Published in final edited form as:
Nanomedicine. 2011 August ; 7(4): 464–471. doi:10.1016/j.nano.2011.01.004.

MICELLAR NANOMEDICINE OF HUMAN NEUROPEPTIDE Y

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Abstract

Human neuropeptide Y (NPY) is an important biologics that regulates multitude of physiological functions and could be amenable to therapeutic manipulations in certain disease states. However, rapid (minutes) enzymatic degradation and inactivation of NPY precludes its development as a drug. Accordingly, we determined whether self-association of NPY with biocompatible and biodegradable sterically stabilized phospholipid micelles (SSM) improves its stability and bioactivity. We found that in saline NPY spontaneously aggregates whereas in the presence of SSM it self-associates with the micelles as monomers. Three NPY molecules self-associate with one SSM at saturation. This process stabilizes the peptide in α-helix conformation, abrogates its degradation by dipeptidyl peptidase-4 and potentiates NPY-induced inhibition of cAMP elaboration in SK-N-MC cells. Collectively, these data indicate that self-association of NPY with SSM stabilizes and protects the peptide in active monomeric conformation, thereby amplifying its bioactivity *in vitro*. We propose further development of NPY in SSM as a novel, long-acting nanomedicine.

Keywords

human neuropeptide Y (NPY) medicine; sterically stabilized micelles (SSM); stability; dipeptydyl peptidase-4; cAMP

Background

Human neuropeptide Y (NPY), a ubiquitous 36-amino acid amphipathic neuropeptide, regulates a range of physiological functions, including food intake, anxiety, cognition, pain and cardiovascular homeostasis.[1–5] In addition, the peptide has been shown to modulate

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The authors declare no conflict of interest.

oxidative fuel selection6, bone homeostasis, intestinal fluid secretion, inflammatory responses and cancer growth.6–10 These responses are mediated, in part, through activation of NPY/ $Y_{(1)}$ receptor subtype in target organs and tissues.11–14 Overexpression of this receptor in malignant neoplasms was reported, particularly in breast cancer.10, 15 Importantly, stimulation of NPY/$Y_{(1)}$ receptor pathway inhibits growth of Ewing sarcoma *in vivo* and several cultured cancer cell lines.16, 17

Taken together, these data suggest that NPY could be developed as a drug for the disease conditions characterized by dysregulation of NPY-dependent metabolic pathway(s). However, clinical development of NPY is hampered by its short half-life (minutes) *in vivo* due to rapid enzymatic degradation predominantly by dipeptidyl peptidase-4 (DPP-4).18, 19 Formation of folded/unfolded peptide monomers, dimers and aggregates in aqueous environment also impedes development of the peptide formulation.20–22

To circumvent NPY instability, we adopted our innovative strategy where native amphipathic peptide drug candidates, such as NPY, self-associate with long-circulating, sterically stabilized, biocompatible and biodegradable phospholipid micelles (SSM).23–25 These micelles (*graphical abstract schema*) are composed of the PEGylated phospholipid molecules, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N- [methoxy(poly-ethyleneglycol)-2000] (DSPE-PEG_{2000}) that self-assemble in aqueous media above its critical micelle concentration (CMC) of 0.5–1 μM.26 Given their very low CMC, SSM are relatively stable upon intravenous administration and dilution in the bloodstream.

Additional advantages of SSM are ease of preparation and ability to be lyophilized without any lyo- or cryoprotectants to provide an acceptable shelf-life23. In our previous studies, using another amphiphilic peptide, vasoactive intestinal peptide (VIP) we found that the peptide bioactivity was prolonged from minutes to hours after systemic administration to laboratory animals.24, 27 This phenomenon was related, in part, to a change in molecular conformation of VIP from degradation-prone random coil in aqueous environment to potentially proteolysis-resistant α-helix in SSM. I*n vivo* circulation time was also prolonged by the stealth property due to the outer polyethylene glycol (PEG) corona of the micellar carrier. In addition, the α-helical conformation of VIP was previously shown to be the preferred conformation for ligand-receptor interactions in target cells.28, 29

However, not all peptide drugs intrinsically possess equal potential to form a successful nanomedicine using SSM and such ability should be tested experimentally for each peptide. Accordingly, the purpose of this study was to determine whether NPY self-associates with SSM, and if so, does this interaction stabilize the peptide and amplifies its bioactivity *in vitro*.

