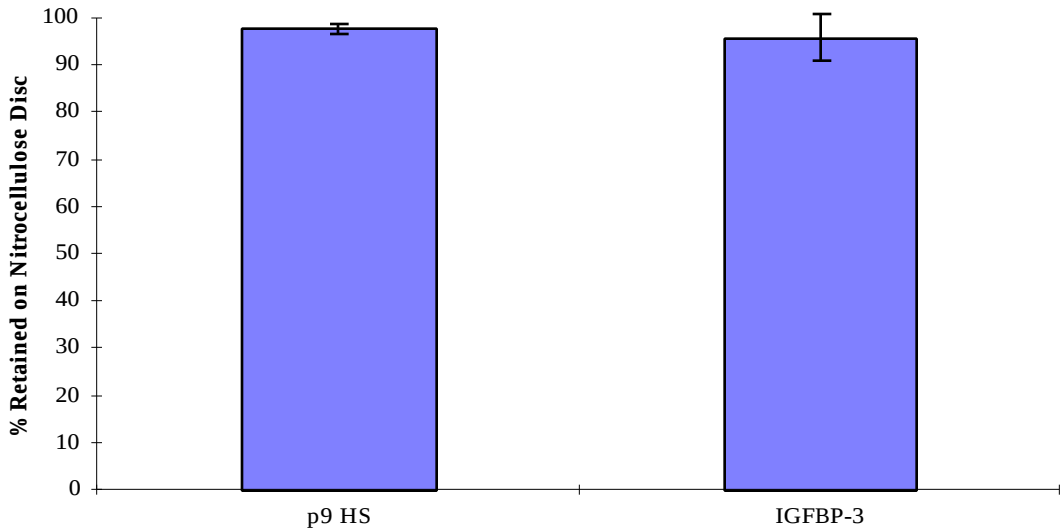

Figure 3-24 35S-p9 HS and 125I-IGFBP-3 retention on nitrocellulose discs after 27 hour drying time at 37*C. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

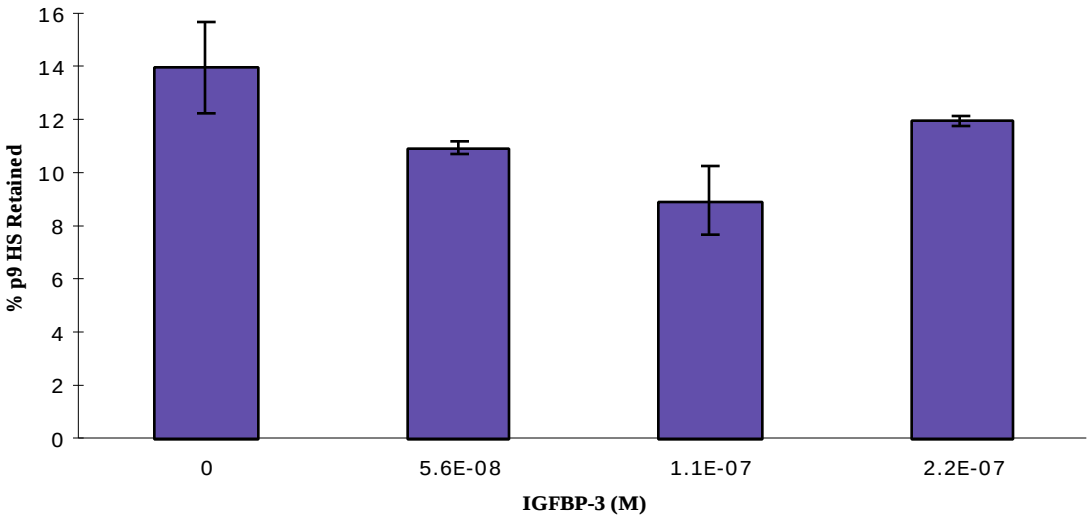

Figure 3-25 Capacity Binding Assay: The capacity of IGFBP-3 to bind 1.8 x 10^{-10} M ^{35}S p9 HS. Results are presented as a mean of triplicate samples and as a percentage of p9 HS that was bound to the nitrocellulose membrane. The bar is the standard error.

