Quantifying Non-Specific Peptide Degradation in Biomaterials

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Statement of Purpose: Peptides are ubiquitously used in synthetic hydrogels to promote cell adhesion, direct differentiation, and enable cell-mediated scaffold degradation. It is generally assumed that peptides present in the system are stable to chemical and enzymatic degradation for the duration of cell culture. However, this is rarely experimentally studied despite it being well known that there is widespread non-specific degradation of therapeutic peptides *in vivo*. This is often due to a class of proteases called exopeptidases, which remove only terminal amino acids from peptides and proteins and can have low specificity for individual amino acids. However, the enzymatic activity of exopeptidases is heavily dependent on the end-group chemistries of peptides.

In this work we have designed a peptide library which contains an RGD analog that has peptides with 19 of the canonical amino acids on the N-terminus (Figure 1A). This was then split three ways to make three different libraries with each of the amino acids and either an N-terminal amine, N-terminal acetyl functional group, or N-terminal β-alanine, an unnatural amino acid which is protease resistant. A second split pool library was created with 19 different amino acids on the C-terminus, and split three ways for the C-terminal COOH, C-terminal amide, and C-terminal β-alanine. This results six total libraries, three amine modifications and three carboxy modifications, each containing 19 pooled peptides with different terminal amino acids, for a total of 114 different peptide-chemistry combinations.

Methods: Peptides were synthesized using split-pool solid-phase peptide synthesis and purified using high performance liquid chromatography (HPLC). Peptides were added to cell culture media and the cultured with macrophages for 24h. The combined concentration of all peptides added to each well is approximately 1 millimolar. Media samples at 0 hours and 24 hours were analyzed by liquid chromatography mass spectroscopy (LCMS) to quantify peptide degradation.

Results: These six peptide libraries were incubated with macrophages for 24 hours and the fraction of each peptide remaining after 24 hours was quantified using LCMS (**Figure 1B**). For every end group the average degradation of each of the 19 different peptides was averaged into a single value. There were undetectable levels of most peptides containing an N-terminal amine after 24 hours, suggesting complete degradation. This indicates that non-specific aminopeptidase activity may be widespread within cell culture systems. Modifications of the N-terminus, either through acetylation, which is common both biologically and in biomaterials, or the unnatural β-alanine, led to significant (acetylation) or almost complete

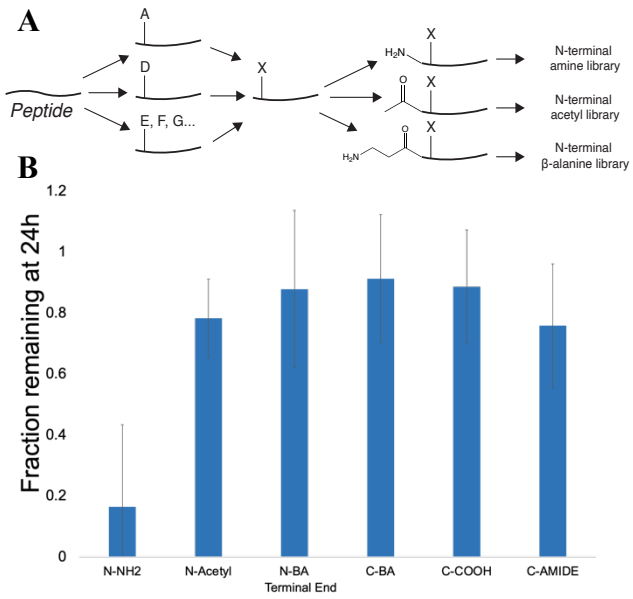

Figure 1. (**A**) A split-pool library of peptides containing 19 different terminal amino acids was synthesized and split into three different N-terminal chemistries: N-terminal amines, N-terminal acetylation, and N-terminal β-alanine. A different library with C-terminal modifications was split into three different chemistries: Carboxylic acid, amide, C-terminal β-alanine. (**B**) At 24 hours there was almost complete degradation of N-terminal amines, while β-alanines prevented degradation.

reduction in non-specific degradation (β-alanine). The C-terminal libraries had less degradation, however peptides with C-terminal amide modifications, which is common in synthesized peptides but uncommon biologically, had approximately 25% degradation at 24 hours.

Since each of these peptide libraries was incubated with the same cell type (macrophages), and some conditions had almost no degradation, it can be assumed that the degradation is solely restricted to exopeptidase degradation of the terminal amino acids and not endopeptidase degradation of interior sequences.

Conclusions: Non-specific degradation of peptide sequences is highly dependent on the chemistry of terminal amino acids. Peptides with N-terminal amines were almost completely degraded at 24 hours irrespective of the terminal amino acid, suggesting that peptides in biomaterials with these chemistries may not be stable during cell culture. These results also indicate that the use of non-natural β-amino acids on the termini almost completely reduces non-specific degradation, and that these can be incorporated into peptide design to improve stability when cultured with cells. Further studies are planned in which peptides are tethered to hydrogel matrices with photo-labile protecting groups to enable quantification of peptides during cell culture.