Methods

Materials

1,2-Distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[methoxy(polyethyleneglycol)-2000] sodium salt (DSPE-PEG_{2000}) was purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Human neuropeptide Y (NPY) was obtained from American Peptide Company (Sunnyvale, CA); sterile saline (0.9% NaCl injection USP) from Baxter Healthcare Corporation (Deerfield, IL), and human dipeptidyl-peptidase 4 from ProSpec Tany TechnoGene (Rehovot, Israel). SK-N-MC cells (HTB-10) were from American Type Culture Collection (Manassas, VA). All other reagents were acquired from Fisher Scientific (Itasca, IL) or Sigma-Aldrich (St. Louis, MO).

Sample preparation

Method to prepare novel NPY-SSM formulation was based on our previous experience with peptide-SSM association.23 Briefly, weighed amount of DSPE-PEG_{2000} was combined with appropriate volume of saline and vortexed for 2 min (Thermolyne Maxi Mix II). The dispersion was equilibrated at 25 °C for 1 hour in dark. This resulted in the formation of sterically stabilized micelles (SSM) at concentrations above lipid critical micelle concentration. Thereafter, measured volume of NPY stock solution in saline was added to SSM dispersion. Resulted NPY-SSM dispersion was further incubated for 2 h at 25 °C in dark. All samples were prepared fresh and diluted with saline according to the experiment.

Particle size analysis

The aggregation behavior of NPY in saline was assessed in presence and absence of DSPE-PEG_{2000} by dynamic light scattering (DLS) using Agilent 7030 NICOMP DLS (Agilent Technologies, Santa Clara, CA). SSM, NPY and NPY-SSM in saline at 5 mM lipid and 20 μM peptide concentrations were evaluated. The mean hydrodynamic particle diameter $d_{\hbar}$ was computed from the diffusion coefficient using Stokes-Einstein equation. Measurements were carried out at 23 °C with autoadjusted light scattering intensity of 300 KHz and fixed detector angel of 90°. Dispersing medium parameters of 0.933 cP for viscosity and 1.33 for refractive index were used. As a minimum of three $d_{\hbar}$ values were averaged for each reported experimental result from the analysis of the autocorrelation function accumulated over at least 15 min.

Fluorescence emission spectroscopy

The maximal self-association number of NPY molecules per SSM was determined by fluorescence spectroscopy as previously described in our laboratory.23

Samples were prepared with varying DSPE-PEG_{2000}: NPY molar ratios ranging from 2:1 to 100:1. Peptide concentration was kept constant at 5 μM while lipid concentration was increased from 0.01 to 0.5 mM. The peak fluorescence emission intensity (Em_{max}) of tyrosine residues of NPY was recorded on SLM Aminco 8000 Spectrofluorimeter (SLM Instruments, Rochester, NY) at excitation wavelength 275 nm. The respective Em_{max} was plotted against corresponding lipid:peptide molar ratio with an assistance of SigmaPlot® (Systat Software Inc., San Jose, CA) and integrated into curve fitting function of the software. The *lipid: NPY molar ratio at saturation* was determined as the lowest lipid: NPY molar ratio at which Em_{max} of NPY was not significantly different from the intensity measured at plateau (in the presence of excessive lipid). Given that approximately 90 lipid monomers form one SSM30 the maximal number of NPY molecules that could associate with each micelle was calculated by the following equation:

$$N_{NPY}=90/x$$

Where N_{NPY} is the number of NPY molecules associated with one SSM and x is the number of lipid molecules at the saturation.

Circular dichroism spectroscopy

The peptide secondary structure in NPY-SSM or NPY in saline (20 μM peptide, 5mM lipid) was determined by circular dichroism (CD) as previously described in our laboratory.28, 31 CD spectra for NPY were recorded on Jasco J-710 spectropolarimeter (Jasco Inc., Easton, MD) calibrated with D-10-camphorsulfonic acid in range 190–260nm at room temperature in 1mm path length fused quartz cuvette. The resulted spectra were corrected for

corresponding SSM or saline scans and smoothed according to manufacturer's instructions by the Savitzky-Golay algorithm.