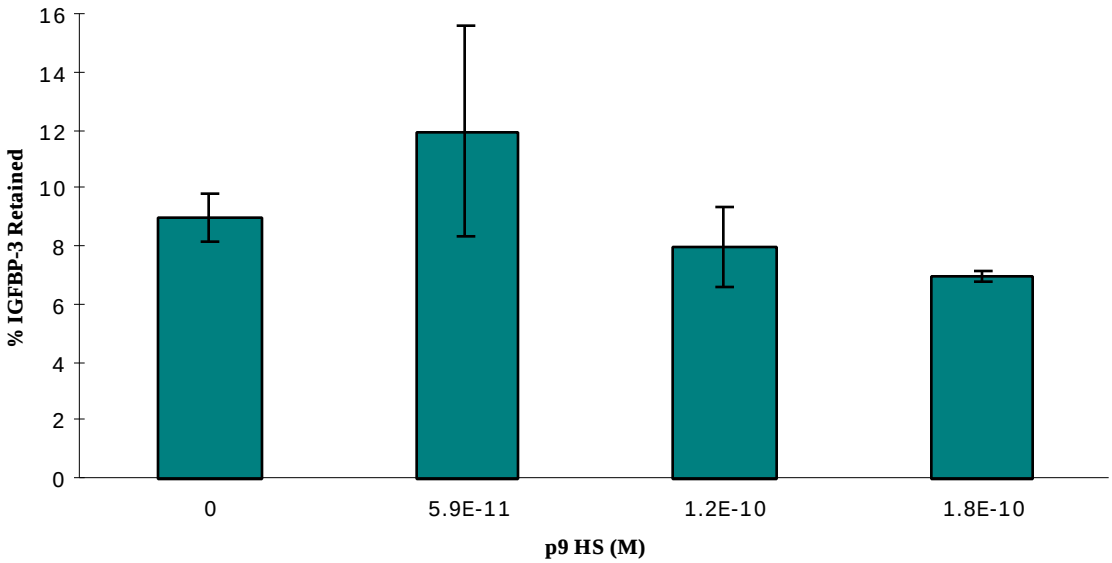

Figure 3-26 Capacity Binding Assay:The capacity of p9 HS to Bind 2000 CPM/200 µL ^{125}I-IGFBP-3. Results are presented as the mean of triplicate samples and as the % of IGFBP-3 that was bound to the nitrocellose membrane. The bar is the standard error.

^{35}S-p9 HS (1.8×10^{-10} M) or ^{125}I-IGFBP-3 (1.4×10^{-9} M) was then added to the discs and incubated at 4°C for 16 hours. Non-specific binding was determined with control discs not containing unlabeled IGFBP-3 or p9 HS. Discs were counted using a scintillation counter. There was no significant ($P \leq 0.05$) specific binding of IGFBP-3 and p9 HS throughout the entire concentration range examined, (Figure 3-25, Figure 3-26).

The capacity binding assay is undefinitive. While the results mimic what was seen with the biodot assay, it is unclear if the low amount of membrane binding was due to actual p9 HS / IGFBP-3 interactions or due to complications of the assay. Future development of this assay may prove to be useful in analyzing IGFBP-3 and p9 HS binding.

E. Future Direction

There are many interesting experiments that could be done in the future in regard to IGF-I, p9 HS and IGFBP-3 cell-free binding. By further separating p9 HS into Peak 1 and Peak 2 (Figure 2-2), investigations on how the two peaks compare or differ

in binding with IGF-I can be investigated. The enzymatic digestion with Heparinase I (Hep I) and Heparintinase III (Hep III) may affect IGF-I / p9 HS binding. Using a β-elimination reaction to free GAG chains from core proteins may also affect IGF-I / p9 HS interactions. Labeling p9 HS with ^{125}I will allow investigations of p9 HS interactions with IGF-I and IGFBP-3 with the charcoal assay. IGF-I and p9 HS binding can be observed with gel chromatography as discussed in Section C. This method was previously used to demonstrate binding between HSPG and bFGF[33].