Mass spectrometry analysis of NPY and its DPP-4 cleaved products in presence and absence of SSM

The purpose of this study was to assess the stability of NPY in self-association with SSM to dipeptidyl peptidase-4 (DPP-4) degradation in comparison to the peptide in saline. DPP-4 is a major blood and tissue associated enzyme involved in regulation of $Y_{(1)}$ to non-$Y_{(1)}$ receptor signaling through NPY truncation at N-terminal penultimate proline.[18, 22, 32]

Stock solutions of NPY in saline and NPY-SSM were prepared as outlined above. Measured volume of stock solutions were added to 1 nM DPP-4 in phosphate buffered saline (pH 7.4) to obtain the final concentrations of 10 μM for NPY and 300 μM for the lipid. The resulting samples were incubated for 1 h at 37 °C followed by analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).[33, 34] Samples were mixed with the matrix solution (10 mg α-cyano-4-hydroxycinnamic acid in 1 ml aqueous solution of 50 % acetonitrile containing 0.1 % trifluoroacetic acid, v/v) at 1:1 ratio. Aliquots of the mixtures (1.3 μl) were spotted onto a MALDI-TOF target and analyzed using Voyager-DE PRO Mass Spectrometer (Applied Biosystems, Foster City, CA) equipped with a 337 nm pulsed nitrogen laser. Peptide mass was measured using positive-ion linear mode over the range m/z 1000–6500.

Bioactivity of NPY-SSM in vitro

The purpose of this experiment was to determine whether NPY in association with SSM retained its biological activity to diminish cAMP accumulation in cultured human SK-N-MC brain neuroepithelioma cells.[35, 36] The selected cell line endogenously expresses neuropeptide $Y_{(1)}$ receptors that upon binding to NPY inhibit intracellular cAMP production.[35, 37]

Stock solutions of NPY-SSM, NPY in saline and their respective controls were prepared as described above with the corresponding NPY and lipid concentrations of 1 μM and 100 μM followed by dilution with saline for further cell culture studies. The method used in this experiment has been previously described in literature.[35] Briefly, cells were seeded in 24-well plates at density 2×10^5 cells per well and cultured in EMEM growth medium supplemented with 10% fetal bovine serum for 24 h at 37 °C in humidified, 5% CO_2 atmosphere. On the day of experiment, growth medium was aspirated and replaced with serum-free medium containing 5 mM theophylline, a non-specific phosphodiesterase inhibitor, and incubated for 1 h at 37 °C in humidified, 5% CO_2 atmosphere. After that, 20 μl of 5 mM DSPE-PEG_{2000} solution were introduced to the wells to be treated with SSM containing solutions to maintain the lipid concentration above CMC. The corresponding amount of saline was added into all other wells to keep up matching well volume. Immediately hereafter, equal volumes of NPY in saline, NPYSSM or their respective controls were added into designated wells to achieve NPY concentration ranging from 0.01–10 nM. Following 3 min incubation, forskolin was added into all wells (100 μl; final concentration, 10 μM) to induce cAMP production. Thereafter, cells were incubated for additional hour at 37 °C in humidified, 5% CO_2 atmosphere. At the conclusion of the incubation period, cells were lysed and cAMP content in cell lysates was determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (cAMP Biotrak EIA Kit, GE Healthcare, Piscataway, NJ). Data were normalized to total protein content measured by BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL).

Data and statistical analyses

Data are expressed as mean ± SD. Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's test. A value of $p<0.05$ was considered statistically significant.

Results

Particle size analysis

The mean particle size and particle distribution of blank SSM, NPY-SSM and NPY in saline were analyzed by dynamic light scattering (Figure 1). Monomodal particle distributions as evaluated by volume- and intensity-weighted Nicomp analysis were observed in all tested samples; number-weighted analysis revealed similar particle allocation (data not shown). Neuropeptide Y (20 μM) in saline formed large soluble aggregates with mean hydrodynamic diameter of 557±100 nm (Figure 1A; volume-weighted averaged value of 3 separate experiments). By contrast, mean hydrodynamic diameter of NPY (20 μM) in SSM (5 mM DSPE-PEG_{2000}) was 14±3 nm (Figure 1B), which was not significantly different from blank SSM 15±3 nm (Figure 1C) (n=3/group; $p>0.05$).

Fluorescence emission spectroscopy

Intrinsic fluorescence of NPY tyrosine residues at constant peptide concentration (5 μM) enhanced with increasing concentration of DSPE-PEG_{2000} until reaching a plateau at lipid:petide molar ratio of 29:1 and beyond (Figure 2). Based on the information that one SSM is formed from ~90 DSPE-PEG_{2000} monomers30 the maximal number of NPY molecules that could self-associate with one SSM was determined to be three.

Circular dichroism spectroscopy

The CD spectra of NPY in SSM and saline were recorded at 20 μm peptide concentration in far-UV range (Figure 3). Notable amount of α-helix for the peptide in saline was detected. Association of NPY with SSM lead to further slight increase in NPY α-helicity, observed by deepening of negative bands at 208 and 222 nm which are characteristics of α-helical peptides. This finding is supported by previously reported NPY potential for enhanced α-helicity in nonpolar environment38, implying that NPY interaction with SSM stabilizes the peptide in its physiologically active helical form.

Mass spectrometry analysis of NPY-SSM in the absence and presence of DPP-4

We found that one hour incubation of $NPY_{(1–36)}$ (molecular mass of 4270 Da) with DPP-4 aminopeptidase at 37 °C in PBS (pH 7.4) generated a considerable amount of secondary product with molecular mass around 4000 Da, indicating production of truncated $NPY_{(3–36)}$ form equivalent in intensity to the parent peptide (Figure 4B). In contrast, such secondary peak was not detectable upon incubation NPY-SSM samples with DPP-4 (Figure 4D), similar to NPY and NPY-SSM samples without the enzyme (Figure 4 A, C) at the same experimental conditions. Splitting of the peptide peak observed for the NPY-SSM mass spectra in the presence and absence of enzyme (Figure 4 C, D) was most probably accounted to variation of the PEG and/or lipid chain length in DSPE-PEG_{2000} lipid of SSM.

Bioactivity of neuropeptide Y in SSM in vitro

Effects of NPY in saline and SSM on cAMP accumulation in human SK-N-MC brain neuroepithelioma cells are depicted in Figure 5. Cyclic AMP content decreased significantly in a concentration-dependent manner in the presence of NPY in SSM and NPY in saline. However, a greater inhibition of cAMP production was observed for the cells treated with NPY-SSM compared to NPY in saline at the same peptide concentration. Cyclic AMP

concentration reduced significantly to 50.2±2.7 % compared to forskolin-stimulated saline-treated cells (positive control; 100 %) in the presence of 0.1 nM NPY-SSM but only to 86.5±14.7 % in the presence of 0.1 nM NPY in saline (Figure 5; n=3/group; $p<0.05$). Likewise, cAMP concentration decreased significantly to 44.7±2.9 % in the presence of 1.0 nM NPY in SSM but only to 59.6±6.8 % at the same NPY concentration in saline (Figure 5; each group n=3; $p<0.05$). Conversely, cellular cAMP level decreased to similar concentration in the presence of 10 nM NPY-SSM and NPY in saline (30.5±3.5 % and 41.6± 6.9 %, respectively; Fig. 5; n=3/group; $p>0.05$). The effect of NPY in micelles at 0.1 nM was similar to the effect of NPY in saline at 10 nM 50.2±2.7 % and 41.6± 6.9 %, respectively (Figure 5; each group, n=3; $p>0.05$), despite 100 times lower peptide content in SSM. SK-N-MC cell incubation with blank SSM, corresponding to the highest lipid concentration of tested NPY-SSM samples, resulted in no significant change of cAMP level in comparison to saline treated control (Figure 5, *inset*).

Discussion

Human neuropeptide Y (NPY) is a neurotransmitter and hormonal peptide that is undergoing intensive investigation for the treatment of cachexia, certain psychiatric disorders and neuropathic pain.[1, 3, 4, 39, 40] Delivery of NPY directly to peripheral tissues possess an additional interest and can be beneficial when peptide action in central nervous system (CNS) is not required. Anti-proliferative and proapoptotic[10] effects of NPY mediated through $Y_{(1)}$ receptor activation can be exploited in treatment of solid tumors overexpressing this receptor subtype. Moreover, targeting of peripheral energy utilization[6] as supplementary treatment of anorexia and downregulation of T-helper 1 hypersensitivity[41] in acute stages of inflammation with NPY are therapeutically beneficial. Besides direct therapeutic application, labeled NPY analogs could serve as a diagnostic tool in imaging. This approach was recently tested in humans to image breast cancer and metastasis with technetium 99 tagged NPY.[42] We anticipate that labeled NPY in SSM should be more effective in imaging due to enhanced accumulation at a tumor site through passive and active targeting.

However, fast proteolytic degradation of NPY upon administration is a major barrier for its successful clinical application.[2] The new findings of this study showed that NPY, which underwent spontaneous self-association with SSM, was protected from enzymatic degradation and its bioactivity was significantly amplified.

Self-association of monomeric NPY with SSM abrogated formation of peptide aggregates in aqueous environment (saline) as shown by particle size analysis (Figure 1). We postulate that intermolecular hydrophobic interactions between NPY molecules at the dimer/tetramer interface are superseded by more energetically-favorable interactions of NPY molecules with PEGylated phospholipid micelles. This was further evidenced by increased fluorescence emission of NPY in the presence of SSM (Figure 2). The enhanced fluorescence intensity was likely due to reduced fluorescence quenching between aggregated NPY molecules.[21] To this end, we found that up to three NPY molecules self-associate with each micelle freely, without interaction with each other.

Furthermore, our circular dichroism study showed that the association of NPY with biocompatible and biodegradable sterically stabilized phospholipid micelles in aqueous environment resulted in stabilization of α-helical peptide confirmation (Figure 3). This was in agreement with the results of another research group on NPY conformational changes with a model membrane system (dodecylphosphocholine micelle). This study revealed a raise in NPY α-helicity, particularly in its C-terminal segment upon interaction with the dodecylphosphocholine micelles.[11] Given that the C-terminal of NPY is critical for binding

to its targets, this conformational change favors peptide-receptor interaction.[11, 20, 43, 44] Increased α-helicity of NPY in SSM is likely contributed, in part, by the adoption of helical conformation at the C-terminal pentapeptide. Moreover, NPY most likely reside within the PEG palisade of PEGylated micellar system, rather than binding to the phospholipid headgroup surface as claimed with dodecylphosphocholine micelles free of PEG corona.[11] Therefore, the PEG layer of SSM should provide an additional steric barrier against physical aggregation and enzymatic degradation of NPY, which would result in further increase in the stability of NPY.

This notion is further supported by our findings that self-association of NPY with SSM abrogated enzymatic peptide cleavage induced by DPP-4 (Figure 4) and potentiated NPY bioactivity as indicated by enhanced suppression of cAMP accumulation in forskolin stimulated SK-N-MC cells (Figure 5). These cells express neuropeptide $Y_{(1)}$ receptors that upon activation reduce intracellular cAMP accumulation which underlies many of the NPY-induced central and peripheral responses.[6, 11–13, 15, 37] Since SK-N-MC cells have been reported to exogenously express DPP-4,[45] enhanced bioactivity of NPY-SSM in these cells could be related, in part, to NPY molecule protection within SSM. However, we believe that such protection is “reversible” and do not interfere with receptor/ligand interaction. This, in turn, enables more intact full length NPY molecules in favorable α-helix conformation to interact with their receptors thereby amplifying peptide bioactivity. Moreover, the peptide will be presumably protected by SSM from the enzymatic cleavage not only in vicinity of their cell targets but also in circulation prolonging its half-life. The possibility of interaction of NPY in SSM with $Y_{(2)}$, $Y_{(4)}$ and $Y_{(5)}$ receptors can not be ruled out. Clearly, additional studies are needed to evaluate this fact.

The results of this study represent the first step in our overall goal to safely and effectively deliver human NPY by the intravenous or intranasal routes. The former path is more applicable for peripheral peptide delivery since our nanomicellar technology most likely will not allow blood brain barrier crossing. From another hand, feasibility of intranasal delivery of peptide drugs has been shown for various nanoparticulate carriers, including liposomes and PEGylated polymeric nanoparticles[46–48] and should be applicable for NPY-SSM as well. Moreover, NPY is currently being tested by intranasal route on humans in a new clinical trial,[49] but not as a nanoparticulate formulation.

In conclusion, we found that self-association of NPY with sterically stabilized phospholipid micelles amplifies its bioactivity *in vitro*. This may be related, in part, to decreased aggregation and increased stability of NPY molecules by the carrier. This, in turn, obviates the need to chemically modify NPY molecule to improve its stability thereby circumventing possible immunogenicity and adverse events.[24, 28, 50] Phospholipid micelles used in this study are composed of PEGylated lipid, which is a component of U.S. FDA approved product Doxil®. The final product, NPY-SSM dispersion is easy to prepare, scale up and freeze-dry for long-term storage. Therefore, we propose further development of NPY in SSM as a novel, long-acting nanomedicine for conditions with peptide deficiency and where NPY/receptor Y(1) pathway mediation is needed.

Acknowledgments

The authors thank Ernest Gemeinhart for his technical assistance in cell culture experiments.

This study was supported, in part, by NIH grants AG024026, CA121797; Parenteral Drug Association Pre-Doctoral Fellowship and VA Merit Review grant. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number CO6RR15482 from the National Center for Research Resources, NIH.

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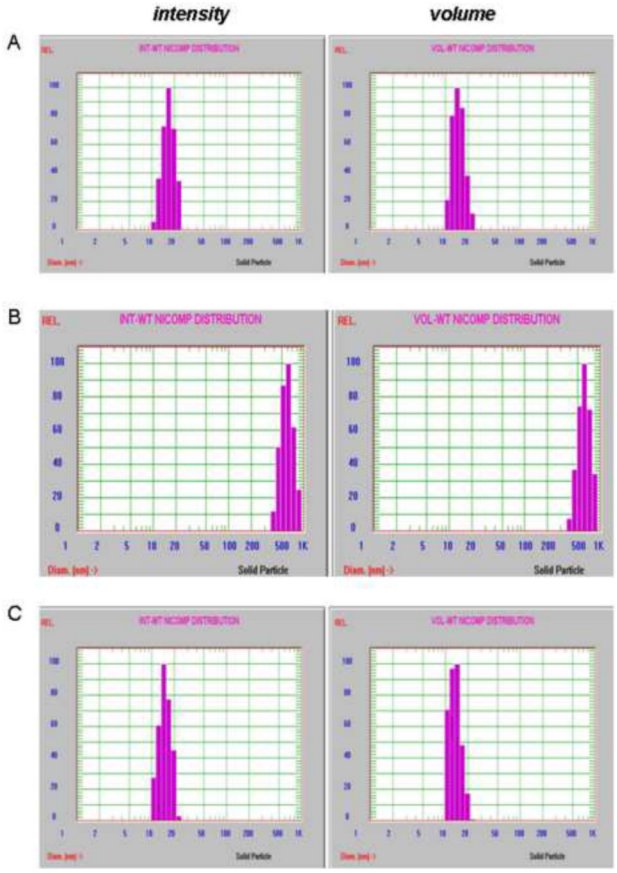

Figure 1.

Representative particle size distribution by intensity- and volume-weighted DLS NICOMP analysis after 2 h incubation of blank (**A**) SSM (100% of the particles form a single peak with average diameter 14 ± 3 nm by volume-weighted analysis), (**B**) neuropeptide Y (NPY) in saline (average hydrodynamic diameter of the aggregates ranged 557 ± 100 nm in a single peak), and (**C**) NPY-SSM (monomodal distribution was observed with mean particle diameter not significantly different from SSM alone, 15 ± 3 nm volume-weighted).

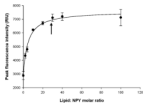

Figure 2.
Peak fluorescence intensity of NPY at varying lipid: NPY molar ratios (each point n=3). The arrow (↑) indicates lipid: NPY saturation molar ratio of 29:1.

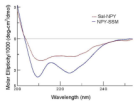

Figure 3.
Representative circular dichroism spectra of neuropeptide Y (NPY; 20 μM) in saline (Sal-NPY) and in SSM (NPY-SSM; 5 mM DSPE-PEG_{2000}).

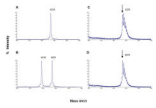

Figure 4.
Representative MALDI-TOF mass spectra of (**A**) NPY (10 μM) in saline (Sal-NPY), (**B**) Sal-NPY + DPP-4 (1 nM), (**C**) NPY-SSM (300 μM DSPE-PEG_{2000}) (**D**) and NPY-SSM + DPP-4. Samples were incubated at 37 °C for 1 h in PBS (pH 7.4) prior the analysis.

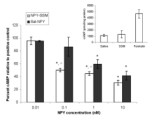

Figure 5.

Cyclic AMP level in forskolin-stimulated SK-N-MC neuroepithelioma cells treated with NPY in saline (Sal-NPY) or NPY-SSM at various peptide concentrations (data normalized against positive control). Each group, n=3 experiments; *p<0.05 in comparison to forskolin-stimulated saline treated cells (NPY 0 nM; positive control); +p<0.05 in comparison to NPY in saline at the same peptide concentration. ***Inset*** figure represents cAMP level in SK-N-MC cells incubated with saline, blank SSM (vehicle control) or forskolin (positive control); n=3/group.